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Mechanisms of Protein Localization

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INTRODUCTION

With the exception of the limited biosynthetic activity exhibited by mitochondria and chloroplasts, the translation of mRNA occurs in the cytoplasm. Nevertheless, all cells synthesize proteins that are exported to various noncytoplasmic locations. In addition, many cells are capable of true protein secretion. These processes of protein localization are selective and efficient in that proteins are strictly compartmentalized to a particular cellular location. During the past decade, considerable effort has been directed towards elucidating the molecular mechanisms by which cells accomplish these processes. One important outcome of this work was the realization that all cells use similar mechanisms of protein localization. This realization has fostered a solid interaction between biologists working with numerous different organisms.

Despite the fact that bacteria are generally considered to be simple organisms, investigators working with eucaryotic systems have led the field with respect to biochemical studies. This is due to the specialization characteristically exhibited by the cells of higher organisms. Eucaryotic cells contain organelles that function specifically in the process of protein localization. Purification of these organelles automatically provides the biochemist with an enriched fraction containing the biological machinery of interest. Procaryotic cells offer no such advantage. Here the number of subcellular compartments is small, and each performs many different functions. On the other hand, investigators working with procaryotic cells have led the field with respect to genetic analysis. Bacteria, such as *Escherichia coli*, are haploid and multiply rapidly. They are well characterized genetically, and techniques used routinely are more sophisticated than those currently available in eucaryotic systems. In this review we describe the genetic techniques that have been applied successfully to the study of protein localization in bacteria. Before beginning, however, we review the relevant biochemical data. This review will familiarize the reader with current concepts, and it will

make apparent the many questions that remain to be answered.

PROTEIN LOCALIZATION IN EUCARYOTIC CELLS

Most of what is currently known about the process of protein localization stems from the work of George Palade. In pioneering studies, Palade traced the intracellular routing of a variety of noncytoplasmic proteins (153). The following key discoveries were made. (i) Proteins for export are synthesized by polysomes that are bound to the membrane of the rough endoplasmic reticulum (185). (ii) Proteins destined for export are never found in completed form in the cytoplasm. They are segregated immediately into the lumen of the rough endoplasmic reticulum (162). (iii) Using autoradiography and cell fractionation, Jamieson and Palade (97, 98) followed pulse-labeled proteins through the export pathway. They found that proteins are transported from the endoplasmic reticulum through the Golgi apparatus to secretory vesicles, where they remain stored until secretion. This intracellular export pathway is often referred to as the Palade pathway.

In the last several years genetic verification of the Palade pathway has been obtained by Novick and Schekman (143), using yeast as a model system. Their elegant work stems from the discovery that many yeast mutants defective in protein export accumulate secretory organelles. This accumulation makes mutant cells more dense than wild-type cells and consequently provides a simple method to enrich for export-defective mutants. Mutant strains that are temperature sensitive and that accumulate exported proteins at the nonpermissive temperature were isolated (142, 143). Morphological examination of many of these mutants has shown that they fall into three classes based on the nature of the accumulated secretory organelle. One class accumulates a structure related to endoplasmic reticulum, another accumulates a structure related to Golgi, and the third accumulates secretory vesicles (142). By constructing a series of double mutants and determining the nature of the secretory organelle that accumulates at the nonpermissive temperature, they have been able to confirm the temporal order of the Palade pathway (141). Genetically, these export-defective mutations define at least 23 different complementation groups (142). This underscores the complexity of the cell's export machinery.

Having elucidated the export pathway, investigators began to examine events at the molecular level to gain insight into the mechanisms of protein localization. Since the actual transfer of the protein across the membrane appeared to occur at the level of the rough endoplasmic reticulum, initial effort was focused here. The experiments of Redman and Sabatini (161) demonstrated that truncated polypeptide chains produced by the action of puromycin were also segregated into the lumen of the rough endoplasmic reticulum. Furthermore, microsomes (right-side-out vesicles of endoplasmic reticulum) can protect nascent chains from proteolytic degradation (170). Experiments such as these led to the conclusion that segregation is the result of vectorial transfer of the nascent polypeptide chain across the membrane into the lumen. In other words, protein transfer is tightly coupled to, if not inseparable from, translation.

The Signal Hypothesis

The question then arose, What is the basis for the observed selectivity of the export process? Where does the information specifying export lie? There were two possibilities: either the information is contained within the mRNA, or it is contained within the structure of the protein. If the latter possibility is correct, then the information must lie near the NH_2-terminal end of the protein since the export process begins before synthesis is complete. The experiments of Redman and Sabatini (161) substantiated this position by showing that the COOH-terminal end of the molecule can be removed without affecting export.

The first experimental evidence to shed light on the nature and location of export information was obtained by Milstein et al. (132). Using a cell-free translation system, they found that immunoglobulin G (IgG) light chain was initially synthesized in larger precursor form with a peptide extension at the NH_2 terminus. Schechter and co-workers (176) verified these findings and presented the first amino acid sequence data for the peptide extension. These results offered suggestive evidence that export information was contained in an NH_2-terminal peptide extension. The assumption did not receive widespread acceptance, however, until the work of Blobel and Dobberstein (11, 12) appeared.

The important breakthrough provided by Blobel and Dobberstein (11, 12) was the development of an in vitro assay for protein transport. Using a cell-free translation system to which microsomal vesicles had been added, they were able to demonstrate protein transport by showing that the protein product was inside the vesicles and thus protease resistant. They also were able to show that, in the absence of membranes, precursor was synthesized. If membranes were present during synthesis, mature product located inside the vesicles was found. However, if membranes were added after synthesis was complete, no processing or transport

could be detected. This evidence led to the formulation of the signal hypothesis.

According to the signal hypothesis, a protein destined to be secreted from cells is synthesized initially as a larger precursor with 15 to 30 additional amino acids at the NH_2-terminal end of the molecule. This peptide extension (the signal sequence) was proposed to initiate binding of the translation complex to the rough endoplasmic reticular membrane, resulting in the formation of a transient pore (an export site) through which the nascent peptide chain passes as synthesis proceeds. The net result is a vectorial transfer of the protein across the rough endoplasmic reticular membrane to the lumen of the organelle. The signal sequence is removed by a specific protease (signal peptidase) probably before synthesis of the secretory protein is completed.

The availability of an assay for protein translocation permits biochemical fractionation and purification of essential components of the export machinery. Two different approaches have been applied successfully. Walter and Blobel (207) purified a membrane-associated protein complex (250,000 daltons) composed of six polypeptide chains. This complex is released from the rough endoplasmic reticular membrane by a high-salt wash. In similar experiments, Meyer and Dobberstein (127) identified a peptide fragment that is released from microsomes by high-salt and limited proteolysis. By raising antibodies against the purified fragment, they were able to demonstrate that the fragment corresponds to a 72,000-dalton membrane protein (128). After some initial confusion regarding the possibility that the 72,000-dalton protein was a component of the 250,000-dalton complex, it has been shown that they are distinct and that they function in sequential steps in the export process. The 250,000-dalton complex has been shown to function by binding to the signal sequence as it emerges from the ribosome and halting further translation. When stripped microsomes (salt washed) are added, translation resumes, and the export process begins (208). This complex, called signal-recognition particle (SRP), functions to couple synthesis and export, a critical activity, because if translation proceeds too far beyond the signal sequence, export cannot occur (167). The 72,000-dalton membrane protein is the component that relieves the SRP-mediated translation block (128) and has been termed docking protein. Similar results have been independently obtained by Gilmore et al. (67), and, on the basis of earlier work, Walter and Blobel (208) termed this protein SRP protein.

Recently, Walter and Blobel (209) showed that 7S cytoplasmic RNA is an integral component of the SRP complex. Although the function of this RNA is not yet known, its discovery raises the possibility that specific nucleic acid base pairing may be required for the interaction between SRP and polysomes. Such an interaction may involve sequences in mRNA or rRNA or both.

Another biochemical approach for identification of the components of the export machinery was to compare the protein composition of rough and smooth endoplasmic reticulum. Such a comparison revealed two glycoproteins (~65,000 and 63,000 daltons) that are greatly enriched in the rough endoplasmic reticulum. Several lines of evidence suggest that these proteins interact with ribosomes. Most persuasive is the finding that these proteins can be cross-linked to ribosomes (111). The proteins, termed ribophorins, may play a role in ribosome binding to the rough endoplasmic reticulum.

The Signal Hypothesis and Membrane Proteins

The signal hypothesis was proposed originally to explain the initial steps of protein secretion. Several lines of evidence demonstrate that many integral membrane proteins that span the lipid bilayer, and contain a hydrophilic domain(s) exposed on both sides, are also routed through the Palade pathway. For example, yeast mutants, which are defective in protein secretion, also appear to be defective in the incorporation of certain proteins into the cytoplasmic membrane (143). Are these membrane proteins exported by a different mechanism from that for other secreted proteins? The available evidence indicates that the answer to this question is no. Rather, the mechanisms seem to be variations of a common theme. Many membrane proteins behave like incompletely secreted proteins. Perhaps the most thoroughly studied example of such a protein is the glycoprotein (G) of vesicular stomatitis virus (VSV) (105, 106, 118, 167; for review, see reference 122). This protein is oriented in the membrane with its glycosylated NH_2-terminal end facing out and a small portion of its COOH-terminal end facing the cytoplasm. The protein is synthesized on membrane-bound ribosomes in a manner analogous to secreted proteins except that the completed protein is never released into the lumen of the organelle. It remains anchored in the membrane by a sequence of 20 to 25 hydrophobic amino acids located near the COOH-terminal end of the molecule. This stretch of hydrophobic amino acids has been given the descriptive term stop transfer sequence (10). Thus, by simply proposing the existence of a second export signal located downstream from the signal sequence in the primary structure of the protein, the signal hypothesis can be adapted to explain the insertion of the G protein into the membrane (Fig. 1).

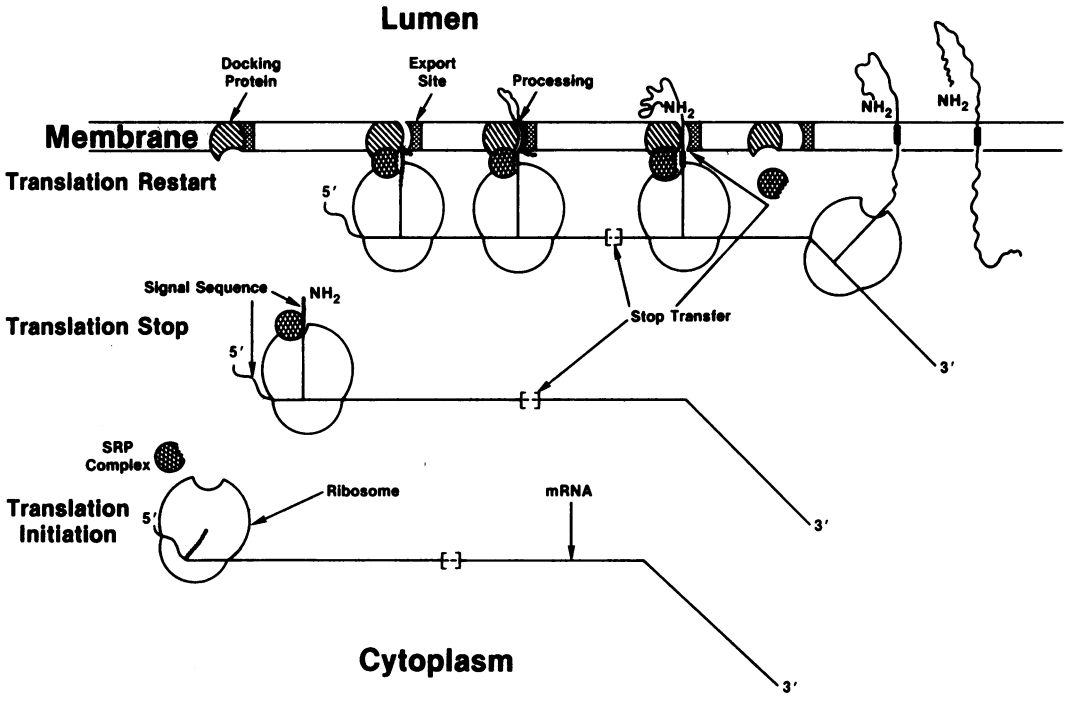

FIG. 1. Schematic illustration of cotranslational export. The export process begins at the bottom of the illustration: the ribosome assembles and initiates translation of the mRNA at the 5′ end. The signal sequence (represented by the wavy line in the mRNA or by small open circles in the protein) emerges from the ribosome and is recognized by the SRP complex, which interacts with the ribosome and the nascent polypeptide chain, stopping translation (middle illustration). This translational block is relieved when the complex (ribosome, SRP, and nascent polypeptide chain) interacts with the membrane-associated docking protein at the export site (top illustration). The signal sequence is composed of two segments, an NH_2-terminal charged segment and a hydrophobic segment. During the initial stages of polysome binding to the export sites, the positively charged segment interacts with either the inner leaflet of the membrane bilayer or a component of the export site. The hydrophobic segment then loops into the bilayer (89) and a functional export site is formed. As translation proceeds, the nascent chain is transferred vectorially across the membrane bilayer. Proteolytic processing of the signal sequence from the polypeptide chain is achieved by a peptidase activity located at the outer face of the membrane. Such processing may occur before synthesis of the protein is complete. The model shows the existence of a second information signal (stop transfer) located within the protein. As this signal emerges from the ribosome, it results in a dissociation of the ribosome from the membrane and release of the SRP complex. Subsequent translation of the mRNA completes the COOH-terminal end of the protein in the cytoplasm, leaving the protein embedded in a transmembrane fashion with the NH_2 terminus facing the lumen and the COOH terminus facing the cytoplasm.

Experimental evidence to support the existence of a stop transfer sequence comes from the study of the μ-chain of IgM. This protein can be found in two forms; one is secreted and the other is membrane bound with an orientation analogous to the G protein. The membrane-bound form is larger than the secreted form, the difference lying at the COOH-terminal portion of the molecule: the secreted form has a 20-amino acid, hydrophilic COOH terminus, whereas the membrane-bound form has a 41-amino acid COOH terminus containing a hydrophobic membrane anchor. Both forms are specified by the same structural gene. The different COOH-terminal portions are the result of different mRNA splicing events (3, 47, 107, 164). In the context of the model of membrane protein insertion described above, we can consider the secreted form of the μ-chain as being deleted for the stop transfer sequence. Similar results have been obtained with the gene coding for the hemagglutinin of the influenza virus. Deletions constructed in vitro by use of techniques of recombinant DNA that remove the COOH-terminal stop transfer sequence cause secretion of the truncated protein into the growth medium (65, 201). Thus, membrane insertion simply requires the presence of the second export signal. Comparison of the

presumptive stop transfer signals, i.e., a membrane-spanning sequence present in many membrane proteins, does not really provide any clue to their molecular function. Moreover, since most membrane-spanning sequences are followed by a stretch of highly charged amino acids (171), it remains to be shown whether the signal encompasses the hydrophobic region, the charged region, or both.

In terms of topology, the G protein, IgM μ-chain, and the influenza hemagglutinin are simple. They span the membrane once with their NH_2 terminus facing out. This topology is clearly not typical of membrane proteins in general. For example, the erythrocyte anion transport protein (band III) is oriented in the membrane in the following manner: a large (~40,000-dalton) NH_2-terminal hydrophilic portion faces the cytoplasm (63, 194, 195). The membrane-bound domain is located at the COOH terminus, and this domain spans the membrane at least twice (45, 160, 195, 217). The protein is glycosylated at a site in the COOH-terminal domain (44, 46, 206). Since the enzymes of protein glycosylation are located within the organelles of the Palade pathway, we must assume that this protein is localized via an intracellular route that is analogous to secreted proteins, VSV G protein, IgM μ-chains, and the influenza hemagglutinin. Furthermore, the kinetics of band III secretion and export are similar to that exhibited by the G protein, and the protein apparently is inserted into the membrane by a cotranslational mechanism (18, 20). Can the signal hypothesis account for the topology of band III? The answer is yes; however, two further assumptions must be made. First, a signal sequence must be able to function from an internal location within the primary structure of a protein. Second, a new export signal, termed insertion sequence (10), must be proposed.

Conflicting evidence has been presented concerning the function of an internal signal sequence. Much of this evidence relates to ovalbumin, a protein which is unusual in terms of protein localization. This egg white protein is synthesized and secreted in typical fashion. However, it is not synthesized in precursor form; i.e., it does not contain a signal sequence that is removed by signal peptidase (154). This result has caused a considerable brouhaha. Does this exception invalidate the signal hypothesis? The debate was seemingly quelled when Blobel and co-workers presented data indicating that ovalbumin contains an internal signal sequence. First, they showed that ovalbumin can compete with preprolactin (a protein containing a typical signal sequence) for an essential export component (120). Next they showed that an internal proteolytic fragment of ovalbumin could also compete (119). These results seemed to indicate an internal signal sequence. However, the last result remains controversial and might be an artifact caused by the extremely high concentrations of fragment required to observe competition. By synchronizing translation and determining the minimum number of amino acids that must be synthesized to allow export to initiate, other investigators have shown that the signal sequence must lie near the NH_2 terminus (20, 126; R. L. Meek, K. A. Walsh and R. Palmiter, Fed. Proc. **39**:1867, 1980). The controversy continues. Talmadge et al. (202) have placed 18 extra amino acids at the NH_2-terminal end of the signal sequence of preproinsulin without affecting export or processing. This seems to support the concept of an internal signal sequence. (Although this study was performed with bacteria, the result is germane; see below.) On the other hand, evidence obtained from the study of Sindbis virus envelope glycoproteins supports the opposite conclusion. These glycoproteins are initially part of a precursor polyprotein. The NH_2 terminus of the polyprotein is the soluble capsid protein. Immediately following is the glycoprotein, p62. The p62 protein is localized after proteolytic removal of the capsid protein by a mechanism analogous to the mechanism that localizes the VSV G protein. The p62 protein resembles ovalbumin, however, in that the signal sequence is not removed (16). Wirth et al. (218) have isolated a temperature-sensitive mutant in which proteolytic removal of the capsid protein from the polyprotein is prevented at the nonpermissive temperature. Under these conditions the p62 signal sequence does not function. This result implies that internal signal sequences cannot be recognized by the export machinery. Thus, the question of internal signal sequences remains open. If they exist, they may not resemble NH_2-terminal signal sequences. The resolution of these questions awaits mutant analysis.

In addition to the signal sequence and the stop transfer sequence, a third signal directing membrane protein topology has been proposed and termed insertion sequence. Such a signal is thought to cause spontaneous membrane insertion, i.e., without the aid of export machinery (10). Such sequences are modeled after the hydrophobic COOH-terminal segment of cytochrome b_5 (151). This protein is synthesized on free polysomes. When the hydrophobic sequence emerges from the ribosomes, it partitions spontaneously into the membrane (Y. Okada, D. Sabatini, and G. Kreibich, J. Cell Biol. **83**:437, 1979). Note that an insertion sequence does not drive protein segments through the membrane; rather, it causes insertion into the membrane. A functioning internal insertion sequence has not been documented.

If these three types of export signal exist (i.e., signal sequences, insertion sequences, and stop transfer sequences) and if these sequences can function from any location within a protein, then the signal hypothesis can account for every conceivable membrane protein topology. All that is required is to arrange these signals in the correct linear order starting at the NH_2 terminus (10, 171).

Intracellular Protein Traffic

To this point our discussions of mechanisms of protein export have focused on the initial steps in the localization process. What about subsequent steps? How are proteins transported from the rough endoplasmic reticulum through the other organelles of the Palade pathway? It is generally assumed that the organelles of the Palade pathway are not physically connected but communicate with each other by forming vesicles that bud from one organelle and fuse with another (153). It is important to note that this process will not alter the topology of membrane proteins. When a protein is inserted into the rough endoplasmic reticular membrane with its COOH terminus facing the cytoplasm, movement through the various organelles by this budding-fusion process will maintain the cytoplasmic location of the COOH terminus. If the protein is destined for the cytoplasmic membrane, the NH_2 terminus will end up facing out. In other words, the lumen of the endoplasmic reticulum is topologically equivalent to the growth medium.

The mechanism of this budding-fusion process remains somewhat mysterious. The discovery of clathrin, a 180,000-dalton protein that can form a regular basket and encapsulate a membrane vesicle (156), made clathrin-coated vesicles a likely candidate for the presumed transport vehicle. Evidence indicating that the VSV G protein is transported between organelles within clathrin-coated vesicles has been presented by Rothman and Fine (166). How the vesicles bud and fuse and why transport through the pathway is unidirectional are important questions that cannot yet be answered.

Sorting Mechanisms

Proteins destined for different cellular locations are routed through the rough endoplasmic reticulum. What are the mechanisms that sort these proteins to the proper cellular compartment? All of the export signals described so far are involved in getting proteins into or through the endoplasmic reticular membrane. These export signals probably will be shared by all proteins that are localized in this manner. Accordingly, yet another set of export signals, i.e., sorting signals, must be proposed.

Proteins that are secreted or exposed at the cell surface, as opposed to cytoplasmic proteins, are glycosylated. It seemed reasonable, therefore, to propose that sorting signals are contained in the carbohydrate portion of the glycoprotein. Evidence to support this proposal comes from the study of lysosomal enzymes. Neufeld and co-workers recognized that cells from patients with I-cell disease secrete lysosomal hydrolases. The enzymes were active; however, they localized incorrectly, leading to the conclusion that the misdirected hydrolases lacked the correct sorting signal (80, 173). Sly and co-workers identified the missing signal as phosphomannosyl residues (58, 104). Furthermore, hydrolases from patients with I-cell disease have been shown to lack these residues (76, 77).

However, not all sorting signals can be present in carbohydrate moieties. First, not all secreted proteins are glycosylated, e.g., albumin, and second, tunicamycin, a drug that blocks glycosylation, does not prevent secretion of several different proteins nor does it prevent protein export to the yeast vacuole (196, 197) nor does it block export of the VSV G protein (66). Tunicamycin does, however, affect the localization of many proteins (121). It may do so simply by altering protein conformation. Interestingly, mutations that prevent localization but not synthesis (9, 136, 223) are relatively common (compare with similar mutations in procaryotic systems; see below). In all cases except for a mutation in the VSV G protein, the mutant protein accumulates in the endoplasmic reticulum, possibly because the sorting signals are not contained in the primary linear sequence but rather in conformational or tertiary sequences. Thus, any mutation or any treatment such as tunicamycin that alters conformation may prevent movement from the endoplasmic reticulum. If true, the effect will complicate genetic analysis of sorting sequences. In the case of the mutant VSV G protein, accumulation occurs in the Golgi. This mutant protein is different from the wild type in that it does not contain covalently attached fatty acids (223). This finding raises the possibility that novel posttranslational modifications may be involved in sorting.

Posttranslational Import: a Second Mechanism

Not all proteins that are localized to intracellular organelles are routed through the Palade pathway. Proteins specified by nuclear DNA destined for localization in mitochondria, or chloroplasts in plants, and proteins destined for peroxisomes are localized by a mechanism different from that seen with the rough endoplasmic reticulum. This was first demonstrated with the chloroplast protein ribulose-1,5-bis-phosphate carboxylase (29, 43, 82). These investiga-

tors showed that the protein is synthesized in precursor form by soluble ribosomes. It is posttranslationally taken up by chloroplasts, and the precursor is processed proteolytically during or immediately after import. The precursor contains an NH_2-terminal peptide extension of 44 amino acids that presumably functions as the uptake signal (179). Similar results have now been obtained for many different chloroplast and mitochondrial proteins (for review, see references 112, 139, 175).

The mitochondrion contains four distinct cellular compartments: the aqueous interior or matrix, an inner membrane, an outer membrane, and an intermembrane space. Generally, proteins destined for the matrix or the inner membrane are synthesized initially in larger precursor form with a peptide extension at the NH_2 terminus. (There are, of course, exceptions; see reference 224.) This extension, which presumably contains export information, is usually much longer than the signal sequence of proteins routed through the endoplasmic reticulum (139). The precursor molecule binds to the exterior surface, i.e., cytoplasmic face, of the outer mitochondrial membrane (79) and is then translocated to the correct cellular location by a mechanism that utilizes energy provided by the electrochemical potential existing across the inner mitochondrial membrane (64, 178). During the transport process, the precursor is processed by a protease that is located in the matrix (15, 125, 135).

Apparently, protein import to the mitochondrial intermembrane space can occur by several mechanisms. Cytochrome b_2, for example, is first made in a larger precursor form which is taken up by mitochondria via a mechanism similar to that described above for inner membrane proteins. Cleavage by the matrix processing enzyme yields an intermediate form, located in the inner membrane. This intermediate is then cleaved by a second processing enzyme to release the final soluble product into the intermembrane space (39, 64). On the other hand, cytochrome *c* is not made in precursor form (225, 226); its import does not require an energized inner membrane, and the information for localization to the intermembrane space lies in the COOH-terminal third of the apoprotein (124). To complete the picture, localization of proteins to the outer mitochondrial membrane appears to occur by a mechanism similar to that described for cytochrome *c*; i.e., it occurs without requiring either processing or energy (62).

Posttranslational import of proteins into the mitochondrial compartments has been convincingly demonstrated both in vivo and in vitro. There is little doubt that import into this organelle can occur posttranslationally. A straightforward prediction of this posttranslational mechanism is that a pool of mitochondrial protein precursors should be detectable in the cytoplasm. This seems to be the case in *Neurospora crassa* (75); however, identification of such a pool has proven to be difficult in yeast because precursor pools can only be clearly shown with *rho*$^-$ cells (contain a large deletion of mitochondrial DNA rendering them nonfunctional) in the presence of the energy uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (163). Apparently, in yeast, proteins are imported into the mitochondria as fast as they are synthesized. The question arises, Is posttranslational import obligatory? Since many mitochondrial proteins, especially those destined for the matrix or the inner membrane, are synthesized initially with an NH_2-terminal peptide extension that presumably contains import information, the possibility of cotranslational import certainly exists. Indeed, numerous investigators have reported the presence of cytoplasmic ribosomes bound to the mitochondrial surface (2, 199). These results are probably not artifactual since the bound polysomes are enriched for mRNAs specifying mitochondrial proteins. Rather, these ribosomes probably are synthesizing proteins that are imported cotranslationally into mitochondria.

The results described above have fueled a controversy: Can protein import into mitochondria occur cotranslationally? As is discussed in a following section, a similar controversy enshrouds protein export in bacteria. These questions may well be moot.

To illustrate this point, let us consider the extreme examples of obligatory co- and posttranslational mechanisms of protein localization. If a protein is localized by an obligatory posttranslational mechanism, it follows that the entire protein or precursor is required for recognition by the export machinery. This could occur, for example, if the recognition signal was a COOH-terminal sequence or some feature of the three-dimensional protein structure that resulted from the folding of the complete polypeptide. Any mutation or treatment that prematurely stops synthesis would be predicted to prevent formation of the signal and thus export initiation. On the other hand, if a protein is localized by an obligatory cotranslational mechanism, the signals that initiate export must be present in an incomplete polypeptide chain and thus be located at or near the NH_2 terminus. In this case, mutations or treatments that prematurely stop synthesis would not be expected to prevent export. Furthermore, if synthesis is completed before export begins, then the protein or precursor may assume a conformation that buries the export signals, thus blocking localization. For protein export via the Palade pathway, the

mechanism involving SRP would specifically prevent this from occurring by halting translation until export begins (Fig. 1).

However, many proteins probably fall between these extremes and can be exported by either co- or posttranslational mechanisms. For instance, a protein normally exported by a cotranslational mechanism may assume, after synthesis, a conformation in which the export signals are still accessible, and thus export could still occur. Conversely, a protein normally exported by a posttranslational mechanism may contain export signals at the NH_2 terminus, and thus export could initiate before synthesis is complete. Since the experimental conditions imposed by the investigator on the physiological state of the living cell can affect the relative rates of protein synthesis and export, the export of a particular protein may occur either during or after synthesis, depending on the conditions. The machinery responsible for protein import into mitochondria, for example, may well be able to do both. In this view, only proteins with the structural constraints described in the preceding paragraph would show an absolute bias toward a particular export pathway.

PROTEIN LOCALIZATION IN *E. COLI*

E. coli is a primitive organism when compared with an animal cell. Basically, this bacterium is cytoplasm surrounded by three concentric envelope layers: an inner (cytoplasmic) membrane, a peptidoglycan cell wall, and an outer membrane. These layers define three noncytoplasmic compartments: the inner and outer membranes and an aqueous space between these two membranes called the periplasm. Intracellular organelles are absent. Considering that organelles play critical roles in protein export in eucaryotic cells, the proposal that *E. coli* and animal cells use similar mechanisms of protein localization may seem far-fetched. However, experimental evidence supports this claim. For example, intragenic information specifying export and processing in a eucaryotic gene (ovalbumin, insulin) can be recognized by *E. coli* and vice versa (alkaline phosphatase, PhoA; β-lactamase, Bla) (60, 137, 165, 203).

For the most part, studies of protein localization in *E. coli* have focused on a relatively small number of key proteins. The relevant characteristics and properties of these proteins are summarized in Table 1. Many of these proteins are known by several different names. To avoid confusion, we will use a similar abbreviation for both the protein and the structural gene; e.g., the *lamB* gene codes for the LamB protein.

Subcellular Fractionation

Reliable methods of subcellular fractionation are required for the analysis of bacterial protein localization. Since many of the experimental results described below are based on cellular fractionation, we will briefly describe the commonly used methods and their limitations.

TABLE 1. Commonly studied exported protein in *E. coli*[a]

Protein	Structural gene	Regulation	Mutant phenotype	Cellular location	Topology
LamB (λ receptor)	*lamB*	Maltose inducible	λ^r Dex^-	Outer membrane	Transmembrane COOH terminus facing out
OmpA (3a,II)	*ompA*	Constitutive	$K3^r$ $TuII^{*r}$ Col^r Con^- $EDTA^s$	Outer membrane	Transmembrane COOH terminus facing in
Lpp (lipoprotein)	*lpp*	Constitutive	Resistance to globomycin	Outer membrane	Integral COOH terminus facing in
MalE (maltose-binding protein)	*malE*	Maltose inducible	Mal^-	Periplasm	
PhoA (alkaline phosphatase)	*phoA*	Phosphate repressible	XP[b]	Periplasm	
Bla (TEM-1 β-lactamase)	*bla*	Constitutive	Ap^s	Periplasm	
fl or M13 major coat	Gene VIII		Defective phage	Inner membrane	Transmembrane COOH terminus facing in
fl or M13 minor coat	Gene III		Defective phage	Inner membrane	Transmembrane COOH terminus facing in

[a] This compilation was obtained from many different sources. For recent reviews, see references 42, 73, 83, 129, 150. Phenotypes are abbreviated as follows: Dex, growth on maltodextrin; ColL, colicin L; Con, conjugation; Mal, growth on maltose; Ap, ampicillin; r, resistance; s, sensitive.

[b] Enzyme activity can be detected with the sensitive indicator bromo-chloro-indolylphosphate (XP).

Centrifugation allows the division of a bacterial lysate into two fractions, a pellet which is composed of the bacterial cell envelope containing the inner membrane, outer membrane, and cell wall components and a supernatant containing cytoplasmic and periplasmic components. The degree of contamination of each fraction with the other is relatively low if the lysate is subjected to high centrifugal forces, i.e., 100,000 × *g*.

Several methods for separating the two bacterial membranes are used (31, 59, 96, 103, 148, 149, 180). In general, cells are converted to spheroplasts by treatment with lysozyme or lysozyme-EDTA and then lysed by osmotic shock, sonication, French press, or detergent. The membranes are collected by centrifugation (100,000 × *g*), washed, and then separated into inner and outer membrane fractions by isopycnic centrifugation in a sucrose gradient (149), by electrophoresis (103), or by selective detergent solubilization (57, 181).

Once cells are broken, cytoplasmic components cannot be distinguished from periplasmic components because both are soluble. To separate the components of these two cellular compartments, one must remove or alter the outer membrane and cell wall without disrupting the inner membrane. Usually this is accomplished by spheroplasting or controlled osmotic shock (138). These methods release periplasmic components, whereas cytoplasmic components remain associated with the cell.

All of these methods rely on a controlled disruption of the bacterial cell to yield fractions enriched for a specific cellular compartment. The conditions for this type of cellular fractionation have been empirically determined. Often these procedures do not work properly when applied to an altered bacterial system. Consequently, one must be cautious about drawing conclusions concerning the location of a particular protein whenever the system is perturbed by unusual environmental conditions, mutation, or adverse treatments such as irradiation or phage infections (32, 95, 109).

To avoid fractionation artifacts, several techniques have been devised that exploit the selective permeability of the various envelope layers. Since the outer membrane is impermeable to large water-soluble molecules such as proteases, antibodies, and bacteriophages, these agents can be used in conjunction with fluorescence or electron microscopy to localize proteins to the cell surface (8, 169). Moreover, by selectively disrupting the outer membrane permeability barrier with EDTA, many of these methods can be expanded to include all proteins that are localized outside the cytoplasm (95). Finally, some chemical agents can cross the outer but not the inner membrane. These agents have been used to selectively label proteins exposed outside of the cytoplasm (192). Because these methods cause little or no cell damage, the possibility of experimentally induced artifact is greatly diminished.

Membrane-Bound Ribosomes

The success of Palade and co-workers in demonstrating the involvement of membrane-bound ribosomes in protein localization in eucaryotic cells prompted many similar studies with *E. coli*. For years, the results of these studies were controversial because membrane-bound and free polysomes could not be distinguished adequately by electron microscopy of thin sections of *E. coli*. The cytoplasmic population of ribosomes is too dense. This high concentration of ribosomes also confuses experiments based on biochemical fractionation. Membrane fractions always contain ribosomes; however, their presence may be the result of artificial trapping (40).

A functional attachment of ribosomes to *E. coli* membranes was first suggested by the experiments of Cancedda and Schlessinger (24). These authors showed that a periplasmic enzyme (Bla) is synthesized preferentially by membrane-bound ribosomes relative to a cytoplasmic enzyme, β-galactosidase. These results were strengthened and extended by Randall and Hardy (159), who provided convincing evidence that membrane-bound ribosomes were involved in the synthesis of both periplasmic (e.g., MalE) and outer membrane (e.g., LamB) proteins.

The most convincing evidence to support the involvement of membrane-bound ribosomes in protein export comes from the work of Smith et al. (192). These investigators designed an elegant experiment to demonstrate directly that the NH_2-terminal end of a growing polypeptide chain protrudes through and is exposed at the outer face of the cytoplasmic membrane. Using spheroplasts, they showed that nascent chains of the periplasmic enzyme PhoA could be labeled with reagents that do not penetrate the cytoplasmic membrane.

Recently, Smith (190) developed an in vitro system for protein export that is analogous to the one developed for eucaryotic cells by Blobel and Dobberstein (11, 12). Smith's system utilizes inside-out inner membrane vesicles. He has shown that, if these inverted vesicles are treated with protease, they lose their ability to transport and process exported proteins. This experiment suggests the presence of an inner membrane protein(s), exposed on the cytoplasmic face of the membrane, that is required for protein export. Presumably this protein(s) is a component of the cellular protein export machinery.

All of the experimental results described in this section conform to predictions of the signal hypothesis. Proteins destined for export to the periplasm or the outer membrane are synthesized by membrane-bound ribosomes and export occurs by a cotranslational mechanism. The cytoplasmic membrane of *E. coli* is functionally analogous to the rough endoplasmic reticulum with respect to protein localization.

Precursors of Exported Proteins

In general, four methods have been used in bacterial systems to demonstrate precursor forms of exported proteins. First, in some cases, proteolytic removal of the signal sequence occurs posttranslationally, i.e., after synthesis of the protein is complete (Bla) (108). Some proteins are processed both co- and posttranslationally (MalE, LamB) (101, 102). For these proteins, precursor can be detected in whole cells by pulse-labeling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, other proteins are processed before synthesis is complete. Thus, the precursor form cannot be detected in whole cells (*ampC*-encoded β-lactamase) (102).

The second and perhaps the most reliable method for detecting a precursor form is to use an in vitro protein-synthesizing system. If a transducing phage or a plasmid containing the cloned structural gene is available, a DNA-dependent system can be used (PhoA) (88). Alternatively, membrane-bound polysomes can be isolated and the translation products can be examined (MalE, LamB) (102).

A third method for detecting precursors involves the use of mini- or maxicells. Minicells are *E. coli* mutants (*minA*) defective in cell division. At some frequency, cell division occurs asymmetrically, causing the formation of small cells that do not contain a chromosome (61). If the mutant strain contains a multicopy plasmid, such as pBR322, the defective cell division will form minicells that contain plasmids but no chromosome. Thus only plasmid-encoded proteins will be synthesized. Maxicells are *recA* strains that have been irradiated by UV. The damage caused by irradiation cannot be repaired in *recA* strains and the chromosome is destroyed (172). Since plasmids such as pBR322 are small and are present at high copy number, many escape damage and destruction. Consequently, only plasmid-encoded proteins are synthesized. Defective processing is generally observed with both mini- and maxicells. For example, Bla, which is specified by a gene on pBR322, accumulates in precursor form in both systems. Similarly, the precursor form can be detected in UV-irradiated cells infected with transducing phages (108). Although the reasons for precursor accumulation with these systems are not clear, the likelihood is that protein synthesis proceeds at a rate exceeding the export capacity of these defective or damaged cells.

A final method for detecting precursors uses one of a variety of different inhibitors. Agents that alter membrane fluidity, such as phenylethyl alcohol (69) or procaine (115), or growth of a fatty acid auxotroph in the presence of eliadate (41, 152) can cause precursor accumulation. The mechanism by which this occurs is unknown. However, these agents do not cause general precursor accumulation. Differential sensitivities are often observed. In a similar vein, precursors can be detected in cells treated with energy uncouplers such as carbonyl cyanide *m*-chlorophenylhydrazone or dinitrophenol (33, 36, 38, 155) or, more significantly, in mutant cells defective in ATPase and deprived of oxygen (56). Unlike membrane perturbants, energy deprivation appears to cause a general defect in protein export. Although these results indicate that proton motive force is required, they offer no insight into the nature of this requirement.

Numerous precursors for periplasmic and outer membrane proteins have been identified, and many of them have been submitted to DNA or amino acid sequence analysis or both. In all cases, the precursor contains a peptide extension at the NH_2-terminal end, i.e., a signal sequence (for review, see reference 129). Indeed, despite reports to the contrary (1), all characterized periplasmic and outer membrane proteins contain an NH_2-terminal signal sequence that is removed during the export process.

In contrast, inner membrane proteins present a more complicated picture with respect to NH_2-terminal signal sequences. The major and minor fl (M13) coat proteins, which are transiently found in the inner membrane, are synthesized initially in precursor form with a typical signal sequence (174, 198). In addition, two inner membrane penicillin-binding proteins, PB5 and PB6, (158), two inner membrane lipoproteins (86), and an inner membrane protein, Tsr, that is a component of the serine chemoreceptor (17) are apparently made in precursor form as well. However, the lactose permease (LacY [48]), the histidine permease (HisP [81]), and two subunits (*b* and *c*) of the F_0 complex of ATPase (140) are not made in precursor form. These proteins may contain a signal sequence which is not removed. A second possibility is that they may not be cotranslationally inserted into the membrane. These proteins are highly hydrophobic, and they may simply partition into the membrane after synthesis is complete. Viewed in terms of the signal hypothesis, these proteins may contain

insertion sequences rather than signal sequences. Remember, insertion sequences cannot drive hydrophilic protein domains through the membrane; they result simply in insertion. Thus, the signal hypothesis predicts that these proteins, even though they may span the bilayer, will not contain a large hydrophilic domain on the outside face of the membrane. A third possibility is that these proteins are exported by a different mechanism. Clearly more work is required to answer these questions.

Signal Sequence Processing

An enzymatic activity that correctly processes the f1 (M13) major coat protein has been identified and localized to the membrane fraction of *E. coli* (26, 123). This enzymatic activity, termed leader (signal) peptidase, has been purified and shown to correctly process the precursor coat protein (228). The endopeptidase also processes the precursor forms of several other exported proteins in vitro (220, 227). By screening a library of cloned *E. coli* DNA, Date and Wickner (37) identified a plasmid that causes a marked overproduction of leader peptidase. With the use of techniques of recombinant DNA, a plasmid has been constructed in which the cloned gene has been placed under the control of a bacteriophage λ promoter, P_r. Strains containing this plasmid overproduce leader peptidase 75-fold. Purification of the enzyme activity yields a protein of 37,000 daltons (220).

DNA sequence analysis indicates that the gene specifying leader peptidase is located in an operon downstream from an open reading frame that would specify a protein of 72,000 molecular weight. Preliminary genetic analysis indicates that this operon maps at 55 min on the *E. coli* chromosome. Moreover, it appears that the gene specifying leader peptidase is essential (35). Although the function of the 72,000-dalton protein is not known, it seems reasonable to propose that this protein is a component of the export machinery as well. The availability of a plasmid containing these structural genes should permit the isolation of mutants, and we can anticipate that this question will soon be answered.

The precise cellular location of leader peptidase is not clear. Genetic studies described below demonstrate that the processing activity is located outside of the cytoplasmic membrane. There is general agreement that the enzyme is bound to the membrane. However, one group has reported that the enzyme is localized to both the inner and outer membranes (123). Since it is unusual for an exported protein to be found in more than one cellular location, these results should be classified as tentative until more convincing fractionation data are presented.

Posttranslational Modifications

As described above, most eucaryotic proteins that are exported from the cytoplasm through the Palade pathway are glycoproteins. As such, they are extensively modified. *E. coli* produces no glycoproteins. Nevertheless, a variety of posttranslational modifications have been described. Perhaps the most extensively modified protein in *E. coli* is the outer membrane protein, lipoprotein (Lpp).

The complete covalent structure of mature Lpp was elucidated by Braun and co-workers (for review, see reference 21). The NH_2-terminal residue is cysteine. To this residue, a diglyceride is attached by thioether linkage and a fatty acid is attached by amide linkage. The COOH-terminal lysine residue is covalently linked by the ε-amino group to peptidoglycan to form the bound form of the protein. This form comprises ca. one-third of the total cellular amount of Lpp. Finally, Lpp, like other outer membrane proteins, is synthesized initially in precursor form with a typical signal sequence at the NH_2-terminal end (92). Clearly these modifications require a variety of modifying enzymes and a series of biochemical reactions.

Insight into the temporal order of the modification pathway came with the discovery of the antibiotic globomycin. This agent prevents proteolytic removal of the Lpp signal sequence. It does not, however, prevent formation of the thioetherdiglyceride (84). Recent evidence shows that signal sequence processing requires prior formation of Lpp diglyceride (85). Most exported proteins are processed without such a modification, and globomycin does not inhibit processing of most exported proteins. Indeed, precursor Lpp, either modified or not, is not processed by leader peptidase (204). Finally, Yamagata et al. (222) have isolated a mutation that exhibits a temperature-sensitive processing activity for Lpp but not for other proteins. The mutation lies outside the *lpp* structural gene. These data strongly suggest that *E. coli* contains more than a single signal sequence processing enzyme.

Lpp is not unique; *E. coli* produces several other proteins localized to the inner and outer membrane that are modified in a similar manner (86). Moreover, the membrane-bound form of penicillinase in *Bacillus licheniformis* is also modified, as described previously (114).

Posttranslational Export, the Membrane Trigger Hypothesis

Eucaryotic cells can use a posttranslational export mechanism for localization to certain organelles. In view of the pronounced similarity between procaryotic and eucaryotic organisms with respect to protein localization, finding that

E. coli also uses a posttranslational mechanism would not be surprising. Indeed, a considerable amount of experimental evidence has been presented to indicate that protein export in *E. coli* can occur posttranslationally. Much of this evidence comes from studies with the f1 (M13) major coat protein.

The major coat protein exists transiently in the inner membrane before its incorporation into phage heads. When present in the inner membrane, it assumes a topology similar to that of the VSV G protein: it spans the membrane once and its NH_2 terminus faces out (214). In almost all respects, this protein is the procaryotic equivalent of a eucaryotic viral glycoprotein.

The mechanism of coat protein export is controversial. Experimental evidence suggests both a cotranslational (26, 27) and a posttranslational (216) mechanism. Recently, Watts et al. (212) have reconstituted a purified preparation of leader peptidase into vesicles composed of *E. coli* phospholipids. They demonstrated that these vesicles could bind, process, and correctly insert radiochemically pure precursor coat protein (189) into the bilayer. These results indicate a posttranslational export mechanism. Furthermore, they suggest that leader peptidase is the only cellular component required for coat protein localization.

To account for the apparent posttranslational export mechanism of coat protein, Wickner (215) has proposed a model for protein localization, called the membrane trigger hypothesis, which is distinctly different from the signal hypothesis. According to this model, the signal sequence promotes the folding of the completed precursor into a soluble export-competent conformation. Exposure to a hydrophobic environment triggers a conformational change in the protein that allows spontaneous membrane insertion. Cleavage of the signal sequence would drive the reaction and make it irreversible. In a recent review, Wickner (216) has applied this model to the export of numerous other proteins.

Further support for a posttranslational export mechanism has come from studies with Bla. As stated earlier, this protein is processed posttranslationally. Using a transducing phage and UV-irradiated cells of *Salmonella typhimurium*, Koshland and Botstein (108) presented data to suggest that the complete precursor protein is synthesized in the cytoplasm before export from the cytoplasm.

Despite the evidence described above, many troubling issues concerning posttranslational export in bacteria remain unresolved. First, experimental demonstration of posttranslational export in vitro is not straightforward. With conventional in vitro systems in *E. coli*, coat protein synthesis cannot be stopped too long before addition of membrane vesicles, or export is not observed. Vesicles must be added within minutes to get efficient export. Indeed, Wickner and co-workers usually add vesicles during synthesis in most of their experiments (68, 212). This is in marked contrast to the posttranslational export observed in eucaryotic cells. Second, clear examples of cotranslational export in *E. coli* have been presented. The signal sequence of these proteins is quite similar to the signal sequences of coat protein and Bla (Fig. 2). This again is in contrast to results with eucaryotic cells. In this case, the cleaved posttranslational export signal is quite different from a signal sequence (179). It seems odd that signal sequences that are so similar would have different functions in different export mechanisms. Indeed, in the case of Bla, studies in a eucaryotic in vitro system showed that the protein is exported by an SRP-dependent cotranslational mechanism (137). It is curious that SRP would recognize a posttranslational export signal.

The essential feature of the membrane trigger hypothesis is protein conformation. The model requires that the protein be synthesized in complete form and that it assume a specific conformation in the cytoplasm before export. Therefore, any mutation that alters this specific conformation should be export defective. In the case of Bla, hundreds of mutations mapping throughout the gene have been isolated. Nevertheless, only those that alter the signal sequence prevent export from the cytoplasm (108, 110). Moreover, when the insulin signal sequence is replaced with the Bla signal sequence, processing and export of insulin to the periplasm still occur (203). A eucaryotic protein that is exported cotranslationally probably would not contain structural information that directs the correct conformation for posttranslational export in bacteria.

It is difficult to meld all of these data and propose a unifying model. One possible explanation is that export and synthesis are not as tightly coupled in procaryotic cells as in eucaryotic cells for export by the endoplasmic reticulum. Accordingly, a fraction of all exported proteins in bacteria may be exported posttranslationally by the cotranslational export machinery, perhaps in a manner analogous to mitochondrial protein import in eucaryotic cells. The fraction could vary from high to low depending on the particular protein. This possibility may be particularly relevant with in vitro systems or systems utilizing damaged cells since under these conditions a coupling factor that normally functions in vivo may be absent or defective. For Bla, this explanation seems reasonable; for coat protein, the experiments of Watts et al. (212), using purified components, are in conflict.

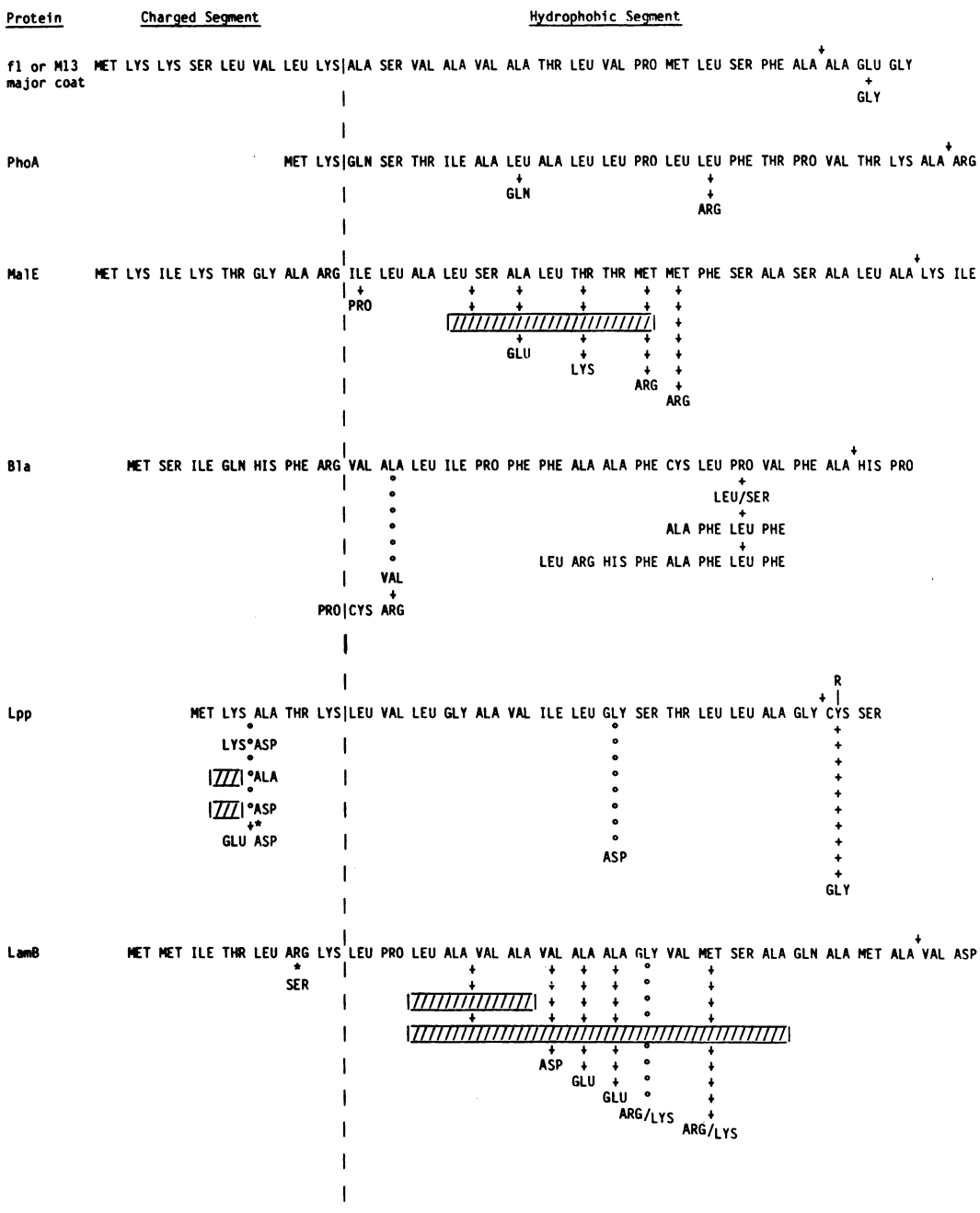

FIG. 2. The amino acids of the signal sequences for the proteins listed in Table 1 are shown. These include the major coat protein of f1 or M13 phage, which is located in the inner membrane (198); the periplasmic proteins PhoA (87), Bla (200), and MalE (6); and the outer membrane proteins Lpp (92) and LamB (78). The (↓) above each of the sequences indicates the processing site. The R group above the last cysteine residue of the Lpp sequence designates the thioetherdiglyceride which is present on the mature protein (21). Specific mutational changes within the signal sequences are shown below each of the wild-type sequences. Mutations that are designated by (↓) block localization of the protein, causing a null phenotype; mutations that are designated by (O) have a less pronounced effect. Deletions are shown by a box; mutations that prevent translation are designated by an asterisk. See the text for a complete description of the phenotypes conferred by each of these mutations.

Cotranslational export requires a cellular export machinery. Even if a protein is exported posttranslationally via this pathway, the export machinery would still be required. The experiments with coat protein suggest that no such machinery is required.

Although strongly suggestive, the experiments of Watts et al. (212) cannot be regarded as conclusive. Neither the preparation of leader peptidase nor that of precursor coat protein was chemically pure. The reconstituted vesicles contained demonstrable amounts of detergent and, in addition, correct insertion of the protein into the bilayer was inefficient. Finally, the coat protein is not a typical membrane protein; it exists in the membrane transiently. The protein also functions in the phage coat, where it almost certainly plays a role in phage infection. Accordingly, it, like its viral glycoprotein counterparts, may promote fusion of the virus with lipid bilayers (213). If the coat protein has such properties, in vitro studies may yield confusing results.

GENETIC ANALYSIS OF PROTEIN EXPORT

The most straightforward genetic approach to the study of protein localization is to isolate a number of export-defective mutants. Analysis of these mutations should reveal the nature and location of intragenic information specifying export, define cellular components of the export machinery, and shed light on their mechanistic roles. Although this approach is conceptually simple, it is technically difficult. The fundamental problem underlying this difficulty is that there is no phenotypic difference between a mutation that prevents export and a mutation that prevents synthesis. Both mutations will confer a null phenotype. In the absence of a selectable or scorable phenotype, one is faced with the necessity of using a brute-force biochemical screen. For example, one might screen a population of mutants that exhibit a null phenotype for those in which accumulation of precursor can be detected. However, besides being labor intensive and time-consuming, historically this approach has not been successful. With very few exceptions, none of the thousands of mutations that are known to cause a null phenotype for an exported protein affect the signal sequence or cause a block in export.

One of the most notable exceptions to this statement is Lpp. This protein, because of its abundance and its small size, is biochemically the best characterized membrane protein in *E. coli*. These studies, however, did not shed much light on its possible function. Accordingly, considerable effort was directed towards mutant isolation. Wu and Lin (221) used a "suicide" selection based on the exclusive biosynthesis of Lpp in the absence of three amino acids not found in the protein. One of the mutants obtained in this manner lacked the diglyceride modification, and consequently the signal sequence was not removed. Subsequent analysis revealed a missense mutation that changed glycine at position 14 of the signal sequence to aspartic acid (Fig. 2). Despite this alteration, protein export to the outer membrane still occurs (116). Why this selection yielded this particular mutation is not clear. Nevertheless, it represents the first example of a signal sequence mutation. These results demonstrate that none of the posttranslation processing reactions observed with Lpp, including removal of the signal sequence, are required for export to the outer membrane.

In the absence of a direct selection for export-defective mutants, a second approach, aimed at defining intragenic export components, has been taken. This approach utilizes a series of nonsense mutations located throughout the gene. This series of chain-terminating mutations produces a collection of truncated polypeptides of various lengths. By determining the cellular location of the truncated peptide with specific antisera, the site of intragenic export information can be defined. This approach has been applied with some success.

Two *malE* nonsense mutations have been studied in detail (95). One produces a peptide that is 90% complete. This protein is processed and exported to the periplasm as evidenced by the fact that it is released by cold osmotic shock. The second mutation produces a peptide one-third the length of wild-type MalE. This peptide is processed, but is not released by osmotic shock. It is, however, exported from the cytoplasm, as shown by finding that the fragment is sensitive to externally added trypsin. These results demonstrate that the COOH-terminal two-thirds of MalE is not required for either export from the cytoplasm or processing.

Koshland and Botstein (108) obtained similar results with Bla. They did not find any peptides that were released by osmotic shock, although all were processed. Originally this finding was interpreted to indicate that the peptides remained in the cytoplasm. However, subsequent experiments with externally added trypsin demonstrated that the peptides are outside of the cytoplasm (110). Here again, the COOH-terminal portion of the protein is not required for export.

The advent of recombinant DNA technology permitted the application of a related experimental approach that also results in the production of truncated peptides. Once the gene for an exported protein has been cloned, portions of the gene coding for the COOH-terminal end of

the protein can be removed by using restriction enzymes. This approach has been extensively applied with the outer membrane protein OmpA (22, 23). The mature protein contains 325 amino acids. Only the 193 amino acids at the NH_2-terminal end are required for export from the cytoplasm and for stable incorporation into the outer membrane. What is remarkable is the fact that these 193 amino acids are functional. This truncated peptide complements all of the *ompA* phenotypes except EDTA sensitivity (Table 1). In contrast, a peptide of 133 amino acids is nonfunctional and very unstable. The production of truncated proteins has also been used to study export of the fl gene III protein (13). Here again, the COOH-terminal portion of the protein was found not to be required for export from the cytoplasm. However, deletions were found that altered the cellular location of the truncated peptide. These results shed light on intragenic export information and are discussed further below.

The results with OmpA emphasize the problems associated with approaches that utilize nonsense fragments or COOH-terminal deletions. Both techniques suffer from the same limitations. First, truncated peptides are often very unstable, and second, short fragments are likely to be nonfunctional and may not cross-react with antisera. Although the results that have been obtained demonstrate that export information must lie at the NH_2-terminal end of the protein, the inherent limitations of this approach preclude precise genetic analysis.

Application of Gene Fusion Technology

A solution to the inherent problems associated with truncated peptides came with the application of gene fusion technology. This technique allows one to specifically label NH_2-terminal fragments of an envelope protein with a marker that is stable and simple to assay. Beckwith and co-workers exploited the sophistication of *lac* genetics and the unusual properties of the cytoplasmic enzyme β-galactosidase to develop techniques that allow fusion of *lacZ* (codes for β-galactosidase) to any gene in *E. coli* (25; for review, see T. J. Silhavy and J. Beckwith, Methods Enzymol., in press and G. M. Weinstock, M. L. Berman, and T. J. Silhavy, *in* T. S. Papas, M. Rosenberg, and J. Chirikjian, ed., *Expression of Cloned Genes in Procaryotic and Eucaryotic Vectors*, in press). Such fusions specify a hybrid protein composed of an NH_2-terminal sequence from the target gene product and a large, functional COOH-terminal portion of β-galactosidase (Fig. 3). By constructing a series of fusions differing only in the amount of target gene DNA contained in the hybrid gene and determining the cellular location of the hybrid protein that is produced, investigators have been able to more accurately define the location of intragenic export information.

Gene fusion technology has been applied extensively to study the localization of two periplasmic proteins, MalE (5) and PhoA (129, 130), and the outer membrane protein LamB (72, 186, 188). To a lesser extent, the technique has also been used to study export of the inner membrane protein MalF (184, 186, 187) and the outer membrane protein OmpF (73).

Results with gene fusions show a clear pattern. Fusions constructed so as to contain a substantial portion of a gene specifying a noncytoplasmic protein produce a hybrid protein that is exported, at least to some degree, from the cytoplasm. Conversely, fusions that contain only a small portion of a gene specifying an exported protein produce a hybrid protein that remains in the cytoplasm. This result demonstrates that export information is contained within the structural gene, and it indicates that the information must lie at a position corresponding to the NH_2-terminal end of the protein.

With the use of gene fusions, it has been shown that β-galactosidase can be exported to both an inner and an outer membrane location. However, it has not been possible to put β-galactosidase into the periplasm. Even fusions that contain nearly all of the gene coding for a periplasmic protein produce a hybrid protein that remains stuck in the cytoplasmic membrane. Apparently, β-galactosidase contains amino acid sequences that are incompatible with passage through the membrane. Attempted export of β-galactosidase to the periplasm jams export sites, causing the intracellular accumulation of precursors of other exported proteins (129, 186).

That β-galactosidase sequences can be exported to the inner or outer membrane but not to the periplasm is significant. It suggests that, although export of periplasmic and membrane proteins may be similar in the early stages, the export pathways diverge before completion. Genes that specify membrane proteins may contain additional export information (i.e., stop transfer sequences) that halts vectorial transfer before synthesis is complete (see Fig. 1), thus preventing β-galactosidase sequences from entering the membrane. If true, then one should be able to fuse the *lacZ* gene to a gene coding for membrane protein such that information specifying export initiation, but not stop transfer, is present. Such a fusion should produce a hybrid protein that is exported in a manner similar to that described for periplasmic proteins. In fact, studies have shown that *lamB-lacZ* fusions that contain ~60% (codes for the signal sequence plus 241 amino acids) of the *lamB* gene produce

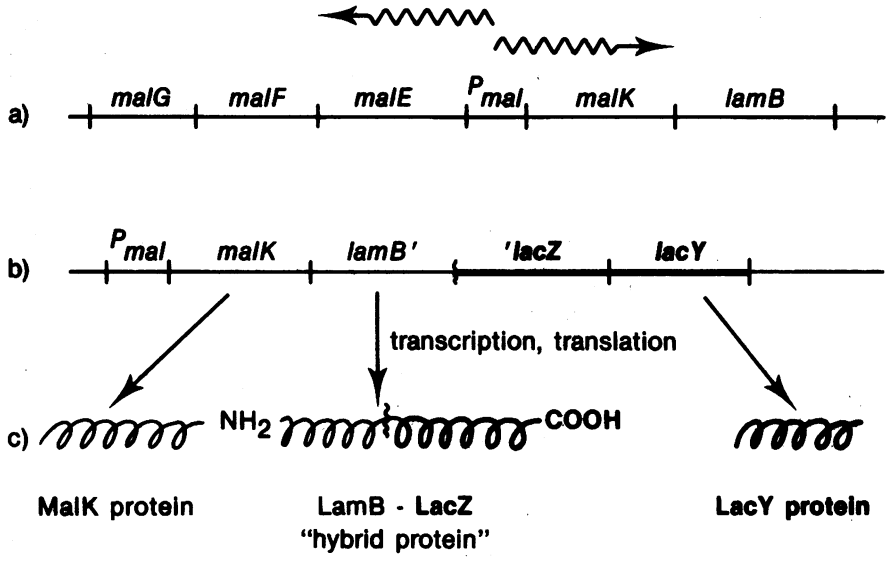

FIG. 3. (a) The two divergent operons that compose the *malB* locus in *E. coli* (83). These two operons specify five proteins, which together make up the active transport system for maltose and maltodextrins. Both operons, and thus the synthesis of all five proteins, are induced by maltose in the growth medium. At least three of these proteins are destined to be localized to noncytoplasmic compartments. The *malE* gene specifies the periplasmic protein MalE; *malF*, *malG*, and *malK* specify inner membrane proteins; and *lamB* specifies the outer membrane protein LamB. Transcription of these two operons is initiated from a central region (P_{mal}). Direction of transcription is shown by wavy arrows. (b) Genetic structure of a *lamB-lacZ* fusion. The fusion joint is designated by a short, vertical, wavy line. Transcription and subsequent translation of the operon shown in (b) results in the production of three proteins. (c) The MalK protein is required for maltose transport. The LacY protein is required for lactose transport. The structure of the LamB-LacZ hybrid protein is shown. The hybrid protein is composed of LamB sequences at the NH_2 terminus and a major functional portion of β-galactosidase at the COOH terminus. All of the hybrid proteins discussed here have essentially identical amounts of β-galactosidase sequences at the COOH terminus. For *E. coli* to express a Lac^+ phenotype, strains must have both LacZ (β-galactosidase) and LacY (lactose transport) protein activities.

a hybrid protein that is exported efficiently to the outer membrane, whereas fusions that contain ~40% (codes for the signal sequence plus 173 amino acids) of *lamB* jam the export machinery and cause precursor accumulation (50, 186).

A final point should be made concerning the function of the signal sequence. Fusions have been constructed that specify a hybrid protein that contains the complete LamB signal sequence plus 15 amino acids of mature LamB. Despite the presence of the signal sequence, the hybrid protein remains in the cytoplasm (134). This finding suggests that, at least for LamB, the signal sequence is not sufficient to cause export from the cytoplasm. Other export information located downstream in the *lamB* gene appears to be required.

Mutant Selections Based on *lacZ* Gene Fusions

The information gained from the biochemical analysis of fusion strains has provided certain insights into the mechanism(s) of protein localization. However, the most significant contribution of gene fusion technology stems from the unusual phenotypes often exhibited by fusion strains. These phenotypes are the consequence of the cell's attempt to export a hybrid protein containing sequences of β-galactosidase, and they can be exploited to isolate export-defective mutants. More importantly, because of the properties of *lacZ* fusions, a mechanism to distinguish mutations that block export from those that prevent synthesis is provided. Although the number of examples are not large, these phenotypes apparently will be exhibited generally.

One unusual phenotype is characteristic of fusion strains that produce a hybrid protein that is incorrectly or inefficiently exported. High-level synthesis of such hybrid proteins is lethal probably because these proteins jam the export machinery, preventing localization of other essential envelope proteins. In the case of *malE-lacZ* or *lamB-lacZ* fusions, this overproduction lethality is observed when maltose is added to the growth medium to induce high-level synthesis of the hybrid protein. Accordingly, such strains are sensitive to maltose (Mal^s). Mutations that relieve the Mal^s phenotype but do not

prevent synthesis of the hybrid protein (Lac^+) are export defective.

A second unusual phenotype is characteristic of fusion strains that produce a hybrid protein that is efficiently localized to a membrane. In such an environment, the β-galactosidase portion of the hybrid protein exhibits reduced enzymatic activity, probably because it cannot assume an active conformation. Accordingly, these fusion strains are unable to grow on lactose (Lac^-). By selecting Lac^+, mutations that prevent export of the hybrid protein can be obtained.

By the selection procedures described, a number of export-defective mutations have been isolated. Such mutations provide the basis for genetic analysis of protein export.

Mutations That Alter the Signal Sequence and Block Export

The selection procedures described in the previous section have been used to isolate mutations that alter the signal sequence of three genes (*lamB*, *malE*, *phoA*). All of these mutations prevent export of the respective hybrid protein. When recombined genetically into an otherwise wild-type gene, most also prevent export of the cognate protein, causing the precursor form to accumulate in the cytoplasm (4, 6, 52–54, 131). In addition to the mutations isolated by using gene fusions, signal sequence mutations have been constructed by in vitro mutagenesis (91, 109, 110). Taken together these mutations prove conclusively that the signal sequence is required for export. Since the mutations cause precursor accumulation in the cytoplasm, we conclude that the signal sequence must function in an early step in the export process. Indeed, the signal sequence apparently is required to initiate protein localization. Many signal sequence mutations, especially those constructed in vitro, cause only a partial export block (55, 91, 109). To distinguish these, we will arbitrarily classify export-defective mutations as those which block export more than 90% and which cause a null phenotype. Many of the signal sequence mutations have been characterized at the level of the DNA sequence. These mutations (Fig. 2) shed some light on the functions performed by the various molecular components of the signal sequence. Because our work deals primarily with the LamB signal sequence, we will use it as a focus for discussion. Like all signal sequences, the LamB signal sequence can be divided into two distinct domains: an NH_2-terminal hydrophilic segment and a central hydrophobic core that extends near to the site of processing. These two domains are generally separated by one or two basic amino acids, especially in procaryotic sequences (Fig. 2). Sequence comparison of all known signal sequences reveals no other striking homologies except for the presence of an amino acid with a small side chain (Ala, Ser, Gly, Cys) at the processing site.

The NH_2-terminal domain, excluding the basic amino acid residues, does not appear to play a critical role in export. Several lines of evidence support this claim. First, no export-defective mutations are known to lie in this region. Second, this sequence varies in composition and varies in size from as small as one (methionine, specified by the initiation codon) to as large as seven. Indeed, Talmadge et al. (202) have placed 18 extra amino acids in this region of the signal sequence of preproinsulin. Export from the cytoplasm of *E. coli* appears to occur normally.

The central hydrophobic core clearly plays an important role in the initiation of protein export. All of the export-defective *lamB* mutations alter this region of the signal sequence (52). Similarly, all export-defective *malE* (6), *phoA* (131), and *bla* (produced by in vitro mutagenesis) (109, 110) mutations alter this region as well. Since no sequence homology can be recognized between various signal sequences and since all export-defective *lamB* mutations, and nearly all others as well, result in the presence of a charged amino acid in this region, one could argue that this sequence functions simply because it is hydrophobic.

Although we do not doubt the importance of hydrophobicity, we believe that certain amino acid residues in this region play a more critical role in export initiation. In the LamB signal sequence, the presence of a charged residue at position 14, 15, 16, or 19 blocks export by >95%. However, a charged residue, either acidic or basic, at position 17 has essentially no effect on the export of LamB to the outer membrane (55). Thus, the position of the alteration, not the presence of a charge, determines the effect of the mutation.

We believe that the residues at positions 14, 15, 16, and 19 define an important recognition site. These four residues probably interact directly with a cellular component of the protein export machinery. If one of these residues is altered by mutation, this critical recognition cannot occur and the export process does not initiate. The result is the accumulation of precursor in the cytoplasm. The data we have obtained by genetic analysis of the LamB signal sequence are consistent with this proposal. All of the export-defective *lamB* mutations (14 base substitutions and 13 deletion mutations) alter one or remove one or more of these critical four residues. The point mutations that do not alter one of these residues do not block export (Fig. 2).

An apparent exception to the statement that

all export-defective *lamB* mutations alter at least one of these critical four residues is the small deletion mutation *lamBS78*. This 12-base pair deletion removes amino acids 10, 11, 12, and 13 from the LamB signal sequence. It blocks export by >95%. Although this deletion certainly does not alter one of the four critical amino acids directly, recent evidence (55a) indicates that the mutation alters the recognition site indirectly by altering the secondary conformation of residues 14, 15, and 16.

Rules for prediction of peptide secondary structure (28) have been used to determine that the hydrophobic core of the LamB signal sequence probably exists in an α-helical conformation (7). Since two amino acids in this core region, proline at position 9 and glycine at position 17, destabilize helical structures, the helix is predicted to terminate in the region of these two residues. According to these rules, none of the point mutations that alter the LamB signal sequence would alter this secondary structure. However, the small deletion mutation *lamBS78*, which removes residues 10, 11, 12, and 13, would alter the secondary structure because in the mutant signal sequence the helix-destabilizing residues, proline and glycine, are too close to each other (three residues apart instead of seven as in the wild-type sequence) to permit a helix to form between them. Consequently, the critical residues 14, 15, and 16 cannot form the helical conformation required for recognition.

The hypothesis that the *lamBS78* deletion alters the secondary structure as described above has been tested genetically. Since the critical recognition site is still intact in the mutant signal sequence, we predicted that function would be restored by a second mutation that permits the critical region to assume an α-helical conformation. Our studies confirmed this prediction. Secondary mutations that change the proline at position 9 to leucine or that change the glycine at position 17 to cysteine restore function to the mutant signal sequence (55a). Both of these changes permit the recognition site to assume an α-helical conformation.

The various molecular components of the LamB signal sequence and the function each appears to perform in initiating protein export can be summarized as follows. (i) The NH_2-terminal domain preceding the basic amino acids does not appear to be required. (ii) The central hydrophobic core is essential. Furthermore, this core must be able to assume an α-helical conformation to allow recognition to occur. (iii) A critical subset of four amino acids contained within the hydrophobic core comprises a recognition site that interacts directly with a component of the cellular export machinery.

The nature of the cellular component that interacts with the recognition site in the hydrophobic core is not known. We presume, however, that the components will be defined genetically by mutations such as *prlA*. Such mutations alter a cellular component and restore recognition of mutationally altered signal sequences (see below). We do not mean to imply that the only function performed by the signal sequence is in the initiation of export. Protein localization is likely to be a multistep process. Conceivably, the signal sequence could function in several of these steps.

The function of the basic amino acid residues that separate the two signal sequence domains remains unclear. None of the export-defective mutations that have been isolated alters one of these residues. However, Schwartz et al. (182) have isolated a mutant in which the arginine at position 6 of the LamB signal sequence is changed to a serine. Studies performed with this mutant suggest that the mutation may interfere with the cellular mechanism that couples export and translation (70, 71). Analogous mutations have been constructed in vitro in the gene coding for Lpp, a major outer membrane protein of *E. coli*, and similar results were obtained (87). These results will be discussed in more detail below.

Mutations That Prevent Processing

One of the most characteristic features of protein export is the proteolytic removal of the signal sequence. Since its initial discovery, this processing step in the export process has posed several important questions. First, what is the basis for pronounced specificity of signal peptidase? In other words, what is the recognition site for the processing enzyme? Second, is processing required for correct cellular localization? Does it, for example, provide thermodynamic energy and render the export reaction irreversible? Third, are precursor molecules biologically active or does the processing step convert an inactive "proenzyme" form of the molecule into an active species in a manner similar to intestinal zymogens? Although the number of examples is not large, there are sufficient data on mutants to allow some of these questions to be answered. Let us deal first with the recognition site for signal peptidase.

As described in the preceding section, mutations that alter the signal sequence and prevent export from the cytoplasm also prevent proteolytic removal of the signal sequence. This correlation between export and processing probably results from the inaccessibility of the internalized precursor molecule to the processing enzyme(s) that appears to be located in the cellular envelope (26, 123, 204). This hypothesis is sup-

ported by the observation that when export of the mutant protein is restored by a suppressor mutation such as *prlA*, processing is restored as well (49, 51; the *prl* suppressor mutations are described in detail in a following section). Thus, most of the export-defective signal sequence mutations do not alter the recognition site for the processing enzyme.

One exception to this statement is the 36-base pair deletion that removes 12 amino acids from the LamB signal sequence (Fig. 2). Even though this deletion ends four amino acids from the processing site, recognition by signal peptidase is prevented (49, 51). A second inframe deletion within the *lamB* gene also appears to prevent processing. This deletion removes amino acids 70 through 200 of the mature protein. Although the shortened LamB protein is localized correctly, it seems not to be processed (8). Thus, for LamB, it appears that the processing site encompasses a rather large NH_2-terminal domain.

Three mutations that alter the Bla signal sequence and affect processing but not export have been characterized (110). Two of these mutations specifically alter the proline at position 20, three amino acids from the processing site. In one mutation the proline is changed to a serine; in the other it is changed to a leucine. In the former case processing occurs at a greatly reduced rate, and in the latter case, it is abolished. The third alteration is a change of four amino acids (18 to 21) two residues from the processing site (Fig. 2). In this case the proline at position 20 is again changed to a leucine and again processing is abolished. Thus, for Bla, the site recognized by the enzyme includes at least the four residues within the signal sequence adjacent to the site of cleavage. Since the mutational alterations that abolish processing all affect proline at position 20, it may be that secondary structure in the region is important.

A mutation that changes the second amino acid of the mature M13 (f1) major coat protein from a glutamic acid to a leucine has been studied in detail (14, 168). This mutant protein is a poor substrate for signal peptidase both in vivo and in vitro. Thus, as with LamB, the recognition site for the processing enzyme for the coat protein appears to extend beyond the signal sequence.

Taken together the results with LamB, Bla, and coat protein indicate that the recognition site for signal peptidase may extend well beyond the processing site in both directions. The results also suggest that secondary conformation may play an important role. Given the pronounced specificity of this protease, these results are perhaps not surprising. However, without more data, accurate description of the substrate of signal peptidase is not possible.

Lpp undergoes an extensive series of posttranslational modifications. Several of these modifications are required to allow proteolytic removal of the signal sequence. Two mutations are known that prevent modification of the cysteine residue at position 1 of mature Lpp to form a thioetherdiglyceride. One mutation changes the glycine at position 14 of the signal sequence to aspartic acid (116). The other changes the cysteine residue itself to glycine (90). Since both mutations prevent the posttranslational modifications, they also prevent removal of the signal sequence. However, because of the requirement for modification, the protease that removes the signal sequence from Lpp probably is not the same as the processing enzyme for other exported proteins that are not modified. Indeed, purified preparations of signal peptidase will not process either modified or unmodified Lpp (204, 205).

Is the requirement for processing necessary either for localization or for activity? With LamB, localization without processing has been observed by three different methods. First, if the export block caused by the deletion that removes amino acids 9 to 20 of the signal sequence is relieved by either a *prlA* or a *prlC* suppressor mutation, the mutant protein is exported to the outer membrane where it functions normally despite the fact that the altered signal sequence has not been removed. Second, the suppressor mutation *prlB* relieves the export block caused by all of the *lamB* signal sequence mutations. In all cases, the mutant protein that is localized to the outer membrane is functional but unprocessed (49, 51). Finally, a deletion that removes amino acids 70 through 200 of mature LamB appears to prevent processing but not export (8). Thus, for LamB, processing appears to be unnecessary.

With Bla, processing is required for secretion of the protein into the aqueous periplasmic space. As with LamB, mutations that prevent processing of Bla do not prevent export from the cytoplasm, nor do they inactivate the enzyme. In this case, however, processing does have a measurable and important effect. Removal of the signal sequence is required for release of the enzyme from the outer face of the cytoplasmic membrane into the aqueous periplasmic compartment (110). This requirement is similar to the situation in the gram-positive organism *B. licheniformis*, in which proteolytic removal of the signal sequence results in secretion of the enzyme into the growth medium (191).

Results with PhoA are somewhat more complicated. The precursor form of the enzyme made in vitro is active (88). However, signal sequence mutations causing the accumulation of precursor in the cytoplasm appear to inactivate

the enzyme. An intriguing explanation for this anomaly is that the disulfide bonds required for producing the active dimer cannot be formed in the relatively reducing environment of the cytoplasm (130). If this explanation is correct, then processing is not required to activate PhoA either.

Since the primary function of Lpp appears to be structural, assessment of the requirement of processing for activity is difficult. Mutations that prevent processing but not export appear to have an Lpp$^-$ phenotype. However, since these mutations also prevent the posttranslational modifications, one cannot determine which event is critical.

All in all, there does not appear to be a good general reason for proteolytic removal of the signal sequence. In light of the results described above, processing is probably not required for export. Moreover, in most cases, it does not appear to be required for activity either. However, processing may have subtle effects on specific activity, and in some cases processing may be essential. Again, more data are required before these questions can be answered.

Intragenic Information Outside the Signal Sequence Specifying Export and Membrane Insertion

The signal sequence functions in the initiation of protein export. Although this sequence is essential, studies with *lamB-lacZ* fusions, for example, suggest that the signal sequence is not sufficient to cause export from the cytoplasm; other information located within *lamB* is also required. Moreover, there must be information within *lamB* and other genes that code for membrane proteins that causes membrane insertion and sorting signals that specify localization to the correct cellular membrane. Since the signal sequence is removed during the export process, such information must lie elsewhere. To determine the nature and location of this additional intragenic export information within *lamB*, techniques to permit the isolation of a series of inframe deletion mutations internal to the structural gene were devised. This experimental approach is described in Fig. 4. Analysis of the effects of these deletions on the export of both a LamB-LacZ hybrid protein and an otherwise wild-type LamB made it possible to more precisely define the location of export information within the *lamB* gene (8).

Ten independent inframe *lamB* deletions were analyzed. Most do not affect the localization of the LamB-LacZ hybrid protein. These deletions have been recombined genetically onto an otherwise wild-type *lamB* gene. With techniques of immunofluorescence, it was demonstrated that

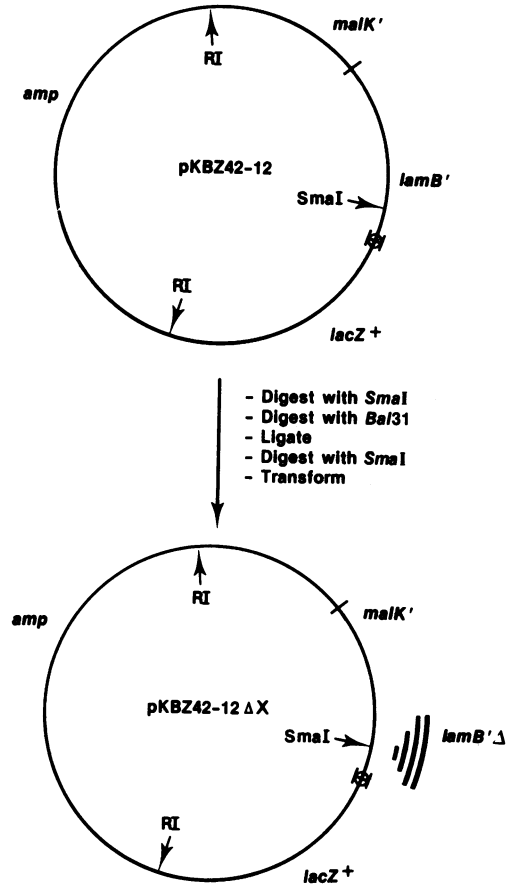

FIG. 4. Scheme for isolating internal inframe deletions. The pKBZ42-12 plasmid provides a means for the in vitro generation of deletions within the *lamB* sequence. This plasmid encodes the COOH terminus of *malK*, a functional (LacZ$^+$) *lamB-lacZ*42-12 hybrid gene, and the selectable pBR322 marker *bla* (ampicillin resistance). A unique *Sma*I restriction site is present at a position corresponding to amino acid 182 of the mature LamB protein. By linearization of the plasmid at this site and then treatment of the DNA with the exonuclease *Bal*31 a random series of small deletions can be generated. After nuclease treatment, the plasmid can be ligated and transformed into an appropriate recipient. DNA molecules that escape the *Bal*31 treatment can be selected against by a second *Sma*I digestion before transformation. Clones containing deletions that retain the correct reading frame for the LamB-LacZ hybrid protein and that do not extend outside the coding sequence for *lamB* can be identified by their blue color in the presence of the chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indoyl-β-D-galactoside.

these deletions also have no detectable effect on the export of LamB. Physical analysis of these deletions showed that they range in size from ~12 to 400 base pairs. Fine-structure mapping

with various restriction enzymes coupled with the known DNA sequence of the *lamB* gene permitted location of the amino acids removed by these deletions to be deduced. Taken as a group, these deletions remove the protein segment from approximately amino acid 70 through 220 of the mature LamB protein. (Mature LamB protein contains 421 amino acids [30].) Since these deletions do not affect export, it was concluded that no export information is located in this region of the *lamB* gene. Furthermore, since the largest of these deletions removes approximately one-third of the protein, it seems reasonable to conclude that overall protein structure is not a driving factor in the localization process.

Two of the 10 inframe *lamB* deletions affect localization of the LamB-LacZ hybrid protein. Although these deletions do not abolish export, they decrease export efficiency; i.e., a significant fraction of the hybrid protein does not reach the outer membrane. Physical analysis of these deletions suggested that they remove DNA close to the fusion joint between *lamB* and *lacZ* (corresponds to amino acid 240 of mature LamB). Taken together, these deletions remove the protein segment from approximately amino acid 100 through, and possibly beyond, 235. Since removal of amino acids 70 through 220 had no effect on export, it would appear that a region that lies close to the fusion joint will increase the efficiency of export of the hybrid protein to the outer membrane. Repeated attempts to recombine these deletions into an otherwise wild-type *lamB* gene were unsuccessful. Apparently these deletions remove most, if not all, of the *lamB* DNA between the promoter-distal end of the deletion and the *lacZ* DNA present at the fusion joint. In the absence of this *lamB* homology, the reciprocal recombination required for incorporation of the deletion onto a wild-type *lamB* gene is prevented. Accordingly, the effect of these deletions on the export of LamB itself cannot be determined. Since both of the deletions that alter the localization efficiency are known to delete DNA very close to and possibly at the fusion joint, the possibility that it is a sequence at the fusion joint that increases the efficiency of localization cannot be eliminated.

The data obtained from the analysis of inframe *lamB* deletions and from the various *lamB-lacZ* fusions are consistent with the hypothesis that discrete regions of the protein play critical roles in the localization process. The signal sequence defines the first discrete region, and it appears to function in the initiation of protein export. A second region is defined by amino acids 15 through 70 of mature LamB. If the signal sequence plus 15 amino acids of mature LamB are attached to a large functional COOH-terminal portion of β-galactosidase, the hybrid protein produced remains in the cytoplasm (134). Conversely, an otherwise identical fusion containing ~175 amino acids of LamB is exported to the outer membrane with an efficiency of ~40%. Clearly, this segment of the *lamB* gene contains sufficient information to direct β-galactosidase to an outer membrane location. A number of inframe deletions demonstrated that amino acids 70 through 220 can be removed without affecting export of either LamB-LacZ hybrid proteins or LamB itself (8). Accordingly, the information required for export from the cytoplasm and incorporation into the outer membrane must be contained in a region of *lamB* corresponding to amino acids 15 to 70. The nature and function of this critical region are a major focus of current research.

A third region of *lamB*, although not required for localization, appears to increase the efficiency of the export process. This region has only been definable through the use of *lamB-lacZ* fusions. Deletion analysis indicates that this region lies after amino acid 235 of LamB. This region of LamB may function as a stop transfer sequence for hybrid protein export (186). Whether this region functions in LamB export remains to be demonstrated. A summary of the intragenic export information within *lamB* is shown in Fig. 5.

Using techniques of recombinant DNA to produce truncated peptides, Boeke and Model (13) have identified a functional stop transfer sequence in the f1 gene III protein. This inner membrane protein spans the bilayer once and has a topology resembling a eucaryotic viral glycoprotein. The COOH terminus is hydrophobic and probably anchors the protein in the membrane. Deletions that produce truncated peptides lacking this COOH-terminal sequence cause export to the periplasm. These results are similar to those obtained with the membrane-bound and secreted forms of IgM μ-chain (164) described above. Here again, removal of a functional stop transfer sequence prevents membrane insertion and allows complete passage of the peptide through the membrane. Further work is required to establish the precise nature and function of the hydrophobic COOH-terminal sequence.

Cellular Components of the Protein Export Machinery

Export-defective mutants. An important goal of studies on protein export is to identify and characterize cellular components of the protein export machinery. One approach to this problem is to isolate mutants that exhibit a pleiotropic export-defective phenotype. Various schemes have been used to identify such mutants. Some

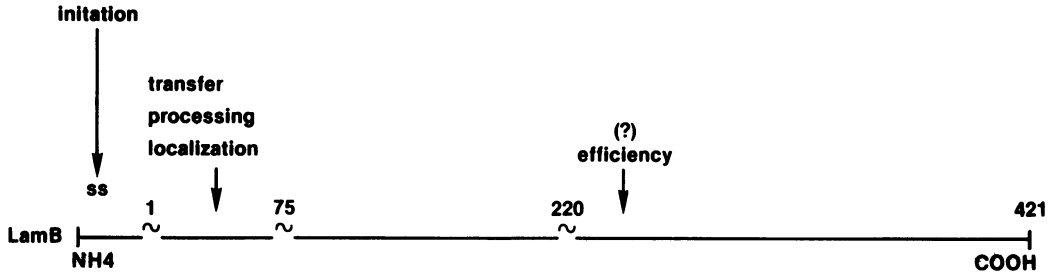

FIG. 5. Location of export information within the LamB protein. The LamB protein is represented by a line. The signal sequence of 25 amino acids is located at the NH_2-terminal end. The mature protein starts at amino acid 1. The various steps of the localization process are positioned above the line; each step is operationally defined by mutation or gene fusion. The first step of the localization process is initiation. We think that at this point the translation complex (mRNA, ribosome, and required factors) has assembled and the nascent polypeptide chain starting with the signal sequence has emerged from the complex, allowing an association with the export machinery in the membrane. Transfer defines the step at which the nascent polypeptide chain becomes firmly associated with the membrane. We think that this association involves the inner membrane because it is this membrane that will be accessible to the translation machinery. We define translocation as the movement of the peptide chain from the site of synthesis and transfer to its final location in the outer membrane. Processing is the proteolytic removal of the signal peptide from the polypeptide chain and occurs at the NH_2-terminal side of the first amino acid of the mature protein. The efficiency signal has been operationally defined through the use of *lamB-lacZ* protein fusions (see text). Its location and function in the mature LamB protein have been extrapolated from these data. We have positioned the steps over the particular region that contains the information determining that step of the process.

of these are based on the assumption that mutations that prevent protein export will be lethal. For example, a collection of conditional-lethal mutants isolated by Hirota that are temperature sensitive for growth has been screened for accumulation of precursors of exported proteins at the nonpermissive temperature without success (K. Ito, personal communication). The failure of such brute-force techniques demonstrates the need for specific selection or screens for export-defective mutants.

Wanner et al. (211) have designed a procedure for the isolation of mutants unable to secrete periplasmic proteins. This procedure is based upon the assumption that some factor(s) exists that is necessary for the secretion of periplasmic proteins, e.g., PhoA, but that is not necessary for the synthesis of cytoplasmic proteins, e.g., β-galactosidase. Mutants are detected on agar medium that includes indicators for both Bla and β-galactosidase syntheses. In addition, since mutations defective in the secretion of a number of envelope proteins may be lethal to the cell, the general approach used by Schedl and Primakoff (177) for the detection of conditional-lethal mutants was adopted. The mutation *perA* was isolated in this manner. Although this mutation has pleiotropic effects, we do not know whether the mutation affects secretion or regulation, or perhaps both. The mutation appears to lie in a gene, *envZ*, which is known to be involved in the transcriptional regulation of the genes specifying the major outer membrane porins OmpF and OmpC (73, 74, 210). In a similar vein, Dassa and Boquet (34) have isolated a mutant, *expA*, that simultaneously depresses expression of 10 periplasmic proteins and at least 1 outer membrane protein. Expression of cytoplasmic proteins is apparently not affected. The mutation maps at 22 min on the *E. coli* chromosome. Here again, it is not clear whether the mutation affects synthesis or export.

Another method for obtaining export-defective mutants requires knowledge of a chromosomal locus known to be involved in protein localization. This technique uses local mutagenesis to increase the frequency of desired mutants. Using this procedure, Ito et al. (96a) isolated a temperature-sensitive mutant that affects protein export. The target for the local mutagenesis was the large ribosomal gene cluster, a locus known to contain the *prlA* mutation (see the following section). All cell manipulations were done at 30°C, and transductants were screened for the inability to grow at 42°C. Temperature-sensitive mutants were screened for an export defect by looking for accumulated precursor MalE after short exposure to the nonpermissive temperature. The precursor protein was identified by antibody precipitation.

A direct selection for mutants affecting the export machinery has been developed based on the Lac$^-$ phenotype exhibited by certain *malE-lacZ* fusion strains. As described above, muta-

tions that prevent export of this hybrid protein confer a Lac$^+$ phenotype. The properties of this strain suggested that only a slight degree of internalization would provide sufficient β-galactosidase activity for growth on lactose. This proved to be the case, and mutations were obtained that define two new genetic loci in *E. coli*, *secA* and *secB* (113, 144, 147).

The *secA* mutation has been characterized in some detail. Strains containing this mutation grow normally at 30°C; at 42°C, growth stops and cells form filaments and accumulate precursor forms of several different exported proteins. However, not all exported proteins are affected by *secA*. Some proteins are localized normally at the nonpermissive temperature. The mutation is not strictly conditional; some precursor can be detected at permissive temperatures.

Subsequent studies have shown that the *secA* gene maps at 2.5 min on the *E. coli* chromosome. Using a *secA* transducing phage and UV-irradiated cells, Oliver and Beckwith (145, 146) have shown that the gene product is a protein of 92,000 daltons. Fusions, *secA-lacZ*, have been constructed, and the hybrid protein has been used to obtain antisera directed against SecA. In addition, a *secA* amber mutation has been isolated. With these reagents, evidence has been obtained indicating that the SecA protein is essential for growth and that it is regulated in response to the secretion requirements of the cell; i.e., if the export machinery is jammed with a hybrid protein, or if export is blocked by a *secA*(Ts) mutation, expression of *secA* is derepressed at least 10-fold.

The *secB* mutants have been more difficult to characterize because no conditional-lethal mutations are known. The mutants accumulate precursors of a variety of exported proteins, and the mutation maps at 80 min on the *E. coli* chromosome (113, 147).

Suppressors of export-defective mutations. As already noted, one approach to identifying cellular components of the export machinery is to isolate mutants that are generally export defective. Another approach is to devise a selection of mutants in which an internalized protein is exported. The mutants in which the precursor of the *lamB* gene product is found in the cytoplasm provide a selection for the export of an internalized protein. These mutant strains do not localize LamB to the outer membrane because the export machinery cannot recognize the mutationally altered signal sequence. Therefore, one should be able to alter the export machinery by mutation to restore recognition of the signal sequence. Such mutations would define components of the export machinery. The availability of mutants in which the export process is altered should allow the identification of important gene products, and this, in turn, should provide a means to analyze the export pathway biochemically.

In mutant strains that produce a LamB protein with a defective signal sequence, the protein is found in soluble form in the cytoplasm with the altered signal sequence still attached. Consequently, such strains exhibit a typical LamB$^-$ phenotype, e.g., inability to utilize maltodextrins as a carbon source and resistance to bacteriophage λ. By using these strains, second-site pseudorevertants were isolated by selecting for the ability to grow on maltodextrins. The mutation responsible for pseudoreversion suppresses the defect caused by the altered signal sequence and restores export and, in most cases, normal processing of the LamB protein. Three different second-site suppressor mutations have been identified. These suppressors, termed *prl* (protein localization) *A*, *-B*, and *-C*, should define cellular components that interact with the signal sequence during the export process (51).

One of the suppressors, *prlA*, has been characterized in some detail. It causes phenotypic suppression of all export-defective signal sequence mutations in *lamB*, *malE* (49), and *phoA* (129, 131). In some cases, *prlA* restores export to levels that are >85% of that seen in wild-type strains. Despite this powerful suppression, *prlA* causes no growth defects nor does it alter normal protein export. Since the *prlA* suppressors restore export of several different proteins with defective signal sequences, the cellular component altered by the *prlA* mutation probably interacts with this sequence during the export process. Genetic mapping shows that the gene altered by the suppressor mutation is a component of the *spc* operon (51). This operon, which maps at 72 min on the *E. coli* chromosome, has been shown to specify 10 different ribosomal proteins (99, 100, 157). By using a combination of genetic, biochemical, and recombinant DNA techniques, it was shown that the *prlA* mutations map at the extreme promoter-distal end of the *spc* operon and do not alter any of the previously known genes (183).

Previous studies had suggested the presence of a rather large DNA sequence at the end of the *spc* operon before the most likely transcription termination site (157). Furthermore, this sequence has been shown to contain an open reading frame capable of expressing a protein of 443 amino acids (P. D. Cerretti, D. Dean, G. R. Davis, D. M. Bedwell, and M. Nomura, Cell, in press). Genetic and biochemical analysis of a cloned *prlA* suppressor demonstrated that the mutation lies in this region. Since the DNA sequence was known, it was possible to fuse sequences from the open reading frame to *lacZ* sequences. The resulting hybrid gene should

specify a hybrid protein comprised of an NH_2-terminal sequence specified by the open reading frame and a large COOH-terminal portion of functional β-galactosidase. The structure of this hybrid gene is such that translation start signals must be provided by the open reading frame. Since expression of the hybrid protein was observed, it can be concluded that functional signals are present and that the open reading frame is expressed in vivo. The protein specified by this heretofore unidentified gene appears to be the cellular component altered by the *prlA* mutations. This new gene has been termed *prlA* (183).

Several lines of evidence suggest that the *prlA* gene does not code for a ribosomal protein. First, the chromosomal location of almost all of the ribosomal protein genes is known. The few remaining genes that have yet to be mapped all specify a protein much smaller than PrlA (93, 219). Finally, the *spc* operon has been expressed in vitro, and the genes specifying each of the known ribosomal proteins have been identified (99, 117). Thus, even though the *prlA* gene product has not been identified, it is unlikely that the gene specifies a known component of the ribosome.

The *prlA* suppressor restores not only export but also processing of every tested signal sequence mutation except one. This result also demonstrates that signal sequence mutations that prevent export do not prevent processing. If the mutant protein can be exported, it can be processed. This finding provides genetic evidence that the processing enzyme must be located outside of the cytoplasm. Indeed, given the problems associated with cellular fractionation techniques, signal sequence processing may provide a more reliable test for export from the cytoplasm.

Several other lines of evidence support the contention that PrlA is a component of the cellular protein export machinery. First, as described above, Ito et al. (96a) have isolated a temperature-sensitive lethal mutation, using techniques of local mutagenesis. At the nonpermissive temperature, the export and processing of several different proteins are drastically reduced. Although the nature of this mutation is not known, it has been shown that expression of the ribosomal protein L15 is greatly reduced at high temperature (96a). This mutation may also decrease expression of PrlA because the *prlA* gene is located adjacent to and promoter distal from the gene specifying L15. Thus, in the absence of PrlA, protein export may be blocked at an early step.

A second line of evidence indicating that PrlA is a component of the export machinery comes from studies with *secA*(Ts). One might anticipate that second-site mutations in genes coding for proteins that interact with SecA could compensate for the export defect. To test this possibility, pseudorevertants of a *secA*(Ts) strain that are no longer temperature sensitive for growth were isolated (E. Brickman, D. Oliver, J. Garwin, C. Kamamato, and J. Beckwith, personal communication). These pseudorevertants no longer exhibit an export-defective phenotype. One of these second-site mutations appears to map in *prlA*. Thus, both SecA and PrlA probably are components of the export machinery. Furthermore, these two proteins may interact at some point during the export process.

The function of the PrlA protein remains to be elucidated. Although the gene does not appear to code for a known ribosomal protein, its location in an operon that specifies 10 other ribosomal proteins suggests that PrlA may be associated with the machinery of protein synthesis. The genetic data suggest that PrlA may be involved in signal sequence recognition. Accordingly, PrlA may function in the coupling of translation and export.

The *prlB* suppressor is unusual in several respects. First, it suppresses only LamB signal sequence mutations. Second, the suppressor restores export of the mutant LamB proteins but not processing (49, 51). Only a single *prlB* allele has been isolated. This mutation is a small deletion (~250 base pairs) in the gene coding for the periplasmic ribose-binding protein that lies at 84 min in the *E. coli* chromosome (J. Garwin and S. Emr, personal communication). It is not clear why a mutation in the gene coding for a protein involved in ribose transport can restore export of a LamB protein with a defective signal sequence. Moreover, since export in the presence of the *prlB* suppressor occurs without processing, the mutant LamB protein may not be localized by the normal route. For these reasons, we suspect that *prlB* is not a component of the export machinery and that it suppresses the *lamB* signal sequence mutations by a bypass mechanism.

Two alleles of *prlC* have been isolated (51; J. Shultz, personal communication). These suppressors resemble *prlA* in that they suppress signal sequence mutations in both *malE* and *lamB* and in that they restore both export and processing (49). The *prlC* mutation maps between 69 and 71 min on the *E. coli* chromosome. This map location provides no clues as to the nature of the cellular component altered by the suppressor mutation.

Recently a fourth suppressor, *prlD*, has been isolated. This was done by selecting Mal^+ pseudorevertants of a strain carrying a deletion that removes amino acids 12 to 18 of the MalE signal sequence. *prlD* suppresses some, but not all, of the *MalE* signal sequence mutations. It has no

effect on the *lamB* signal sequence mutations. In the cases where *prlD* is effective, it restores both export and processing. The suppressor maps near, but not in, *secA*. A particularly intriguing result is that *prlA prlD* double mutants exhibit a generalized export-defective phenotype, causing the accumulation of precursor forms of many envelope proteins (V. Bankaitis, B. Rasmussin, and P. Bassford, personal communication). These results provide important evidence that both PrlA and PrlD are components of the export machinery. Moreover, they suggest that the two gene products may interact directly.

A Mechanism to Couple Protein Synthesis and Export

Studies with eucaryotic cells indicate a biochemical mechanism to couple translation and export. This mechanism involves SRP and docking protein (Fig. 1). Evidence currently available in procaryotic systems suggests the existence of a similar mechanism. The first experimental results to support this coupling mechanism came from the analysis of mutations that alter one of the basic amino acids in the hydrophilic portion of the LamB signal sequence. The manner in which these mutants were isolated is complicated and warrants some discussion. Starting with a *lamB-lacZ* fusion, Schwartz et al. (182) were able to isolate mutations that prevent translation of the hybrid gene by selecting for mutants in which expression of LacZ, but not LacY, activity is decreased. Some of these mutations appear to decrease translation of the hybrid gene by stabilizing a stem-loop structure at the beginning of the LamB mRNA that includes the ribosome-binding site. Second-site mutations that destabilize the stem-loop structure and restore translation were then obtained by selecting Lac^+. These second-site mutations were shown by DNA sequence analysis to lie within the signal sequence region of *lamB* and to change the arginine codon at position 6 to a serine codon (Fig. 2) (71). Using a *lamB-lacZ* fusion that contains the LamB signal sequence but produces a hybrid protein that remains in the cytoplasm, Hall et al. (70) have shown that translation is restored to wild-type levels in the double mutant. However, when the mutations are recombined into a *lamB-lacZ* fusion that specifies an exported hybrid protein, expression is reduced markedly. Moreover, when the mutations are present in an otherwise wild-type *lamB* gene, expression of LamB is reduced as well. Recently, S. Inouye et al. (91) constructed similar mutations in Lpp, using in vitro techniques. These mutations also appear to affect expression. The correlation between export and synthesis suggests that these two processes may be coupled in vivo.

Further evidence to support a mechanism that couples synthesis and export comes from studies with the *secA* nonsense mutation. When this mutation is present in a strain carrying a temperature-sensitive nonsense suppressor, expression of SecA can be controlled conditionally. Oliver and Beckwith (146) have shown that synthesis of MalE is greatly reduced when this strain is incubated at high temperature, in contrast to results described previously with *prlA*. In this case, absence of the gene product appears to prevent export but not synthesis since precursor accumulation can be demonstrated (Ito et al., in press).

It is tempting to speculate that in *E. coli* SecA performs a function related to that of docking protein, whereas PrlA may be a component of SRP. If SecA is not present, the translation block cannot be removed. Conversely, if PrlA is absent, the block cannot occur and this prevents export and processing. Although more work is obviously required, it appears likely that synthesis and export may be coupled in *E. coli*.

If a mechanism to couple synthesis and export exists, then biochemical experiments that utilize pulse-labeling techniques must be assessed cautiously. Such experiments are based on the assumption that translation proceeds at a constant rate, and this assumption may not be valid. Similarly, genetic selections for export-defective mutants that require synthesis of the exported protein may not yield the desired mutation. Indeed, the selection that was used to isolate *secA*(Ts) utilized a *malE-lacZ* fusion and required synthesis of the hybrid protein (Lac^+). Mutations in *secA* that destroy function cannot be isolated in this manner because such a mutation would prevent synthesis of β-galactosidase. The *secA*(Ts) allele must produce an altered gene product.

CONCLUSIONS/PROJECTIONS

Results described in this review demonstrate the enormous progress that has been made in the last decade towards understanding the molecular mechanisms of protein localization. We have learned that the genetic information specifying correct cellular localization is contained within the structural gene. In many cases, this export information is composed of small discrete sequences arranged in a defined order in the primary structure of the protein. Each of these export signals mediates a particular step in the localization process. Alteration or removal of these signals by mutation causes the protein to be incorrectly localized to a different cellular compartment. Moreover, by using genetics or recombinant DNA techniques or both, these signals can be grafted onto different proteins so

as to cause export to a particular cellular location.

Specific selection procedures have been described that have permitted the identification of several genetic loci whose products are components of the cellular protein export machinery. With existing technology, this knowledge can be exploited to identify the gene products and raise specific antisera. Such reagents will allow protein isolation and biochemical characterization. Information gained in such studies will form the basis for reconstituting the export machinery in vitro from purified components.

Despite these advances, much remains to be done. Although we have identified intragenic export signals, our knowledge is descriptive. The mechanisms by which the various molecular components of these export signals function remain to be elucidated. With respect to the cellular export machinery, we have only scratched the surface. In all probability, most of the essential components have yet to be identified. Solutions to these problems will require more extensive mutant analysis and increasingly sophisticated in vitro systems. Both genetic and biochemical approaches are complicated by the nature of the export process itself. The series of reactions that comprise the process of protein localization are oriented not only in time, but also in space. In vitro reconstitution will undoubtedly require sealed membrane vesicles that define different aqueous compartments. Replacing the various components of the export machinery into the correct compartment or into the membrane in the correct topology will require considerable expertise. A further complication stems from the observed coupling between export and protein synthesis. Apparently protein export requires numerous cellular components working in concert, a situation that may be difficult to mimic in vitro. This complexity hinders genetic analysis as well. In such cases, distinguishing the direct effects of a particular mutation from misleading secondary effects is often troublesome. Furthermore, the coupling of synthesis and export complicates mutant selection. To date, all selections for export-defective mutations require synthesis of the protein in question. Novel selection procedures that are not so limited are required to understand the phenomenon.

Perhaps the most exciting result of work in the field of membrane biogenesis is the realization that all cells use similar mechanisms of protein localization. This realization has fostered solid relationships among scientists working in areas as diverse as eucaryotic cell biology and procaryotic molecular genetics. As we have seen, the fruits of these unusual collaborations have been plentiful.

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LITERATURE CITED

1. **Achtman, M., P. A. Manning, C. Edelbluth, and P. Herrlich.** 1979. Export without proteolytic processing of inner and outer membrane proteins encoded by F sex factor *tra* cistrons in *Escherichia coli* minicells. Proc. Natl. Acad. Sci. U.S.A. **76**:4837–4841.
2. **Ades, I. Z., and R. A. Butow.** 1980. The products of mitochondria-bound cytoplasmic polysomes in yeast. J. Biol. Chem. **255**:9918–9924.
3. **Alt, F. W., A. L. M. Bothwell, M. Knapp, E. Siden, E. Mather, M. Koshland, and D. Baltimore.** 1980. Synthesis of secreted and membrane-bound immunoglobulin Mu heavy chains is directed by mRNAs that differ at their 3′ ends. Cell **20**:293–301.
4. **Bassford, P., and J. R. Beckwith.** 1979. *Escherichia coli* mutants accumulating the precursor of a secreted protein in the cytoplasm. Nature (London) **277**:538–541.
5. **Bassford, P. J., T. J. Silhavy, and J. R. Beckwith.** 1979. Use of gene fusion to study secretion of maltose-binding protein into *Escherichia coli* periplasm. J. Bacteriol. **139**:19–31.
6. **Bedouelle H., P. J. Bassford, A. V. Fowler, I. Zabin, J. Beckwith, and M. Hofnung.** 1980. Mutations which alter the function of the signal sequence of the maltose binding protein of *Escherichia coli*. Nature (London) **285**:78–81.
7. **Bedouelle, H., and M. Hofnung.** 1981. Functional implications of secondary structure analysis of wild-type and mutant bacterial signal peptides, p. 309–403. *In* D. Oxender et al. (ed.), Membrane transport and neuroreceptors. A. R. Liss, Inc., New York.
8. **Benson, S., and T. Silhavy.** 1983. Information within the mature LamB protein necessary for localization to the outer membrane of *Escherichia coli* K-12. Cell **32**:1325–1335.
9. **Berg, N. O., and S. Erikson.** 1972. Liver disease in adults with alpha$_1$-antitrypsin deficiency. N. Engl. J. Med. **287**:1264–1267.
10. **Blobel, G.** 1980. Intracellular protein topogenesis. Proc. Natl. Acad. Sci. U.S.A. **77**:1496–1500.
11. **Blobel, G., and B. Dobberstein.** 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. **67**:835–851.
12. **Blobel, G., and B. Dobberstein.** 1975. Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components. J. Cell Biol. **67**:852–862.
13. **Boeke, J. D., and P. Model.** 1982. A prokaryotic membrane anchor sequence: carboxyl terminus of bacteriophage f1 gene *III* protein retains it in the membrane. Proc. Natl. Acad. Sci. U.S.A. **79**:5200–5204.
14. **Boeke, J. D., M. Russel, and P. Model.** 1980. Processing of filamentous phage pre-coat protein. J. Mol. Biol. **144**:103–116.
15. **Böhni, P., S. Gasser, C. Leaver, and G. Schatz.** 1980. A matrix-localized mitochondrial protease processing cytoplasmically-made precursors to mitochondrial proteins, p. 423–433. *In* A. M. Kroon and C. Saccone (ed.), The organization and expression of the mitochondrial genome. Elsevier/North-Holland, Amsterdam.
16. **Bonatti, S., and G. Blobel.** 1979. Absence of a cleavable signal sequence in Sindbis virus glycoprotein PE$_2$. J. Biol. Chem. **254**:12261–12264.

17. **Boyd, A., K. Kendall, and M. Simon.** 1983. Structure of the serine chromoreceptor in *Escherichia coli*. Nature (London) **301**:623–626.
18. **Braell, W. A., and H. F. Lodish.** 1981. Biosynthesis of the erythrocyte anion transport protein. J. Biol. Chem. **256**:11337–11344.
19. **Braell, W. A., and H. F. Lodish.** 1982. The erythrocyte anion transport protein is cotranslationally inserted into microsomes. Cell **28**:23–31.
20. **Braell, W. A., and H. F. Lodish.** 1982. Ovalbumin utilizes an NH_2-terminal signal sequence. J. Biol. Chem. **257**:4578–4582.
21. **Braun, V.** 1975. Covalent lipoprotein from the outer membrane of *Escherichia coli*. Biochim. Biophys. Acta **415**:335–377.
22. **Bremer, E., E. Beck, I. Hindennach, I. Sonntag, and U. Henning.** 1980. Cloned structural gene *ompA* for an integral outer membrane protein of *Escherichia coli* K-12 localization on hybrid plasmid PTU-100 and expression of a fragment of the gene. Mol. Gen. Genet. **179**:13–20.
23. **Bremer, E., S. T. Cole, I. Hindennach, U. Henning, E. Beck. C. Kurz, and H. Schaller.** 1982. Export of a protein into the outer membrane of *Escherichia coli* K12. Eur. J. Biochem. **122**:223–231.
24. **Cancedda, R., and M. J. Schlessinger.** 1974. Localization of polyribosomes containing alkaline phosphatase nascent polypeptides on membranes of *Escherichia coli*. J. Bacteriol. **117**:290–301.
25. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* operon to selected promoters in *E. coli* using bacteriophage λ and Mu. J. Mol. Biol. **104**:541–555.
26. **Chang, C. N., G. Blobel, and P. Model.** 1978. Detection of prokaryotic signal peptidase in an *Escherichia coli* membrane fraction: endoproteolytic cleavage of nascent F1 pre-coat protein. Proc. Natl. Acad. Sci. U.S.A. **75**:361–365.
27. **Chang, C. N., P. Model, and G. Blobel.** 1979. Membrane biogenesis: cotranslational integration of the bacteriophage F1 coat protein into an *Escherichia coli* membrane-fraction. Proc. Natl. Acad. Sci. U.S.A. **76**:1251–1255.
28. **Chou, P. Y., and G. D. Fasman.** 1978. Empirical predictions of protein conformation. Annu. Rev. Biochem. **47**:251–276.
29. **Chua, N.-H., and G. W. Schmidt.** 1978. Posttranslational transport into intact chloroplasts of a precursor to the small subunit of ribulose 1.5-biphosphate carboxylase. Proc. Natl. Acad. Sci. U.S.A. **75**:6110–6114.
30. **Clement, J. M., and M. Hofnung.** 1981. Gene sequence of the λ receptor, an outer membrane protein of *E. coli* K12. Cell **27**:507–514.
31. **Collins, M. L., and R. Niederman.** 1976. Membranes of *Rhodospirillum rubrum*: isolation and physicochemical properties of membranes from aerobically grown cells. J. Bacteriol. **126**:1316–1326.
32. **Crowlesmith, I., K. Gamon, and U. Henning.** 1981. Precursor proteins are intermediates in vivo in the synthesis of two major outer membrane proteins the OmpA and OmpF proteins, of *Escherichia coli* K-12. Eur. J. Biochem. **113**:375–380.
33. **Daniels, C. J., D. G. Bole, S. C. Quay, and D. L. Oxender.** 1981. Role of membrane potential in the secretion of protein into the periplasm of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **78**:5396–5400.
34. **Dassa, E., and P. L. Boquet.** 1981. *expA*: a conditional mutation affecting the expression of a group of exported proteins in *Escherichia coli* K-12. Mol. Gen. Genet. **181**:192–200.
35. **Date, T.** 1983. Demonstration by a novel genetic technique that leader peptidase is an essential enzyme of *Escherichia coli*. J. Bacteriol. **154**:76–83.
36. **Date, T., J. M. Goodman, and W. T. Wickner.** 1980. Procoat, the precursor of M13 coat protein, requires an electrochemical potential for membrane insertion. Proc. Natl. Acad. Sci. U.S.A. **77**:4669–4673.
37. **Date, T., and W. Wickner.** 1981. Isolation of the *Escherichia coli* leader peptidase gene and effects of leader peptidase overproduction in vivo. Proc. Natl. Acad. Sci. U.S.A. **78**:6106–6110.
38. **Date, T., C. Zwizinski, S. Ludmerer, and W. Wickner.** 1980. Mechanisms of membrane assembly: effects of energy poisons on the conversion of soluble M13 coliphage procoat to membrane-bound coat proteins. Proc. Natl. Acad. Sci. U.S.A. **77**:827–831.
39. **Daum, G., S. M. Gasser, and G. Schatz.** 1982. Import of proteins into mitochondria. J. Biol. Chem. **257**:13075–13080.
40. **Davis, B. D., and P. C. Tai.** 1980. Mechanism of protein secretion across membranes. Nature (London) **283**:433–438.
41. **DiRienzo, J. M., and M. Inouye.** 1979. Lipid fluidity-dependent biosynthesis and assembly of the outer membrane proteins of *E. coli*. Cell **17**:155–161.
42. **DiRienzo, J. M., K. Nakamura, and M. Inouye.** 1978. The outer membrane proteins of gram-negative bacteria biosynthesis assembly and functions. Annu. Rev. Biochem. **47**:481–532.
43. **Dobberstein, D., G. Blobel, and N.-H. Chua.** 1977. In vitro synthesis and processing of a putative precursor for the small subunit of ribulose-1,5-biphosphate carboxylase of *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. U.S.A. **74**:1082–1085.
44. **Drickamer, L. K.** 1976. Fragmentation of the 95,000-dalton transmembrane polypeptide in human erythrocyte membranes. J. Biol. Chem. **251**:5115–5123.
45. **Drickamer, L. K.** 1977. Fragmentation of the band 3 polypeptide from human erythrocyte membranes. J. Biol. Chem. **252**:6909–6917.
46. **Drickamer, L. K.** 1978. Orientation of the band 3 polypeptide from human erythrocyte membranes. J. Biol. Chem. **253**:7242–7248.
47. **Early, P., J. Rogers, M. Davis, K. Calame, M. Bond, R. Wall, and L. Hood.** 1980. Two mRNAs can be produced from a single immunoglobulin μ gene by alternative RNA processing pathways. Cell **20**:313–319.
48. **Ehring, R., K. Beyreuther, J. K. Wright, and P. Overath.** 1980. In vitro and in vivo products of *E. coli* lactose permease gene are identical. Nature (London) **283**:537–540.
49. **Emr, S. D., and P. J. Bassford, Jr.** 1982. Localization and processing of outer membrane and periplasmic proteins in *Escherichia coli* strains harboring export-specific suppressor mutations. J. Biol. Chem. **257**:5852–5860.
50. **Emr, S. D., M. N. Hall, and T. J. Silhavy.** 1980. A mechanism of protein localization: the signal hypothesis and bacteria. J. Cell Biol. **86**:701–711.
51. **Emr, S. D., S. Hanley-Way, and T. J. Silhavy.** 1981. Suppressor mutations that restore export of a protein with a defective signal sequence. Cell **23**:79–88.
52. **Emr, S. D., J. Hedgpeth, J. M. Clement, T. J. Silhavy, and M. Hofnung.** 1980. Sequence analysis of mutations that prevent export of lambda receptor. An *Escherichia coli* outer membrane protein. Nature (London) **285**:82–85.
53. **Emr, S. D., M. Schwartz, and T. J. Silhavy.** 1978. Mutations altering the cellular localization of the phage λ receptor, an *Escherichia coli* outer membrane protein. Proc. Natl. Acad. Sci. U.S.A. **75**:5802–5806.
54. **Emr, S. D., and T. J. Silhavy.** 1980. Mutations affecting localization of an *Escherichia coli* outer membrane protein, the bacteriophage λ receptor. J. Mol. Biol. **141**:63–90.
55. **Emr, S. D., and T. J. Silhavy.** 1982. The molecular components of the signal sequence that function in the initiation of protein export. J. Cell Biol. **95**:689–696.
55a. **Emr, S. D., and T. J. Silhavy.** 1983. Importance of secondary structure in the signal sequence for protein secretion. Proc. Natl. Acad. Sci. U.S.A. **80**:4599–4603.
56. **Enequist, H. G., T. R. Hirst, S. Harayama, S. J. S. Hardy, and L. L. Randall.** 1981. Energy is required for

maturation of exported proteins in *Escherichia coli*. Eur. J. Biochem. **116**:227–233.

57. **Filip C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. J. Bacteriol. **115**:717–722.
58. **Fischer, H. D., A. Gonzalez-Noriega, and W. S. Sly.** 1980. β-Glucuronidase binding to human fibroblast membrane receptors. J. Biol. Chem. **255**:5069–5074.
59. **Fox, C. F., J. H. Law, N. Tsukagoshi, and G. Wilson.** 1970. A density label for membrane. Proc. Natl. Acad. Sci. U.S.A. **67**:598–605.
60. **Fraser, T. H., and B. J. Bruce.** 1978. Chicken ovalbumin is synthesized and secreted by *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **75**:5936–5940.
61. **Frazer, A. C., and R. Curtiss III.** 1975. Production, properties and utility of bacterial minicells. Curr. Top. Microbiol. Immunol. **69**:1–84.
62. **Freitag, H., M. Janes, and W. Neupert.** 1982. Biosynthesis of mitochondrial porin and insertion into the outer mitochondrial membrane of *Neurospora crassa*. Eur. J. Biochem. **126**:197–202.
63. **Fukuda, M., Y. Eshdat, G. Tarone, and V. T. Marchesi.** 1978. Isolation and characterization of peptides derived from the cytoplasmic segment of band 3, the predominant intrinsic membrane protein of the human erythrocyte. J. Biol. Chem. **253**:2419–2428.
64. **Gasser, S. M., A. Ohashi, G. Daum, P. Böhni, J. Gibson, G. Reid, T. Yonetani, and G. Schatz.** 1982. Imported mitochondrial proteins cytochrome b_2 and cytochrome c_1 are processed in two steps. Proc. Natl. Acad. Sci. U.S.A. **79**:267–271.
65. **Gething, M.-J., and J. Sambrook.** 1982. Construction of influenza haemagglutinin genes that code for intracellular and secreted forms of the protein. Nature (London) **300**:598–599.
66. **Gibson, R., S. Schlesinger, and S. Kornfeld.** 1979. The nonglycosylated glycoprotein of vesicular stomatitis virus is temperature-sensitive and undergoes intracellular aggregation at elevated temperatures. J. Biol. Chem. **254**:3600–3607.
67. **Gilmore, R., P. Walter, and G. Blobel.** 1982. Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. J. Cell Biol. **95**:470–477.
68. **Goodman, J. M., C. Watts, and W. Wickner.** 1981. Membrane assembly: posttranslational insertion of M13 procoat protein into *E. coli* membranes and its proteolytic conversion to coat protein in vitro. Cell **24**:437–441.
69. **Halegoua, S., and M. Inouye.** 1979. Translocation and assembly of outer membrane proteins of *Escherichia coli*. Selective accumulation of precursors and novel assembly intermediates caused by phenethyl alcohol. J. Mol. Biol. **130**:39–61.
70. **Hall, M. N., J. Gabay, M. Debarbouille, and M. Schwartz.** 1982. A role for mRNA secondary structure in the control of translation initiation. Nature (London) **295**:616–618.
71. **Hall, M. N., J. Gabay, and M. Schwartz.** 1983. Evidence for a coupling of synthesis and export of an outer membrane protein in *Escherichia coli*. EMBO J. **2**:15–19.
72. **Hall, M. M., M. Schwartz, and T. J. Silhavy.** 1982. Sequence information within the *lamB* gene is required for proper routing of the bacteriophage λ receptor protein to the outer membrane of *Escherichia coli* K-12. J. Mol. Biol. **156**:93–112.
73. **Hall, M. N., and T. J. Silhavy.** 1981. Genetic analysis of the major outer membrane proteins of *Escherichia coli*. Annu. Rev. Genet. **15**:91–142.
74. **Hall, M. N., and T. J. Silhavy.** 1981. The *ompB* locus and the regulation of the major outer membrane porin proteins of *Escherichia coli* K-12. J. Mol. Biol. **146**:23–43.
75. **Hallermayer, G., R. Zimmerman, and W. Neupert.** 1977. Kinetic studies on the transport of cytoplasmically synthesized proteins into the mitochondria in intact cells of *Neurospora crassa*. Eur. J. Biochem. **81**:523–532.
76. **Hasilik, A., and E. Neufeld.** 1980. Biosynthesis of lysosomal enzymes in fibroblasts: synthesis as precursors of higher molecular weight. J. Biol. Chem. **255**:4937–4945.
77. **Hasilik, A., and E. Neufeld.** 1980. Biosynthesis of lysosomal enzymes in fibroblasts—phosphorylation of mannose residues. J. Biol. Chem. **255**:4946–4950.
78. **Hedgpeth, J., J. M. Clement, C. Marchal, D. Perrin, and M. Hofnung.** 1980. DNA-sequence encoding the NH_2-terminal peptide involved in transport of lambda receptor. An *Escherichia coli* secretory protein. Proc. Natl. Acad. Sci. U.S.A. **77**:2621–2625.
79. **Hennig, B., and W. Neupert.** 1981. Assembly of cytochrome *c*. Apocytochrome *c* is bound to specific sites on mitochondria before its conversion to holocytochrome *c*. Eur. J. Biochem. **121**:203–212.
80. **Hickman, S., L. J. Shapiro, and E. F. Neufeld.** 1974. A recognition marker for uptake of a lysosomal enzyme by cultured fibroblasts. Biochem. Biophys. Res. Commun. **51**:55–61.
81. **Higgins, C. G., P. D. Haag, H. Nikaido, F. Ardeshir, G. Garcia, and G. F. L. Ames.** 1982. Complete nucleotide sequence and identification of membrane components of the histidine transport operon of *S. typhimurium*. Nature (London) **298**:723–727.
82. **Highfield, P. E., and R. J. Ellis.** 1978. Synthesis and transport of the small subunit of chloroplast ribulose biophosphate carboxylase. Nature (London) **271**:420–424.
83. **Hofnung, M. (ed.).** 1982. The maltose system as a tool in molecular biology. Masson Publishing, Paris. (Reprinted from Ann. Microbiol. **133**:1–8.)
84. **Hussain, M., S. Ichihara, and S. Mizushima.** 1980. Accumulation of glyceride-containing precursor of the outer membrane lipoprotein in the cytoplasmic membrane of *Escherichia coli* treated with globomycin. J. Biol. Chem. **255**:3707–3712.
85. **Hussain, M., S. Ichihara, and S. Mizushima.** 1982. Mechanism of signal peptide cleavage in the biosynthesis of the major lipoprotein of the *Escherichia coli* outer membrane. J. Biol. Chem. **257**:5177–5182.
86. **Ichihara, S., M. Hussain, and S. Mizushima.** 1981. Characterization of new membrane lipoproteins and their precursors of *Escherichia coli*. J. Biol. Chem. **256**:3125–3129.
87. **Inouye, H., W. Barnes, and J. Beckwith.** 1982. Signal sequence of alkaline phosphatase of *Escherichia coli*. J. Bacteriol. **149**:434–439.
88. **Inouye, H., and J. Beckwith.** 1977. Synthesis and processing of an *Escherichia coli* alkaline phosphatase precursor in vitro. Proc. Natl. Acad. Sci. U.S.A. **74**:1440–1444.
89. **Inouye, M., and S. Halegoua.** 1979. Secretion and membrane localization of proteins in *Escherichia coli*. Crit. Rev. Biochem. **7**:339–371.
90. **Inouye, S., T. Franceschini, M. Satl, K. Itakura, and M. Inouye.** 1982. Prolipoprotein signal peptidase of *Escherichia coli* requires a cysteine residue at the cleavage site. Cell **32**:789–797.
91. **Inouye, S., X. Soberon, T. Franceschini, K. Nakamura, K. Itakura, and M. Inouye.** 1982. Role of positive charge on the amino-terminal region of the signal peptide in protein secretion across the membrane. Proc. Natl. Acad. Sci. U.S.A. **79**:3438–3441.
92. **Inouye, S., S. Wang, J. Sekizawa, S. Halegoua, and M. Inouye.** 1977. Amino acid sequence for the peptide extension on the prolipoprotein of the *Escherichia coli* outer membrane. Proc. Natl. Acad. Sci. U.S.A. **74**:1004–1008.
93. **Isono, K.** 1980. Genetics of ribosomal proteins and their modifying and processing enzymes in Escherichia coli, p. 641–670. *In* G. Chambliss, G. R. Craven, J. Davies, K. Davies, L. Kahan and M. Nomura (ed.), Ribosomes: structure, function, and genetics. University Park Press, Baltimore.

94. **Ito, K.** 1982. Purification of the precursor form of maltose-binding protein, a periplasmic protein of *Escherichia coli*. J. Biol. Chem. **257**:9895–9897.
95. **Ito, K., and J. R. Beckwith.** 1981. Role of the mature protein sequence of maltose-binding protein in its secretion across the *E. coli* cytoplasmic membrane. Cell **25**:143–150.
96. **Ito, K., T. Sato, and T. Yura.** 1977. Synthesis and assembly of the membrane proteins in *E. coli*. Cell **11**:551–559.
96a. **Ito, K., W. Wittekind, M. Nomura, A. Miura, K. Shiba, T. Yura, and H. Nashimoto.** 1983. A temperature-sensitive mutant of *E. coli* exhibiting slow processing of exported proteins. Cell **32**:789–797.
97. **Jamieson, J. D., and G. E. Palade.** 1967. Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of peripheral elements of the golgi complex. J. Cell Biol. **34**:577–597.
98. **Jamieson, J. D., and G. E. Palade.** 1967. Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transport to condensing vacuoles and zymogen granules. J. Cell Biol. **34**:597–615.
99. **Jaskunas, S. R., A. M. Fallon, and M. Nomura.** 1977. Identification and organization of ribosomal protein genes of *Escherichia coli* carried by λ*fus*2 transducing phage. J. Biol. Chem. **252**:7323–7336.
100. **Jaskunas, S. R., A. M. Fallon, M. Nomura, B. G. Williams, and F. R. Blattner.** 1977. Expression of ribosomal protein genes cloned in charon vector phages and identification of their promoters. J. Biol. Chem. **252**:7355–7364.
101. **Josefsson, L., and L. L. Randall.** 1981. Different exported proteins in *Escherichia coli* show differences in the temporal mode of processing in vivo. Cell **25**:151–157.
102. **Josefsson, L., and L. L. Randall.** 1981. Processing in vivo of precursor maltose-binding protein in *Escherichia coli* occurs post-translationally as well as co-translationally. J. Biol. Chem. **256**:2504–2507.
103. **Joseleau-Petit, D., and A. Kepes.** 1975. A novel electrophoretic fractionation of *Escherichia coli* envelopes. Biochim. Biophys. Acta **406**:2504–2507.
104. **Kaplan, A., D. T. Achord, and W. S. Sly.** 1977. Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. Proc. Natl. Acad. Sci. U.S.A. **74**:2026–2030.
105. **Katz, F. N., J. E. Rothman, D. M. Knipe, and H. F. Lodish.** 1977. Membrane assembly: synthesis and intracellular processing of the vesicular stomatitis viral glycoprotein. J. Supramol. Struct. **7**:353–370.
106. **Katz, F. N., J. E. Rothman, V. R. Lingappa, G. Blobel, and H. F. Lodish.** 1977. Membrane assembly in vitro: synthesis, glycosylation and asymmetric insertion of a transmembrane protein. Proc. Natl. Acad. Sci. U.S.A. **74**:3278–3282.
107. **Kehry, M., S. Ewald, R. Douglas, C. Sibley, W. Raschke, D. Fambrough, and L. Hood.** 1980. The immunoglobulin μ chains of membrane-bound and secreted IgM molecules differ in their C-terminal segments. Cell **21**:393–406.
108. **Koshland, D., and D. Botstein.** 1980. Secretion of beta-lactamase requires the carboxy end of the protein. Cell **20**:749–760.
109. **Koshland, D., and D. Botstein.** 1982. Evidence for post-translational translocation of β-lactamase across the bacterial inner membrane. Cell **30**:893–902.
110. **Koshland, D., R. T. Sauer, and D. Botstein.** 1982. Diverse effects of mutations in the signal sequence on the secretion of β-lactamase in *Salmonella typhimurium*. Cell **30**:903–914.
111. **Kreibich, G., M. Czako-Graham, R. Grebenau, W. Mok, E. Rodriguez-Boulan, and D. Sabatini.** 1978. Characterization of the ribosomal binding site in rat liver rough microsomes: ribophorins I and II, two integral membrane proteins related to ribosome binding. J. Supramol. Struct. **8**:279–302.
112. **Kreil, G.** 1981. Transfer of proteins across membranes. Annu. Rev. Biochem. **50**:317–348.
113. **Kumamato, C., and J. Beckwith.** 1983. Mutations in a new gene, *secB*, cause defective protein localization in *Escherichia coli*. J. Bacteriol. **154**:253–260.
114. **Lai, J.-S., M. Sarvas, W. J. Brammar, K. Neugebauer, and H. C. Wu.** 1981. *Bacillus licheniformis* penicillinase synthesized in *Escherichia coli* contains covalently linked fatty acid and glyceride. Proc. Natl. Acad. Sci. U.S.A. **76**:3506–3510.
115. **Lazdunski, C., D. Baty, and J. M. Pages.** 1979. Procaine, a local anesthetic interacting with the cell membrane, inhibits the processing of precursor forms of periplasmic proteins in *Escherichia coli*. Eur. J. Biochem. **96**:49–57.
116. **Lin, J. J.-C., H. Kanazawa, J. Ozols, and H. C. Wu.** 1978. *Escherichia coli* mutant with an amino-acid alteration within signal sequence of outer membrane prolipoprotein. Proc. Natl. Acad. Sci. U.S.A. **75**:4891–4895.
117. **Lindahl, L., L. Post, J. Zengel, S. F. Gilbert, W. A. Strycharz, and M. Nomura.** 1977. Mapping of ribosomal protein genes by in vitro protein synthesis using DNA fragments of λ*fus*3 transducing phages as templates. J. Biol. Chem. **252**:7365–7383.
118. **Lingappa, V. R., F. N. Katz, H. F. Lodish, and G. Blobel.** 1978. A signal sequence for the insertion of a transmembrane glycoprotein. Similarities to the signals of secretory proteins in primary structure and function. J. Biol. Chem. **253**:8667–8670.
119. **Lingappa, V. R., J. R. Lingappa, and G. Blobel.** 1979. Chicken ovalbumin contains an internal sequence. Nature (London) **281**:117–121.
120. **Lingappa, V. R., D. Shields, S. L. C. Woo, and G. Blobel.** 1978. Nascent chicken ovalbumin contains the functional equivalent of a signal sequence. J. Cell Biol. **79**:567–572.
121. **Lodish, H. F.** 1981. Post-translational modification of proteins. Enzyme Microb. Technol. **3**:178–188.
122. **Lodish, H. F., and J. E. Rothman.** 1979. The assembly of cell membranes: the two sides of a biological membrane differ in structure and function; studies of animal viruses and bacteria have helped to reveal how this asymmetry is preserved as the membrane grows. Sci. Am. **240**:48–63.
123. **Mandel, G., and W. Wickner.** 1979. Translational and post-translational cleavage of M13 procoat protein: extracts of both the cytoplasmic and outer membranes of *Escherichia coli* contain leader peptidase activity. Proc. Natl. Acad. Sci. U.S.A. **76**:236–240.
124. **Matsuura, M., M. Arpin, C. Hannum, E. Margoliash, D. D. Sabatini, and T. Morimoto.** 1981. In vitro synthesis and posttranslational uptake of cytochrome *c* into isolated mitochondria. Role of specific addressing signal in the apocytochrome. Proc. Natl. Acad. Sci. U.S.A. **78**:4368–4372.
125. **McAda, P. C., and M. G. Douglas.** 1982. A neutral metallo endoprotease involved in the processing of an F_1-ATPase subunit precursor in mitochondria. J. Biol. Chem. **257**:3177–3182.
126. **Meek, R. L., K. A. Walsh, and R. D. Palmiter.** 1982. The signal sequence of ovalbumin is located near the NH_2 terminus. J. Biol. Chem. **257**:2245–2251.
127. **Meyer, D. I., and B. Dobberstein.** 1980. Identification and characterization of a membrane component essential for the translocation of nascent proteins across the membrane of the endoplasmic reticulum. J. Cell Biol. **87**:503–508.
128. **Meyer, D. I., E. Krause, and B. Dobberstein.** 1982. Secretory protein translocating across membranes—the role of the 'docking protein.' Nature (London) **297**:647–650.
129. **Michaelis, S., and J. Beckwith.** 1982. Mechanism of incorporation of cell envelope proteins in *Escherichia coli*. Annu. Rev. Microbiol. **36**:435–464.
130. **Michaelis, S., L. Guarente, and J. Beckwith.** 1983. In vitro construction and characterization of *phoA-lacZ* gene fusions in *Escherichia coli*. J. Bacteriol. **154**:356–

365.
131. **Michaelis, S., H. Inouye, D. Oliver, and J. Beckwith.** 1983. Mutations that alter the signal sequence of alkaline phosphatase in *Escherichia coli*. J. Bacteriol. **154**:366–374.
132. **Milstein, C., G. G. Brownlee, T. M. Harrison, and M. B. Mathews.** 1972. A possible precursor of immunoglobulin light chains. Nature (London) New Biol. **239**:117–120.
133. **Miura, T., and S. Mizushima.** 1968. Separation by density gradient centrifugation of two types of membranes from spheroplast membrane of *Escherichia coli* K12. Biochim. Biophys. Acta **150**:159–161.
134. **Moreno, F., A. V. Fowler, M. Hall, T. J. Silhavy, I. Zabin, and M. Schwartz.** 1980. A signal sequence is not sufficient to lead beta-galactosidase out of the cytoplasm. Nature (London) **286**:356–359.
135. **Mori, M., S. Miura, M. Tatibana, and P. P. Cohen.** 1980. Characterization of a protease apparently involved in processing of pre-ornithine transcarbamylase of rat liver. Proc. Natl. Acad. Sci. U.S.A. **77**:7044–7048.
136. **Mosmann, T. R., and A. R. Williamson.** 1980. Structural mutations in a mouse immunoglobulin light chain resulting in failure to be secreted. Cell **20**:283–292.
137. **Müller, M., I. Ibrahimé, C. N. Chang, P. Walter, and G. Blobel.** 1982. A bacterial secretory protein requires signal recognition particle for translocation across mammalian endoplasmic reticulum. J. Biol. Chem. **257**:11863–11864.
138. **Neu, H. C., and L. A. Heppel.** 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. **240**:3685–3692.
139. **Neupert, W., and G. Schatz.** 1981. How proteins are transported into mitochondria. Trends Biochem. Sci. **6**:1–4.
140. **Nielsen, J., G. H. Flemming, J. Hoppe, P. Friedl, and K. von Meyenburg.** 1981. The nucleotide sequence of the *atp* genes coding for the F_0 subunits a, b, c and the F_1 subunit δ of the membrane-bound ATP synthase of *Escherichia coli*. Mol. Gen. Genet. **184**:33–39.
141. **Novick, P., S. Ferro, and R. Schekman.** 1981. Order of events in the yeast secretory pathway. Cell **25**:461–469.
142. **Novick, P., C. Field, and R. Schekman.** 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell **21**:205–215.
143. **Novick, P., and R. Schekman.** 1979. Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. **76**:1858–1862.
144. **Oliver, D. B., and J. Beckwith.** 1981. *Escherichia coli* mutant pleiotropically defective in the export of secreted proteins. Cell **25**:765–772.
145. **Oliver, D. B., and J. Beckwith.** 1982. Identification of a new gene (*secA*) and gene product involved in the secretion of envelope proteins in *Escherichia coli*. J. Bacteriol. **150**:686–691.
146. **Oliver, D. B., and J. Beckwith.** 1982. Regulation of a membrane component required for protein secretion in *Escherichia coli*. Cell **30**:311–319.
147. **Oliver, D., C. Kumamoto, M. Quinlan, and J. Beckwith.** 1982. Pleiotropic mutants affecting the secretory apparatus of *Escherichia coli*. Ann. Microbiol. (Paris) **133**:105–110.
148. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962–3972.
149. **Osborn, M. J., and R. Munson.** 1974. Separation of the inner (cytoplasmic) and outer membranes of gram-negative bacteria. Methods Enzymol. **31**:642–653.
150. **Osborn, M. J., and H. C.-P. Wu.** 1980. Proteins of the outer membrane of gram-negative bacteria. Annu. Rev. Microbiol. **34**:369–422.
151. **Ozols, J., and C. Gerard.** 1977. Primary structure of the membranous segment of cytochrome b_5. Proc. Natl. Acad. Sci. U.S.A. **74**:3725–3729.
152. **Pages, J. M., M. Plovant, S. Varenne, and C. Lazdunski.** 1978. Mechanistic aspects of the transfer of nascent periplasmic proteins across the cytoplasmic membrane in *Escherichia coli*. Eur. J. Biochem. **86**:589–602.
153. **Palade, G. E.** 1975. Intracellular aspects of the process of protein synthesis. Science **189**:347–358.
154. **Palmiter, R. D., J. Gagnon, and K. A. Walsh.** 1978. Ovalbumin: a secreted protein without a transient hydrophobic leader sequence. Proc. Natl. Acad. Sci. U.S.A. **75**:94–98.
155. **Palva, E. T., R. T. Hirst, S. J. S. Hardy, J. Holmgren, and L. Randall.** 1981. Synthesis of a precursor to the B subunit of heat-labile enterotoxin in *Escherichia coli*. J. Bacteriol. **146**:325–330.
156. **Pearse, B. M. F.** 1976. Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. Proc. Natl. Acad. Sci. U.S.A. **73**:1255–1259.
157. **Post, L. E., A. E. Arfsten, G. R. Davis, and M. Nomura.** 1980. DNA sequence of the promoter region for the α ribosomal protein operon in *Escherichia coli*. J. Biol. Chem. **255**:4653–4659.
158. **Pratt, J. M., I. B. Holland, and B. G. Spratt.** 1981. Precursor forms of penicillin-binding proteins 5 and 6 of *E. coli* cytoplasmic membrane. Nature (London) **293**:307–310.
159. **Randall, L. L., and S. J. S. Hardy.** 1977. Synthesis of exported proteins by membrane-bound polysomes from *Escherichia coli*. Eur. J. Biochem. **75**:43–53.
160. **Rao, A., and R. A. F. Reithmeier.** 1979. Reactive sulfhydryl groups of the band 3 polypeptide from human erythrocyte membranes. J. Biol. Chem. **254**:6144–6150.
161. **Redman, C. M., and D. D. Sabatini.** 1966. Vectorial discharge of peptides released by puromycin from attached ribosomes. Proc. Natl. Acad. Sci. U.S.A. **56**:608–615.
162. **Redman, C. M., P. Siekevitz, and G. E. Palade.** 1966. Synthesis and transfer of amylase in pigeon pancreatic microsomes. J. Biol. Chem. **241**:1150–1158.
163. **Reid, G. A., and G. Schatz.** 1982. Import of proteins into mitochondria. J. Biol. Chem. **257**:13062–13067.
164. **Rogers, J., P. Early, C. Carter, K. Calame, M. Bond, L. Hood, and R. Wall.** 1980. Two mRNAs with different 3′ ends encode membrane-bound and secreted forms of immunoglobin μ chain. Cell **20**:303–312.
165. **Roggenkamp, R., B. Kustermann-Kuhn, and C. Hollenberg.** 1981. Expression and processing of bacterial β-lactamase in the yeast *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. **78**:4466–4470.
166. **Rothman, J. E., and R. E. Fine.** 1980. Coated vesicles transport newly synthesized membrane glycoproteins from endoplasmic reticulum to plasma membrane in two successive stages. Proc. Natl. Acad. Sci. U.S.A. **77**:780–784.
167. **Rothman, J. E., and H. F. Lodish.** 1977. Synchronized transmembrane insertion and glycosylation of a nascent membrane protein. Nature (London) **269**:775–780.
168. **Russel, M., and P. Model.** 1981. A mutation downstream from the signal peptidase cleavage site affects cleavage but not membrane insertion of phage coat protein. Proc. Natl. Acad. Sci. U.S.A. **78**:1717–1721.
169. **Ryter, A., H. Shuman, and M. Schwartz.** 1975. Integration of the receptor for bacteriophage lambda in the outer membrane of *Escherichia coli*: coupling with cell division. J. Bacteriol. **122**:295–301.
170. **Sabatini, D. D., and G. Blobel.** 1970. Controlled proteolysis of nascent polypeptides in rat liver cell fractions. II. Location of the polypeptides in rough microsomes. J. Cell Biol. **45**:146–157.
171. **Sabatini, D. D., G. Kreibich, T. Morimoto, and M. Adesnik.** 1982. Mechanisms for the incorporation of proteins in membranes and organelles. J. Cell Biol. **92**:1–22.

172. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-coded proteins. J. Bacteriol. **137**:692–693.
173. **Sando, G. N., and E. F. Neufeld.** 1977. Recognition and receptor-mediated uptake of a lysosomal enzyme, α-L-iduronidase by cultured human fibroblasts. Cell **12**:619–627.
174. **Schaller, H., E. Beck, and M. Takanami.** 1978. Sequence and regulatory signals of the filamentous phage genome, p. 139–153. *In* D. Denhardt, D. Dressler, and D. Ray (ed.), The single stranded DNA phages. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
175. **Schatz, G., and R. A. Butow.** 1983. How are proteins imported into mitochondria? Cell **32**:316–318.
176. **Schechter, I., D. J. McKean, R. Guyer, and W. Terry.** 1975. Partial amino acid sequence of the precursor of immunoglobin light chain programmed by messenger RNA in vitro. Science **188**:160–162.
177. **Schedl, P., and P. Primakoff.** 1973. Mutants of *Escherichia coli* thermosensitive for the synthesis of transfer RNA. Proc. Natl. Acad. Sci. U.S.A. **70**:2091–2095.
178. **Schleyer, M., B. Schmidt, and W. Neupert.** 1982. Requirement of a membrane potential for the posttranslational transfer of proteins into mitochondria. Eur. J. Biochem. **125**:109–116.
179. **Schmidt, G. W., A. Devillers-Thiery, H. Desruisseaux, G. Blobel, and N.-H. Chua.** 1979. NH_2-terminal amino acid sequences of precursor and mature forms of the ribulose-1,5-bisphosphate carboxylase small subunit from *Chlamydomonas reinhardtii*. J. Cell Biol. **83**:615–622.
180. **Schnaitman, C. A.** 1970. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. **104**:890–901.
181. **Schnaitman, C. A.** 1971. Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolated cell walls of *Escherichia coli*. J. Bacteriol. **108**:553–563.
182. **Schwartz, M., M. Roa, and M. Debarbouille.** 1981. Mutations that affect *lamB* gene expression at a posttranscriptional level. Proc. Natl. Acad. Sci. U.S.A. **78**:2937–2941.
183. **Shultz, J., T. J. Silhavy, M. L. Berman, N. Fiil, and S. D. Emr.** 1982. A previously unidentified gene in the *spc* operon of *Escherichia coli* K-12 specifies a component of the protein export machinery. Cell **31**:227–235.
184. **Shuman, H. A., T. J. Silhavy, and J. R. Beckwith.** 1980. Labeling of proteins with beta-galactosidase by gene fusion—identification of a cytoplasmic membrane component of the *Escherichia coli* maltose-transport system. J. Biol. Chem. **255**:168–174.
185. **Siekevitz, P., and G. E. Palade.** 1960. A cytochemical study on the pancreas of the guinea pig. V. In vivo incorporation of leucine-1-C^{14} into the chymotrypsinogen of various cell fractions. J. Biophys. Biochem. Cytol. **7**:619–631.
186. **Silhavy, T. J., P. J. Bassford, Jr., and J. R. Beckwith.** 1979. A genetic approach to the study of protein localization in *Escherichia coli*, p. 203–254. *In* M. Inouye (ed.), Bacterial outer membranes: biogenesis and functions. John Wiley & Sons, Inc., New York.
187. **Silhavy, T. J., M. J. Casadaban, H. A. Shuman, and J. R. Beckwith.** 1976. Conversion of β-galactosidase to a membrane-bound state by gene fusion. Proc. Natl. Acad. Sci. U.S.A. **73**:3423–3427.
188. **Silhavy, T. J., H. A. Shuman, J. Beckwith, and M. Schwartz.** 1977. Use of gene fusions to study outer membrane protein localization in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **74**:5411–5415.
189. **Silver, P., C. Watts, and W. Wickner.** 1981. Membrane assembly from purified components. I. Isolated M13 procoat does not require ribosomes or soluble proteins for processing by membranes. Cell **25**:341–345.
190. **Smith, W. P.** 1980. Cotranslational secretion of diphtheria toxin and alkaline phosphatase in vitro: involvement of membrane protein(s). J. Bacteriol. **141**:1142–1147.
191. **Smith, W. P., P.-C. Tai, and B. D. Davis.** 1981. *Bacillus licheniformis* penicillinase: cleavages and attachment of lipid during cotranslational secretion. Proc. Natl. Acad. Sci. U.S.A. **78**:3501–3505.
192. **Smith, W. P., P.-C. Tai, R. C. Thompson, and B. D. Davis.** 1977. Extracellular labeling of nascent polypeptides traversing the membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **74**:2830–2834.
193. **Steck, T. L.** 1978. The band 3 protein in the human red cell membrane. J. Supramol. Struct. **8**:311–324.
194. **Steck, T. L., J. J. Koziarz, M. K. Singh, G. Reddy, and H. Köhler.** 1978. Preparation and analysis of seven major, topographically defined fragments of band 3, the predominant transmembrane polypeptide of human erythrocyte membranes. Biochemistry **17**:1216–1222.
195. **Steck, T. L., B. Ramos, and E. Strapazon.** 1976. Proteolytic dissection of band 3, the predominant transmembrane polypeptide of the human erythrocyte membrane. Biochemistry **15**:1154–1161.
196. **Stevens T., E. Brent, and R. Schekman.** 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. Cell **30**:439–448.
197. **Struck, D. K., P. B. Siuta, M. D. Lane, and W. J. Lane.** 1978. Effect of tunicamycin on the secretion of serum proteins by primary cultures of rat and chick hepatocytes. J. Biol. Chem. **253**:5332–5337.
198. **Sugimoto, K., H. Sugisaki, T. Okamoto, and M. Takanami.** 1977. Studies on bacteriophage fd DNA. IV. The sequence of messenger RNA for the major coat protein gene. J. Mol. Biol. **110**:487–507.
199. **Suissa, M., and G. Schatz.** 1982. Import of proteins into mitochondria. Eur. J. Biochem. **257**:13048–13055.
200. **Sutcliffe, J. G.** 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc. Natl. Acad. Sci. U.S.A. **75**:3737–3741.
201. **Sveda, M. M., L. J. Markoff, and C.-J. Lai.** 1982. Cell surface expression of the influenza virus hemagglutinin requires the hydrophobic carboxy-terminal sequences. Cell **30**:649–656.
202. **Talmadge, K., J. Brosius, and W. Gilbert.** 1981. An internal signal sequence directs secretion and processing of proinsulin in bacteria. Nature (London) **294**:176–178.
203. **Talmadge, K., S. Stahl, and W. Gilbert.** 1980. Eukaryotic signal sequence transports insulin antigen in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **77**:3369–3373.
204. **Tokunaga, M., J. M. Loranger, P. B. Wolfe, and H. C. Wu.** 1982. Prolipoprotein signal peptidase in *Escherichia coli* is distinct from the M13 procoat protein signal peptidase. J. Biol. Chem. **257**:9922–9925.
205. **Tokunaga, M., H. Tokunaga, and H. C. Wu.** 1982. Posttranslational modification and processing of *Escherichia coli* prolipoprotein in vitro. Proc. Natl. Acad. Sci. U.S.A. **79**:2255–2259.
206. **Tsuji, T., T. Irimura, and T. Osawa.** 1980. The carbohydrate moiety of band-3 glycoprotein of human erythocyte membranes. Biochem. J. **187**:677–686.
207. **Walter, P., and G. Blobel.** 1980. Purification of a membrane-associated protein complex required for protein translocation across the endoplasmic reticulum. Proc. Natl. Acad. Sci. U.S.A. **77**:7112–7116.
208. **Walter, P., and G. Blobel.** 1981. Translocation of proteins across the endoplasmic reticulum. III. Signal recognition protein (SRP) causes signal sequence-dependent and site-specific arrest of chain elongation that is released by microsomal membranes. J. Cell Biol. **91**:557–561.
209. **Walter, P., and G. Blobel.** 1982. 7S small cytoplasmic RNA is an integral component of the signal recognition particle. Nature (London) **299**:691–698.
210. **Wandersman, C., F. Moreno, and M. Schwartz.** 1980. Pleiotropic mutations rendering *Escherichia coli* K-12 resistant to bacteriophage TP-1. J. Bacteriol. **143**:1374–1383.
211. **Wanner, B. L., A. Sarthy, and J. Beckwith.** 1979. *Esche-*

richia coli pleiotropic mutant that reduces amounts of several periplasmic and outer membrane proteins. J. Bacteriol. **140**:229–239.

212. **Watts, C., P. Silver, and W. Wickner.** 1981. Membrane assembly from purified components. 2. Assembly of M13 procoat into liposomes reconstituted with purified leader peptidase. Cell **25**:347–353.
213. **White, J., and A. Helenius.** 1980. pH-dependent fusion between the Semliki-forest virus membrane and liposomes. Proc. Natl. Acad. Sci. U.S.A. **77**:3273–3277.
214. **Wickner, W.** 1976. Asymmetric orientation of phage M13 coat protein in *Escherichia coli* cytoplasmic membranes and in synthetic lipid vesicles. Proc. Natl. Acad. Sci. U.S.A. **73**:1159–1163.
215. **Wickner, W.** 1979. Assembly of proteins into biological membranes: membrane trigger hypothesis. Annu. Rev. Biochem. **48**:23–45.
216. **Wickner, W.** 1980. Assembly of proteins into membranes. Science **210**:861–868.
217. **Williams, D. G., R. E. Jenkins, and M. J. A. Tanner.** 1979. Structure of the anion-transport protein of the human erythrocyte membrane: further studies of the fragments produced by proteolytic digestion. Biochem. J. **181**:477–493.
218. **Wirth, D. F., H. F. Lodish, and P. W. Robbins.** 1979. Requirements for the insertion of the Sindbis envelope glycoproteins into the endoplasmic reticulum membrane. J. Cell Biol. **81**:154–162.
219. **Wittmann, H. G., J. A. Littlechild, and B. Wittmann-Liebold.** 1980. Structure of ribosomal proteins, p. 51–88. *In* G. Chambliss, G. R. Craven, J. Davies, K. Davies, L. Kahan and M. Nomura (ed.), Ribosomes: structure, function, and genetics. University Park Press, Baltimore.
220. **Wolfe, P. B., P. Silver, and W. Wickner.** 1982. The isolation of homogeneous leader peptidase from a strain of *Escherichia coli* which overproduces the enzyme. J. Biol. Chem. **257**:7898–7902.
221. **Wu, H. C., and J. J.-C. Lin.** 1976. *Escherichia coli* mutants altered in murein lipoprotein. J. Bacteriol. **126**:147–156.
222. **Yamagata, H., C. Ippolito, M. Inukai, and M. Inouye.** 1982. Temperature-sensitive processing of outer membrane lipoprotein in an *Escherichia coli* mutant. J. Bacteriol. **152**:1163–1168.
223. **Zilberstein, A., M. D. Snider, M. Porter, and H. F. Lodish.** 1980. Mutants of vesicular stomatitis virus blocked at different stages in maturation of the viral glycoprotein. Cell **21**:417–427.
224. **Zimmerman, R., and W. Neupert.** 1980. Transport of proteins into mitochondria. Eur. J. Biochem. **109**:217–229.
225. **Zimmerman, R., U. Paluch, and W. Neupert.** 1979. Cell-free synthesis of cytochrome *c*. FEBS Lett. **108**:141–146.
226. **Zitomer, R. S., and B. D. Hall.** 1976. Yeast cytochrome *c* messenger RNA. J. Biol. Chem. **251**:6320–6326.
227. **Zwizinski, C., T. Date, and W. Wickner.** 1981. Leader peptidase is found in both the inner and outer membranes of *Escherichia coli*. J. Biol. Chem. **256**:3593–3597.
228. **Zwizinski, C., and W. Wickner.** 1980. Purification and characterization of leader (signal) peptidase from *Escherichia coli*. J. Biol. Chem. **255**:7973–7977.