A Mechanism of Protein Localization: The Signal Hypothesis and Bacteria

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ABSTRACT We are studying the molecular mechanism of cellular protein localization. The availability of genetic techniques, such as gene fusion in *Escherichia coli*, has made this problem particularly amenable to study in this prokaryote. We have constructed a variety of strains in which the gene coding for an outer membrane protein is fused to the gene coding for a normally cytoplasmic enzyme, β-galactosidase. The hybrid proteins produced by such strains retain β-galactosidase activity; this activity serves as a simple biochemical tag for studying the localization of the outer membrane protein. In addition, we have exploited phenotypes exhibited by certain fusion strains to isolate mutants that are altered in the process of protein export. Genetic and biochemical analyses of such mutants have provided evidence that the molecular mechanism of cellular protein localization is strikingly similar in both bacteria and animal cells.

"What is true for *E. coli* is true for elephants, only more so."
J. Monod

The complex machinery responsible for the synthesis of proteins invariably is located in the cytoplasm of the cell. Nevertheless, proteins are localized to a variety of subcellular compartments. Furthermore, many cells are capable of true protein secretion. The processes of cellular protein localization and secretion are selective and efficient. Proteins indigenous to a particular cellular location rarely, if ever, are found in another. Thus, all cells must have mechanisms for sorting and distributing proteins to their correct cellular locations. The basic goal of our work is to characterize these mechanisms in molecular terms.

Essentially everything currently known about the process of protein localization stems from the work of George Palade. In pioneering studies, Palade and his co-workers traced the intracellular routing of a variety of noncytoplasmic proteins. They demonstrated, for example, that proteins destined to be secreted are synthesized initially by ribosomes tightly bound to the rough endoplasmic reticulum. Such proteins are found in the lumen of this organelle immediately after synthesis is completed. Subsequently, the protein is routed to the Golgi apparatus before secretion (22).

In molecular terms, perhaps the most difficult step to envision in the process of protein secretion is the transfer of a large, water-soluble protein through a hydrophobic membrane. Palade's work suggested that this step occurs at the level of the rough endoplasmic reticulum membrane. Furthermore, it became apparent that this process occurs in a manner tightly coupled to, if not inseparable from, translation. A number of ways in which this step might be accomplished have been proposed (for recent review, see reference 10).

The best known of these is the "signal hypothesis." Although evidence suggesting this hypothesis had been accumulating for several years, widespread interest in it was not aroused until the work of Blobel and Dobberstein appeared in 1975 (6). In their work, the basic features of the signal hypothesis and supporting experimental evidence were presented. An updated version of the signal hypothesis is shown in Fig. 1.

According to the signal hypothesis, a protein destined to be secreted from cells is synthesized initially as a larger precursor with 15–30 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 reticulum membrane. This binding results in the formation of a transient pore through which the nascent peptide chain passes as synthesis proceeds. The net result is a vectorial transfer of the protein across the rough endoplasmic reticulum 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 signal hypothesis is attractive for several reasons, but we mention only two. First, it does not require the existence of a special class of "membrane-bound" ribosomes. According to the model, it is the signal sequence, not the ribosome, that initiates binding of the translation complex to the membrane. This is consistent with the fact that no special "membrane-bound" class of ribosomes has been identified, despite an extensive search. Second, and more importantly, the model makes a specific, experimentally testable prediction, i.e., proteins destined for secretion should be seen as larger precursors

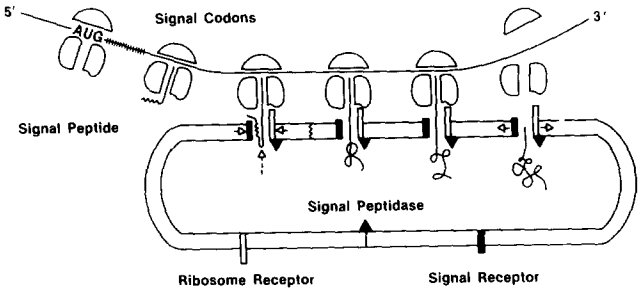

FIGURE 1 Schematic illustration of the signal hypothesis for the translocation of secretory proteins across the rough endoplasmic reticulum membrane. Various stages of the translation of an mRNA for a secretory protein on a membrane-bound polysome are indicated. The polysome contains six ribosomes. The first two ribosomes near the 5′ end of mRNA are not yet bound to the membrane. The nascent chain is indicated to grow in a tunnel in the large ribosomal subunit. The signal peptide portion is indicated as a zig-zagged line at the amino-terminus of the nascent chain (see second and third ribosomes) or cleaved from the nascent chain and located in the membrane (see membrane between ribosome 3 and 4). The signal-peptidase site of the nascent chain is indicated by an upward-pointing arrowhead connected to a dashed line. Ribosome-receptor and signal-receptor activity are represented arbitrarily by two different integral membrane proteins. Alternatively, these two activities could be represented as two separate domains on one integral membrane protein (reference 7; source: Günter Blobel, The Rockefeller University).

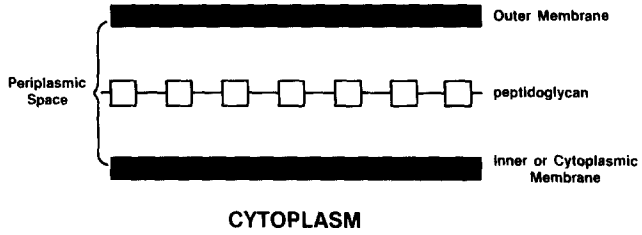

FIGURE 2 Major envelope layers of a gram-negative bacteria such as *E. coli*. The envelope layer exposed to the external environment is called, appropriately enough, the outer membrane. Although it resembles a typical biological membrane in the electron microscope, this membrane is structurally and functionally quite different. The outer membrane is high in protein content; however, there are relatively few different protein species. The major proteins of this membrane function as pores through which small water-soluble molecules can diffuse and/or they play structural roles. Certain other proteins are involved in the transport of specific substrates. Most, if not all, serve as receptors for various different bacteriophages and/or certain proteins, e.g., colicins. The outer membrane is nearly devoid of enzymatic activity. Only a phospholipase and a protease are known to be located here. The characteristic lipid of gram-negative bacteria, lipopolysaccharide, is found solely in the outer leaflet of the outer membrane bilayer. The peptidoglycan is a linear polysaccharide polymer cross-linked by short peptide bridges. It forms a rigid net surrounding the bacteria protecting against osmotic lysis in a dilute environment. The inner or cytoplasmic membrane is the true permeability barrier of the cell. This membrane is very similar to other biological membranes. Most common membrane-associated enzymatic activities, ATPase, lipid biosynthesis, active transport, etc., are located here. The inner and outer membranes delimit a zone of controlled permeability. This aqueous compartment, the periplasm, contains a variety of different protein species. Generally speaking, these proteins are either degradative enzymes, i.e., alkaline phosphatase or RNase, or binding proteins involved in the active transport of certain substrates, e.g., maltose-binding protein. (for reviews, see references 13 and 17).

(with the signal sequence still attached) when made "in vitro," in the absence of the rough endoplasmic reticulum and the associated processing activity. This has now been demonstrated for a variety of proteins. This surge of supporting evidence prompted a general acceptance of the hypothesis. (Variants of the model have appeared to explain protein localization to the cytoplasmic membrane, lysosomes, etc. [5, 7, 20, 27].) However, though the existence of a plethora of precursors admittedly is compelling, it cannot be taken as conclusive proof for the signal hypothesis. Such evidence is only circumstantial.

As bacterial geneticists, we are struck by yet another aspect of Blobel's work. The in vitro system employed by Blobel and co-workers is a concoction of disparate elements. It contains translation factors from plants, ribosomes from rabbits, and rought endoplasmic reticulum from dogs. Nevertheless, it works. Does this mean that the process of protein localization is conserved throughout the animal and plant kingdoms? Can a bacterium such as *Escherichia coli* employ a mechanism of localization similar to that of higher organisms?

E. coli is a primitive organism when compared to an animal cell. The cellular envelope of *E. coli* can be depicted rather accurately by a three-line drawing (Fig. 2). Basically, this bacterium is a cytoplasm surrounded by three concentric envelope layers: an inner membrane (cytoplasmic membrane), a peptidoglycan cell wall, and an outer membrane. Intracellular organelles such as nucleus, mitochondria, endoplasmic reticulum, Golgi complex, etc. are absent. Considering that intercellular organelles play a critical role in the process of protein localization in eukaryotic cells, it may seem far-fetched to propose that *E. coli* and animal cells employ a similar mechanism of protein localization. However, a considerable amount of experimental evidence supports this claim (7, 10, 20, 31, 34).

Bacteria appear to have delegated certain functions of eukaryotic organelles to the cytoplasmic membrane. For example, ATP synthesis, a function of mitochondria, occurs in the cytoplasmic membrane of bacteria. Lipid biosynthesis, a function of the endoplasmic reticulum, occurs in the cytoplasmic membrane of bacteria. Furthermore, the inner and outer membranes of *E. coli* delimit a zone of controlled permeability called the periplasmic space (Fig. 2). It has been suggested that this cellular compartment is an evolutionary precursor of lysosomes (12). It is believed that the cytoplasmic membrane of bacteria is functionally analogous to the rough endoplasmic reticulum with respect to protein localization.

We have used a bacterium, *E. coli*, to study protein localization. In particular, we are studying the mechanism of localization of the receptor for the bacteriophage λ. This protein, coded for by the gene *lamB*, is an integral outer membrane protein. Mutants lacking this protein are resistant to infection and killing by phage λ (25). In addition, this protein functions in the transport of maltose (37) and is essential for the transport of maltodextrins (32). Mutants lacking this protein are capable of growth on maltose (Mal^+) but are incapable of growth on maltodextrins (Dex^-). Its role in maltose transport is reflected by the fact that synthesis of the protein is induced by maltose (11).

Randall et al. (24) have shown that λ receptor is synthesized preferentially as a larger precursor by ribosomes bound to the cytoplasmic membrane. Recent studies involving DNA sequencing of the *lamB* gene and amino acid analysis of the precursor protein have revealed that the precursor contains a 25 amino acid extension at the NH_2-terminal end of the

molecule. This additional sequence (signal sequence) contains a high proportion of hydrophobic amino acids.[1] All of these results are consistent with predictions made by the signal hypothesis.

Rationale for the Use of Gene Fusions to Study Cellular Protein Localization

The signal hypothesis states that the information specifying a noncytoplasmic location is contained within the structural gene of the exported protein. This part of the structural gene codes for the signal sequence. Genetically, this aspect of the hypothesis can be tested in several ways. One way is to isolate mutations that alter the signal sequence and to demonstrate that these mutations block localization. Another way is to alter the cellular location of a normally cytoplasmic protein by attaching the DNA coding for a signal sequence to the structural gene of this protein. If a "hybrid" gene could be constructed so that the resulting "hybrid" protein was synthesized with an intact signal sequence at the NH_2-terminus, the signal hypothesis would predict that this protein should be exported.

Using techniques developed by Malcolm Casadaban (8), it is possible to fuse (i.e., create a hybrid gene) the gene coding for the cytoplasmic enzyme β-galactosidase (*lacZ*) to any gene in *E. coli*. This technique allows one to fuse a portion of the gene coding for a noncytoplasmic protein to a consistently reproducible portion of the gene *lacZ*. All such fusions are identical in that each results in the production of a hybrid protein comprised of an NH_2-terminal sequence of the noncytoplasmic protein in question (λ receptor) and a major, functional portion of the COOH-terminal sequence of β-galactosidase (Fig. 3). By constructing a series of *lamB-lacZ* fusions differing only in the amount of *lamB* DNA contained in the hybrid gene and determining the cellular location of the hybrid protein produced, we have been able to define regions within the *lamB* structural gene that must be involved in determining cellular location.

Properties of Various lamB-lacZ Fusions

A series of *lamB-lacZ* fusions have been constructed. Genetic mapping has revealed that these hybrid genes contain varying amounts of *lamB* DNA. This genetically determined variation in size is corroborated by the molecular weights of the hybrid proteins produced by these gene fusions (Fig. 4). Based on these results, the *lamB-lacZ* fusions have been grouped into four distinct size classes. The properties of representative *lamB-lacZ* fusions from each of the four size classes are summarized in Table I.

Class I fusions are the smallest and the most frequently obtained. Fusion 61-4 is the most extensively studied of this group. This fusion contains such a small amount of *lamB* DNA that the fusion joint maps earlier in *lamB* (corresponds to the extreme NH_2-terminal end of the λ receptor) than any known mutation in the gene. The apparent molecular weight of this fusion protein, 115,000, is slightly smaller than that of wild-type β-galactosidase. The hybrid protein synthesized by strains carrying fusions of this class are localized exclusively in the cytoplasm (32). In collaboration with Maxime Schwartz and Audree Fowler, we have purified the 61-4 hybrid protein and determined the amino acid sequence of the NH_2-terminal portion of the molecule. Only three amino acids[2] coded for by the *lamB* gene are present in the hybrid protein (21). The cytoplasmic location of this hybrid protein, therefore, is not surprising.

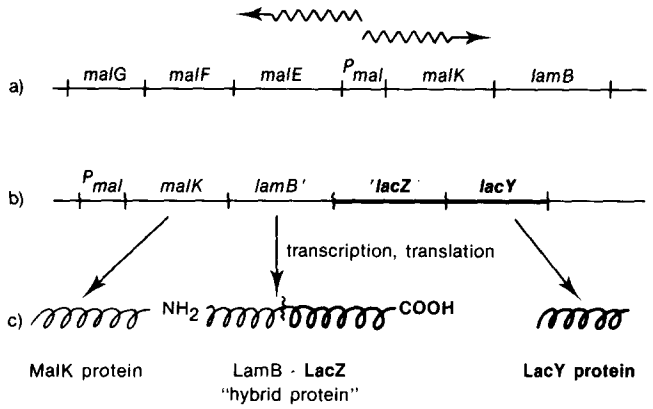

FIGURE 3 (a) The two divergent operons that comprise the *malB* locus in *E. coli* (16). These two operons specify five proteins, which together make up the active transport system for maltose and maltodextrins. Both operons, and thus the synthesis of all five proteins, are induced by maltose in the growth media (11). At least three of these proteins are destined to be localized to noncytoplasmic compartments. The *malE* gene specifies the periplasmic MBP (19), *malF* specifies an inner membrane protein (30), and *lamB* specifies the outer membrane protein, λ receptor (25). The *malK* and *malG* gene products have not been identified. Transcription of these two operons is initiated from a central region (Pmal). 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* result 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 comprised of λ receptor 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. In order for *E. coli* to express a Lac^+ phenotype, strains must have both LacZ (β-galactosidase) and LacY (lactose transport) protein activities.

The one class II fusion strain that exists is similar to the class I fusions. This fusion, 52-4, contains slightly more *lamB* DNA and produces a hybrid protein of mol wt 118,000, which is slightly larger than β-galactosidase. Again, in collaboration with Schwartz and Fowler, we have purified the 52-4 hybrid protein and determined the amino acid sequence of the amino-terminal portion of the molecule. Thirty-nine amino acids corresponding to the NH_2-terminal end of precursor λ receptor are present in the hybrid protein. This protein, therefore, contains the entire λ receptor signal sequence plus an additional 15 amino acids. Although evidence presented below suggests

[1] Hedgpeth, J., J.-M. Clement, S. Marchal, D. Perrin, and M. Hofnung. 1980. DNA sequence encoding the NH_2-terminal peptide involved in transport of λ receptor, an *Escherichia coli* secretory protein. *Proc. Natl. Acad. Sci. U. S. A.* 77:2621–2625.

[2] According to the DNA sequence, the λ receptor protein should begin with the amino acids met-met . . . (footnote 1). However, the two hybrid proteins that have been sequenced begin with only a single met (21). We do not know if this is caused by initiation at the second, rather than the first, AUG codon of the mRNA, or if a proteolytic cleavage removing the first met occurs in vivo. To avoid confusion, we will assume for the purposes of this article that the *lamB* gene product begins with two successive met residues.

that this hybrid transiently binds to the membrane, it does remain in the cytoplasm (21). Apparently, a signal sequence alone is not sufficient to specify export. This fusion will be discussed in greater detail below.

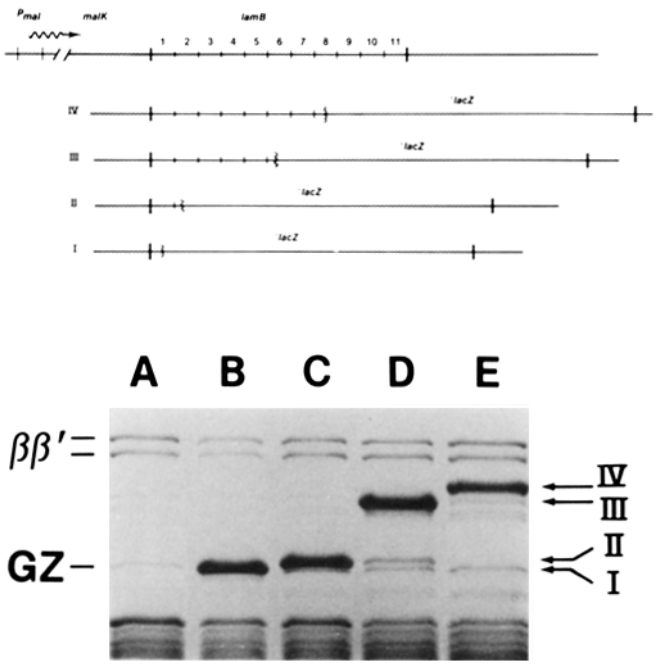

FIGURE 4 *lamB-lacZ* fusion size classes. The genetically determined fusion size classes are depicted schematically above. The *lamB* gene has been divided into 11 deletion groups (23). The deletion segments with which the fusions of each size class recombine are indicated on each fusion. The amount of *lacZ* coded protein, β-galactosidase, is constant in all fusions. Below the schematic drawing is an SDS polyacrylamide gel demonstrating the variation in size of the hybrid proteins made by the different fusion classes. The hybrid proteins are of higher molecular weight than the vast majority of *E. coli* proteins. Thus, proteins can be visualized simply by electrophoresing a whole cell extract of a fusion strain. Shown here is the high molecular weight portion of the gel. Channel *A* is the parental strain in which all fusions were constructed. Channels *B*–*E* are fusion strains of size classes I through IV, respectively. The position of β-galactosidase on such a gel is indicated. Also indicated are the *ββ'* subunits of RNA polymerase.

Class III fusions contain approximately one-half of the *lamB* gene and produce a hybrid protein with an apparent mol wt of 137,000. These hybrid proteins are localized to the outer membrane, but not efficiently. Fusion 42-1, the best characterized class III fusion, produces a hybrid protein that is found in the cytoplasm, the inner membrane, and the outer membrane in approximately equal amounts. Strains containing fusions of this size class also exhibit a very characteristic maltose-sensitive (Mal^s) phenotype (32). When synthesis of large amounts of hybrid protein is induced by the addition of maltose to the growth medium, cells lyse after a few hours. We believe that this Mal^s phenotype is a direct result of the cells' inability to localize efficiently large amounts of the hybrid protein (see the following section).

Class IV fusions contain more *lamB* DNA than any other *lamB-lacZ* fusion class and, accordingly, produce the largest hybrid proteins (141,000). In view of our results with class III *lamB-lacZ* fusions, we expected that class IV fusions would be extremely Mal^s as a result of their larger size. This, however, is not the case. Although the class IV fusion strains do exhibit some maltose sensitivity, this sensitivity is much less than that observed with class III fusion strains.

Determining the cellular location of the class IV hybrid proteins has proven to be difficult for several reasons. The β-galactosidase activity produced by these fusions is much lower than that of other *lamB-lacZ* fusions. The amount of hybrid protein produced, however, is roughly equivalent to the amount produced by class III fusions. Thus, we conclude that the specific activity of the class IV hybrid proteins, relative to β-galactosidase, is very low. This is quite unusual, as no other hybrid protein class that we have studied has such a low specific activity, even though all of the fusions we have studied contain a nearly identical portion of β-galactosidase. This low specific activity has forced us to look directly at the hybrid protein in each cellular fraction by SDS gel electrophoresis, rather than simply assaying for β-galactosidase activity. If this is done, we find that essentially all of the hybrid protein is membrane-bound. Our major problem, at present, is to determine, quantitatively, the amount of hybrid protein present in the inner and outer membranes. We have found that different methods give different results. Clearly, more careful fractionation studies must be performed. Nevertheless, we feel confident in saying that the majority of the class IV hybrid protein

TABLE I

Relevant Properties of Various lamB-lacZ Fusion Strains and Hybrid Proteins

Fusion class	Example	Phenotype	Induced β-galactosidase activity	Cellular hybrid protein localization: Inner membrane	Outer membrane	Periplasm	Cytoplasm	Approximate hybrid protein mol wt	Approximate amount of *lamB* DNA present in hybrid gene*
			U	%					
I	61-4	Mal^+Lac^+	900–1,100	<10	<1	<1	90	115,000	<1/11
II	52-4	Mal^+Lac^+	2,000–3,000	<15	<1	<1	85	118,000	1/11
III	42-1	Mal^sLac^+	1,000–1,300	25	30	2	43	137,000	5/11
IV	42-12	Mal^+Lac^-	20–50	<10	~80	NT	<10‡	141,000	7/11

NT, not tested

* The *lamB* gene has been divided into 11 segments by deletion mapping. The value listed represents the fraction of segments that are present in the hybrid gene and that were not removed during construction (23). It should be noted that this is a genetic result, not a physical result, and accordingly these data are only an estimate. If deletion endpoints are nonrandom, it is possible that these estimates are substantially incorrect.

‡ Exact fractionation data with these strains are difficult to give because of the extremely low specific activity of the hybrid protein. These values represent estimates from gels of the various cellular fractions.

is localized to the outer membrane. The efficient localization of the class IV fusion suggests that it contains most, if not all, of the information specifying an outer membrane location.

Intragenic Information Specifying Cellular Location: Involvement of the Signal Sequence in the Export Process

The class III *lamB-lacZ* fusions, in particular fusion 42-1, have been used to identify information within the *lamB* gene that is essential for normal export of the λ receptor protein. As stated above, strains containing these fusions exhibit a characteristic Mal^s phenotype. When grown in the presence of maltose (conditions that cause synthesis of large amounts of the hybrid protein), the cells filament and ultimately lyse. We have demonstrated that this Mal^s phenotype is the result of the cells' inability to efficiently export this hybrid protein (15). Synthesis of large amounts of the hybrid protein results in a lethal jamming of the export machinery. This is supported by the observation that, under conditions in which large amounts of the hybrid protein are synthesized, precursors of many envelope proteins can be detected accumulating in the moribund cell (footnote 3, reference 3, and Fig. 5). Consequently, we believe that this hybrid protein contains substantial, but incomplete, export information.

Because the Mal^s phenotype is a consequence of the defective export of the hybrid protein, then selecting a maltose-resistant (Mal^r) phenotype should yield mutants in which export of the hybrid protein is blocked. We have isolated several Mal^r mutants. To date, 26 have been analyzed genetically, biochemically, and physically. All of these mutants fail to export the hybrid protein. In each of the mutant strains, the hybrid protein is found in soluble form in the cytoplasm.[3] The genetic lesion in each of the 26 mutants maps very early in the *lamB* portion of the hybrid gene, in a region corresponding to the NH_2-terminal end of the λ receptor precursor.

To determine the effect of the mutations on an otherwise wild-type λ receptor protein and to do further genetic analysis, these mutations were recombined from the *lamB-lacZ* hybrid gene into a wild-type *lamB* gene. We find that all of these mutations confer a typical LamB$^-$ phenotype to wild-type strains, i.e., the inability to grow on maltodextrins (Dex$^-$) and resistance to phage λ (λ^r). Taking advantage of these properties, we were able to perform reversion analyses and recombinational crosses with the different mutants by selecting Dex$^+$ revertants or recombinants. These studies enabled us to identify the mutations as 12 deletion and 14 point mutations (Fig. 7). DNA restriction analysis has shown that the deletions range in size from 12 to 500 base pairs. The point mutations have been mapped to four genetically distinct sites. (In other words, even though all of these mutations were isolated independently, many of them are identical.) All of the mutations lie in a very small, well-defined region, extremely early in the *lamB* gene (Fig. 7).

The effect of these mutations on the localization of the λ receptor protein was determined by fractionating the mutant cells into the four cellular compartments (cytoplasm, periplasm, inner membrane, and outer membrane). Immune precipitation of each of these fractions with anti-λ receptor serum has shown

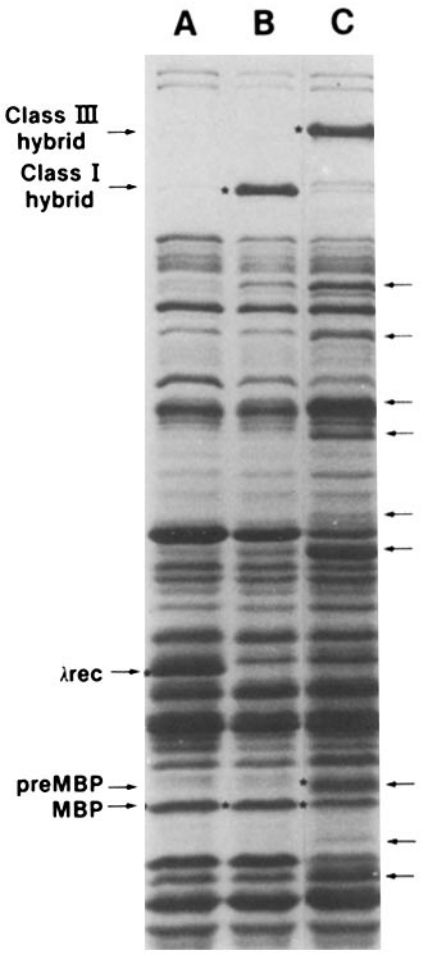

FIGURE 5 Jamming of the export machinery. Shown is an SDS polyacrylamide gel of whole cell extracts from maltose-induced strains (induced for synthesis of the LamB-LacZ hybrid protein); lane (*A*) a wild-type strain; (*B*) a class I fusion strain, 61-4; (*C*) a class III fusion strain, 42-1. The accumulating precursors of several envelope proteins in the induced class III fusion strain (lane C) are indicated by arrows to the right of the gel. Such precursors do not appear in the wild-type strain (lane *A*) or in a class I fusion strain (lane *B*) when treated in the same way. As is shown, one of these accumulating precursors, the precursor maltose-binding protein (preMBP), has been identified. A similar pattern of precursor accumulation has been found with *malE-lacZ* fusion strains (3).

that the mutant λ receptor precursor is found in the cytoplasmic fraction of these cells (Fig. 6). Presumably, in this location, the protein is sequestered from the signal peptidase, which is necessary for cleavage of the signal sequence from the precursor. These results demonstrate that early *lamB* mutations can prevent export of both the *lamB-lacZ* hybrid protein and the λ receptor protein.

All of the evidence described above is consistent with the idea that the mutations lie in the portion of the *lamB* gene that codes for the signal sequence and that the amino acid alterations in this sequence prevent a step in the export of the λ receptor protein to the outer membrane. Presumably, this must be an early step, as the mutant proteins remain in the cytoplasm. DNA sequencing, done in collaboration with Joe Hedgpeth and Maurice Hofnung (14; see Fig. 7), demonstrate that all of the mutations cause alterations in the λ receptor signal sequence. These mutations, therefore, provide conclusive proof

[3] 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.* In press.

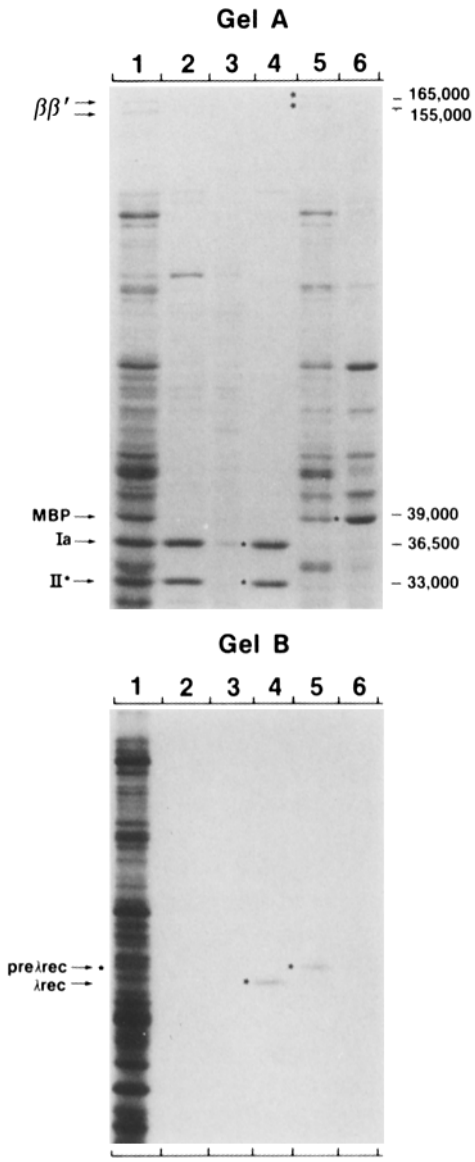

FIGURE 6 Accumulation of precursor λ receptor in the cytoplasm of the *lamB* signal sequence mutants. In gel *A*, ^{14}C-labeled samples from each of the cellular fractions from a representative signal sequence mutant were subjected to SDS polyacrylamide gel electrophoresis. Samples from each of the cellular fractions also were subjected to immune precipitation with rabbit anti-λ receptor serum and to gel electrophoresis as is shown in gel *B*. Gel *A*; lane (*1*) whole cell extract; (*2*) total membrane fraction (inner and outer); (*3*) inner membrane; (*4*) outer membrane; (*5*) total soluble fraction (cytoplasm and periplasm); (*6*) periplasm. Marker proteins known to be localized to specific cellular compartments are indicated. These include: the outer membrane proteins Ia and II*; the cytoplasmic RNA polymerase subunits (β and β'); and the periplasmic MBP. Gel *B*; lane (*1*) whole cell extract; (*2*) anti-λ receptor precipitation from the inner membrane fraction; (*3*) anti-λ receptor precipitation from the outer membrane fraction; (*4*) marker wild-type λ receptor protein; (*5*) anti-λ receptor precipitation from the soluble fraction; (*6*) anti-λ receptor precipitation from the periplasmic fraction.

for at least one aspect of the signal hypothesis: a functional signal sequence is required for export.

A comparison of known prokaryotic and eukaryotic precursor sequences (signal sequences) indicates that they share several similar characteristics. The seven known prokaryotic signal sequences (Fig. 7) all can be broken down into two principal segments, a short NH_2-terminal hydrophilic basic segment followed by a predominantly hydrophobic segment of amino acids that extends up to the site of processing. There are one to three basic amino acids (arg and/or lys) in the basic segment of these signal sequences. It has been suggested that these positively charged residues play a role in the initial attachment of the polysomes to the negatively charged inner surface of the cytoplasmic membrane (13). The hydrophobic segment directly follows the last basic residue of the initial hydrophilic segment. Charged amino acids, basic or acidic, are completely absent from this portion of the signal sequence. It has been suggested that this hydrophobic region loops into the membrane lipid bilayer or becomes associated with specific membrane protein(s); transmembrane transfer of the polypeptides then is initiated (13; Fig. 1).

The mutations in the λ receptor signal sequence described here argue strongly that the hydrophobic segment of the signal sequence plays a critical role in the initiation of the export process. The four different single amino acid changes in the λ receptor signal sequence are all changes from hydrophobic (val, ala, met) amino acids to hydrophilic, charged amino acids (asp, glu, arg), and all of these changes are in the hydrophobic segment of the signal sequence (14; footnote 3). These mutations and similar mutations that have been isolated in the signal sequence region of the *malE* gene provide strong support for a requirement of the hydrophobicity of this segment (2–4). The possibility that other features of the signal sequence are essential for its function, however, must be considered. For example, the secondary structure or the NH_2-terminal basic segment of this sequence may be important. At present, we do not have sufficient mutant data to comment on these features of the signal sequence.

The fact that all of the export-defective mutations characterized to date lead to alterations in the signal sequence and not elsewhere in the protein suggests that the signal sequence is the only component required for attachment of ribosomes to the cytoplasmic membrane and for initiation of passage of the protein through the membrane. This, however, does not mean that the signal sequence is all that is required for the secretion of a protein.

Other Information within the lamB Gene Specifying Cellular Location

The signal sequence mutants described above demonstrate that an intact signal sequence is necessary to initiate protein export. We now can ask if the signal sequence alone is sufficient for export. Results discussed in this section provide strong evidence that this is not the case.

The most compelling evidence we have to support the contention that a signal sequence alone is not sufficient for export comes from the amino acid sequence analysis of the class II *lamB-lacZ* fusion 52-4 (21). Until residue 39, the NH_2-terminal sequence of the 52-4 protein is that of the λ receptor protein precursor. Residue 41 of the hybrid protein corresponds to residue 20 of β-galactosidase. Therefore, the 52-4 protein not only possesses the complete signal sequence of the λ receptor protein precursor but also the first 15 amino acids of the mature λ receptor protein. In spite of this, the 52-4 protein is cytoplasmic. This result plus the fact that classes III and IV *lamB-lacZ* fusions are exported demonstrate that, for β-galactosidase at

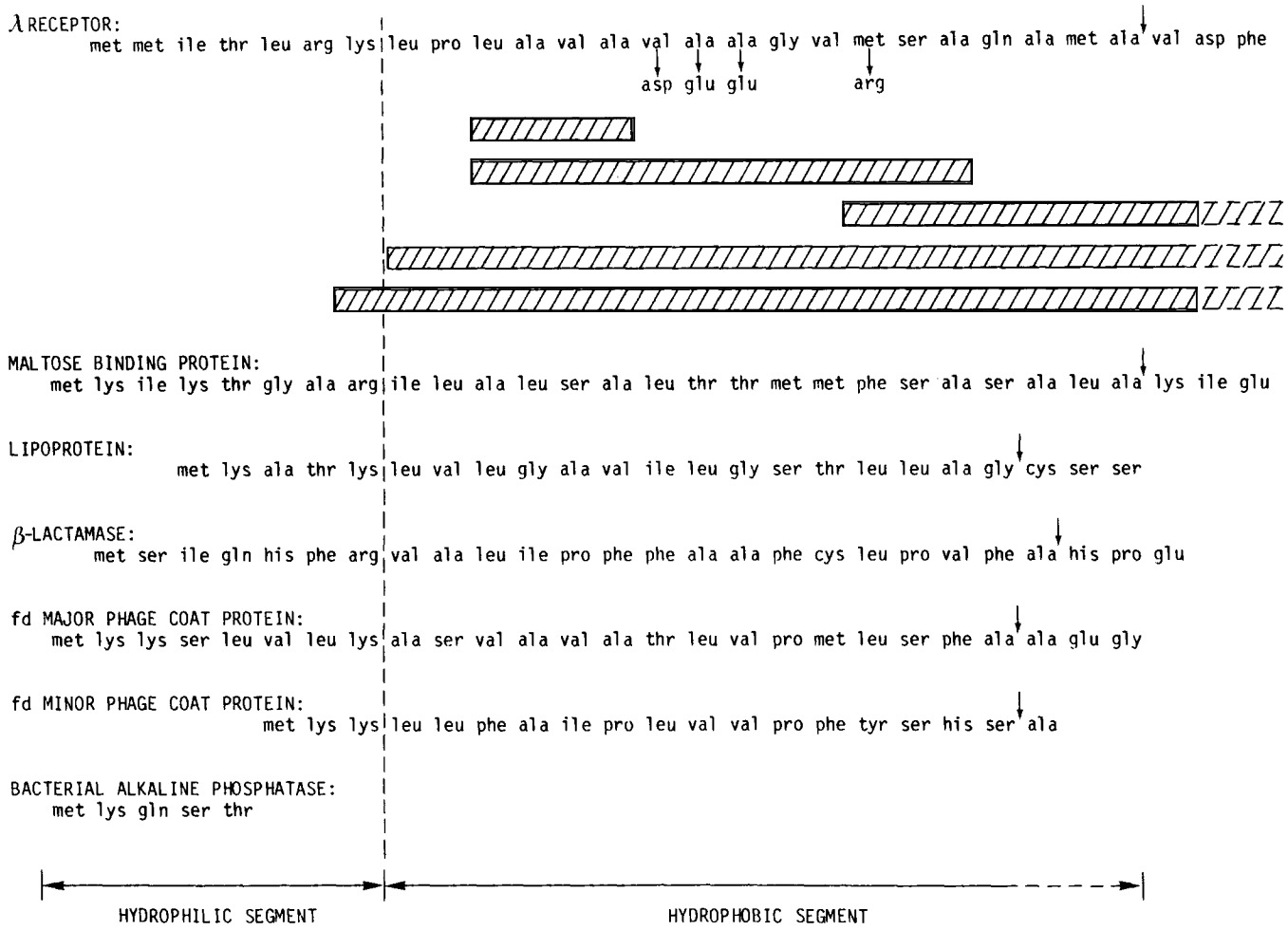

FIGURE 7 The amino acid sequence of 7 known prokaryotic signal sequences is shown. These include λ receptor,[1] MBP (4), lipoprotein (18), β-lactamase (36), fd major phage coat protein (35), fd minor phage coat protein (29), and alkaline phosphatase (28). Mutational alterations (deletions and point mutations) that have been isolated in the λ receptor signal sequence are indicated below the wild-type sequence (14). The site of processing for these signal sequences is indicated above each sequence by an arrow.

least, a signal sequence alone is not sufficient for export. Other information within the *lamB* gene must be required.

In addition to the information within the *lamB* gene that specifies export from the cytoplasm, there must be information to specifically direct the protein product to the outer membrane, as opposed to the periplasm, for example. The nature of the distinct phenotypes exhibited by class III and class IV *lamB-lacZ* fusion strains provides evidence for this additional information in λ receptor. This information is present, presumably, only in fusions of the larger size class. These fusions appear to be localized efficiently to the outer membrane, and these cause only a mild form of maltose sensitivity. Conversely, class III fusions are localized poorly to the outer membrane. A fully induced class III fusion also is lethal to the cell. To account for these different phenotypes, we have proposed the existence of a "dissociation sequence." The role of the dissociation sequence is shown in Fig. 8. It serves as a signal for the release of the translating ribosome from the inner membrane, thus stopping vectorial transfer of the nascent polypeptide. The shorter class III fusions, according to the model, are deleted for this sequence. The "crippled" class III hybrid protein, therefore, jams the cellular export machinery as the ribosome attempts to feed β-galactosidase sequences into the membrane, causing the accumulation of certain envelope protein precursors, loss of membrane integrity, and cell death. Because the larger class IV hybrid protein does contain the dissociation sequence, it is localized efficiently and does not inhibit cell growth drastically. Other investigators working with different systems have proposed the existence of similar "stop transfer" sequences (5, 7, 9, 27).

A dissociation sequence explains how the cells' export machinery can differentiate between a nascent polypeptide destined for the periplasm and one destined for the outer membrane. A periplasmic protein does not contain a dissociation sequence; thus, the entire protein is transferred concurrently with translation, through the inner membrane and into the periplasm. Results described elsewhere (3) with fusions of β-galactosidase to the maltose-binding protein (MBP), a periplasmic protein, support this model. None of these fusions produce a hybrid protein that is located in the periplasm (even though, in one fusion, nearly all of the gene that codes for MBP, *malE*, is present in the hybrid gene). Large MalE-LacZ hybrid proteins remain stuck in the cytoplasmic membrane. Consequently, all *malE-lacZ* fusions that contain an intact MBP signal sequence confer a Mal^s phenotype. As stated above, we believe that this Mal^s phenotype is a result of the jamming of the export machinery with β-galactosidase sequences. According to the model, these fusions could not

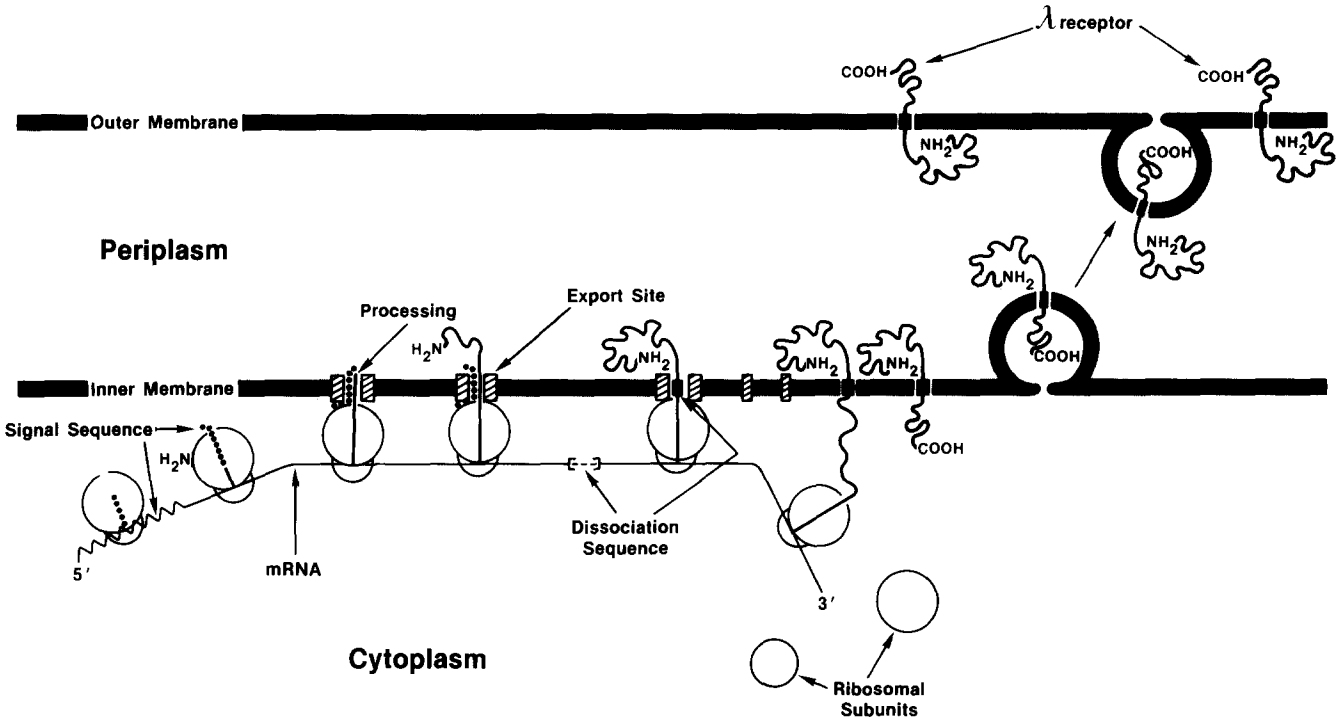

FIGURE 8 Schematic illustration of the export of a newly synthesized λ receptor to its normal outer membrane location. The export process, as depicted here, begins on the left and proceeds to the right. The signal sequence, represented by a jagged line at the 5′ end of the mRNA and by small closed circles at the NH_2-terminal end of the nascent polypeptide chain, emerges from the ribosome and, acting in concert with the ribosome, initiates attachment of the polysome to export sites (▨▨) located within the inner or cytoplasmic membrane. The signal sequence is comprised of two segments, an NH_2-terminal basic segment and a hydrophobic segment. During initial stages of polysome binding to the export sites, the positively charged basic segment interacts either with the inner leaflet of the membrane bilayer or with a component of the export site (▨). The hydrophobic segment then "loops" into the bilayer, 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 catalyzed by a peptidase activity (a component of the export site) located at the outer face of the membrane. Such processing probably occurs before synthesis of the protein is complete. This model postulates the existence of a second informational signal located within the λ receptor protein (■) necessary for membrane localization. This signal is called "dissociation sequence" or "stop-transfer" sequence. As this sequence emerges from the ribosome, dissociation of the ribosome from the membrane occurs. This stops the vectorial transfer. Subsequent translation of the mRNA completes the COOH-terminal end of the protein in the cytoplasm, leaving the λ receptor embedded in the inner membrane with its NH_2-terminus facing the periplasm and the COOH-terminus facing the cytoplasm. Additional information, presumably in the λ receptor protein, allows the cell to recognize and thus transport the protein to its final outer membrane location. The mechanism by which this process occurs is unknown. We have depicted it as occurring via a vesicle intermediate. This transport mechanism predicts that the protein is located in the outer membrane in a transmembrane fashion with the NH_2-terminal end facing the periplasm and the COOH-terminal end facing the external environment.

contain a signal for the release of the membrane-bound ribosome. Sensitivity to maltose would not be relieved, therefore, regardless of the size of the hybrid protein.

At present, we have no direct biochemical evidence to support the existence of a dissociation (stop transfer) sequence. Also lacking is evidence concerning the process by which the cell discriminates outer membrane proteins from inner membrane proteins. Clearly, such information must exist; yet this step in the export process remains a mystery.

Cellular Components Involved in the Process of Protein Localization

All models for protein localization predict the involvement of other cellular components at various stages of the process (see Fig. 1). With regard to λ receptor export, we know that a signal peptidase activity must exist because precursor processing is observed. Although we have no direct biochemical data to suggest the involvement of yet additional cellular components, we think it highly likely. We have two lines of evidence to support such a contention. One comes from the analysis of the Mal^s *lamB-lacZ* fusion. As is discussed above, induction of this fusion causes the accumulation of precursors of certain envelope proteins (Fig. 5). This suggests the existence of a finite number of discrete export sites within the cytoplasmic membrane. The LamB-LacZ hybrid protein jams these sites, thereby inhibiting export of envelope proteins.

The class II *lamB-lacZ* fusion 52-4 provides a second line of evidence supporting the existence of discrete export sites. The experiment providing this evidence is as follows: a strain has been constructed in which only ~1–5% of wild-type levels of λ receptor are exported.[3] This strain, nevertheless, is λ^s. When a transducing phage carrying the 52-4 fusion lysogenizes this strain such that it is merodiploid for the λ receptor signal sequence (the strain now contains both a *lamB* gene and a *lamB-lacZ* hybrid gene), the strain becomes λ^r. If the same experiment is performed with a transducing phage carrying the 61-4 fusion (a fusion without a complete signal sequence), the

strain remains λ^s. Because both hybrid proteins are located in the cytoplasm, we conclude that the 52-4 hybrid protein becomes bound transiently to export sites in the membrane and competitively inhibits binding of the signal sequence attached to λ receptor, thereby inhibiting export of the λ receptor and conferring a λ^r phenotype on the strain. Apparently, the signal sequence present in the 52-4 hybrid protein is recognized by the export machinery, but is insufficient to complete vectorial transfer. The simplest interpretation of these results is that signal sequences can compete for binding to a limited number of discrete export sites.

Mutations that alter the localization of a protein (or a group of proteins) but are not in the structural gene for the protein itself should help identify additional components of the export machinery. For example, if ribosome-membrane interactions are important, it should be possible to mutationally alter the ribosome so that this interaction could not occur, hence preventing protein export. Alternatively, it may be possible to mutate the membrane component and cause a similar effect. Mutations may be found that alter a pore or channel through which proteins pass during the localization process or that block the proteolytic precursor processing activity. Genetic and biochemical characterization of such unlinked mutations would provide us with insight into the nature of these other components and the mechanism of the export process.

The selection procedures employed to obtain mutations that block export of λ receptor have yielded mutations of the signal sequence only. Accordingly, other genetic approaches may be required to uncover new genetic loci involved in the export process. In particular, one approach involves seeking mutants that are generally export-defective, i.e., mutants that fail to export a number of envelope proteins (38). Although such mutants would be quite useful, this approach is technically difficult, and success in this regard has been limited. Another approach is to devise selections for mutants in which an internalized protein is exported.

The mutants we have isolated 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 λ receptor because the export machinery cannot recognize the mutationally altered signal sequence. We reasoned, therefore, that it should be possible to mutationally alter the export machinery to restore recognition of the signal sequence. Such mutations would define components of the export machinery. These mutations could be obtained by selecting reversion of a signal sequence mutation. Reversion can be selected by growth on dextrin. The mutation responsible for reversion must restore export of λ receptor to the outer membrane. Many such revertants appear to be "true revertants," as the reversion mutation is at the site of the original mutation. Such true revertants produce normal amounts of λ receptor protein and exhibit wild-type sensitivity to phage λ. A second class of revertants produces barely detectable levels of λ receptor in the outer membrane. Genetic mapping studies revealed that this class of reversion mutations is not linked to the *lamB* gene. At present, three different second site mutations have been identified.[4] Only one has been characterized genetically in detail. This mutation maps at ~72 min on the current *E. coli* linkage map in a region of the chromosome commonly referred to as the "ribosomal gene cluster." In this tightly linked cluster, there are 27 genes coding for proteins that are a part of both the large and small ribosomal subunits. In light of these mapping results, we suspect that these reversion mutations lie within a gene coding for a ribosomal protein. We favor the idea that the mutation alters some aspect of the localization process, thus permitting export of λ receptor even though the signal sequence of the protein is altered mutationally. This contention is strengthened by the observation that this ribosomal mutation also phenotypically suppresses several of the *malE* signal sequence mutations (P. Bassford, unpublished observations).

Although several alternative explanations can be entertained, we believe that the signal sequence and the ribosome act in concert to initiate protein transfer through the membrane. Perhaps both a ribosomal protein and a functional signal sequence are needed for binding of the translation complex to the export machinery located in the inner membrane. Certain mutations within the signal sequence could, of course, prevent this binding. However, this defect might be overcome if the ribosome were altered so as to increase binding between it and the export machinery. Alternatively, the signal sequence itself may interact directly with the ribosome. Such an interaction could put the signal sequence (or the ribosome) in the correct secondary or tertiary structure for recognition by the export machinery. Whatever the mechanism, we believe that these mutations and other, different mutations that allow export of an internalized protein will provide powerful tools for identifying and characterizing other cellular components involved in the export process.

CONCLUSIONS AND PROJECTIONS

We have presented a summary of the studies that we and our collaborators have made on the molecular mechanism of λ receptor export to the outer membrane of *E. coli*. Evidence indicates that the process of protein localization is strikingly similar in both prokaryotic and eukaryotic organisms. The model we present to describe the process of outer membrane protein export in *E. coli* is indistinguishable, at least in the early steps, from models (e.g., the signal hypothesis) describing either protein secretion from, or protein export to the cytoplasmic membrane of, eukaryotic cells (compare Fig. 1 and Fig. 8; see references 5, 7, 20, and 27). These models portray synthesis of noncytoplasmic proteins as occurring on membrane-bound ribosomes. They depict the signal sequence as playing a critical role in the binding of the polysome to the membrane and initiating the process of vectorial transfer of the nascent polypeptide chain. Furthermore, in the case of membrane proteins, these models predict the existence of a second informational signal (dissociation or stop-transfer sequence) that functions to embed the protein within the membrane bilayer.

By studying the process in bacteria, we have been able to utilize effectively the wealth of knowledge and the variety of techniques that are currently available to the *E. coli* geneticist to isolate and characterize a number of mutations that specifically prevent export of the outer membrane protein, λ receptor. DNA sequence analysis of these mutations has revealed that all the mutations cause alterations in the λ receptor signal sequence. This result demonstrates the essential role of the signal sequence in the initial stages of the export process. In addition, we have been able to use these signal sequence

[4] Emr, S. D., S. Hanley-Way, and T. J. Silhavy. Genetic evidence for the involvement of the ribosome in the export of the λ receptor protein to the outer membrane of *Escherichia coli*. Manuscript submitted for publication.

mutations to obtain evidence for ribosomal involvement in the export process. We believe that this result provides genetic evidence that export of the λ receptor protein occurs in a cotranslational manner. Membrane-bound ribosomes and vectorial transfer of the nascent polypeptide chain are central features of the signal hypothesis and related models of protein localization.

Using techniques of gene fusion, we have shown that information within the *lamB* structural gene is sufficient for export of even the large normally cytoplasmic enzyme, β-galactosidase, to the outer membrane. The concept that information specifying cellular location is incorporated within the structure of the exported protein itself is yet another aspect of the signal hypothesis and related models verified by our work. Although many models imply that the signal sequence alone is sufficient to specify export, our evidence indicates that this is not the case. However, this should not be viewed as evidence against the signal hypothesis. Several explanations can be made for our result that require only minor modifications of the model. For example, it well may be that the true export signal is larger than the peptide removed by the signal peptidase.

Although it is clear that enormous strides have been taken in recent years towards an understanding of the process of protein localization, it is also clear that much remains unknown. Despite sequencing data obtained from a plethora of exported proteins and despite the existence of a number of mutations, we still do not understand how the signal sequence functions. The nature of the additional intragenic export information also is unknown, as is information other than that specifying export which must be present to direct the protein to the correct cellular location. Available evidence indicates that proteins destined for several different cellular locations may be exported in a similar manner. Clearly, such export pathways must diverge at some point.

What about cellular components? Are there membrane pores or channels through which proteins pass during the export process? At present, we simply do not know the answers to these questions. Finally, are all proteins localized via a common mechanism? Even if we confine ourselves solely to outer membrane proteins in *E. coli*, this does not seem likely. Many such proteins contain an NH_2-terminal signal sequence that is removed during export (13). Many other outer membrane proteins are not processed (1). Certain newly synthesized outer membrane proteins appear in the membrane at the septal region of dividing cells (26). Others appear at sites located throughout the cell surface (33). Also suggestive of multiple export pathways is the observation described here that export of certain, but not all, proteins appears to be prevented by the synthesis of large amounts of certain *lamB-lacZ* hybrid proteins. Answers to many of these questions will come from the isolation and characterization of mutants altered in each step of the complex process of protein localization.

We would like to express our gratitude to Jon Beckwith. He, more than any other person, is responsible for applying the techniques of gene fusion to the study of protein localization. Without his expertise and foresight, none of the work described here would have been possible. We also would like to thank our many collaborators: in particular, Maxime Schwartz, who taught us the ins and outs of *malB* genetics and biochemistry; Maurice Hofnung and Joe Hedgpeth for their help with DNA sequencing; and Audree Fowler for her work on protein sequencing. We also would like to thank Phil Bassford and Howard Shuman for sharing their results with us and for many hours of fruitful discussion.

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