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Protein Secretion and Export in Bacteria

Edited by
Henry C. Wu and Phang C. Tai

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With 34 Figures

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Preface

The last decade has witnessed rapid progress in our understanding of the mechanisms of protein export and secretion in both prokaryotic and eukaryotic cells. Studies of protein secretion across the membranes of the rough endoplasmic reticulum have led to the formulation of the now-classic signal hypothesis, which has stimulated many discussions and new ideas, and the identification of the signal recognition particle as an organelle in the initiation of the export process. However, more recent work pertaining to intragenic information related to targeting specific proteins for either secretion or membrane localization, the energetics of protein secretion, the timing of synthesis versus the initiation of export, structural requirements for the processing of precursor proteins, and the identification of the processing enzymes (signal peptidases), has been the result of a combined biochemical and genetic approach to the study of protein localization in bacteria.

While reviews on the biochemistry and genetics of protein secretion have appeared frequently in recent years, this book attempts to summarize the current status and the future perspectives of this rapidly moving field in a single volume. Topics covered in this book include the genetics of protein secretion in *E. coli*, biochemical analysis of protein export in vitro, signal peptidases, excretion of colicins and hemolysin in *E. coli*, protein secretion in *Bacillus*, and protein secretion cloning vectors. Approaches encompassing classical bacterial genetics, membrane biochemistry, bioenergetics, and recombination DNA technology are utilized in a concerted effort for the elucidation of the molecular mechanisms of protein secretion in bacteria. It is our hope that this book will be valuable, not only to those actively engaged in studies of protein secretion in bacteria, but also to those who are interested in protein targeting and protein trafficking in general.

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Past Triumphs, Future Challenges

B.D. Davis

The study of protein export, into or across membranes, has clearly become one of the liveliest areas in cell biology today, with rapid advances being made both in prokaryotes and in eukaryotic cells. But as a microbiologist, aware that the simplicity and the ease of genetic manipulability of prokaryotes made them the organisms of choice in the early explorations of many of the other universal properties of cells, I have been intrigued by the fact that studies of membrane structure and function have always lagged in prokaryotes compared with eukaryotes.

It may surprise students today that, for example, until the 1950s most microbiologists doubted the possibility, in a cell as small as a bacterium, of a selectively permeable membrane with a variety of specific transport systems. Thus in the first International Congress that I attended – Biochemistry, in Paris in 1952 – Krampitz presented excellent isotopic and enzymatic evidence for the presence of the Krebs tricarboxylic acid cycle in *Escherichia coli*; but Sir Hans Krebs himself, chairing the session, was skeptical, because the intact cells could not utilize exogenous citrate. And a decade later, Monod, finding that *E. coli* cells could concentrate a lactose analog, hesitated in his initial paper to suggest that this effect might be due to active transport (like that already known in animal and plant cells) rather than to specific adsorbing units within the cell.

Studies of the aspects of membranes taken up in this volume similarly lagged in prokaryotes. One reason was difficulty in visualization. Ribosomes are tightly packed in bacteria, and so a decade after Palade had demonstrated membrane-bound ribosomes in liver cells and postulated that they function in protein secretion, it was still not clear whether any of the ribosomes in bacteria were bound to membranes. Another reason was that the cytoplasmic membrane in the simple prokaryotes is, paradoxically, particularly complex, for it combines many functions that are divided among differentiated membranes in eukaryotes.

In support of the Palade model, Redman and Sabatini soon presented evidence that incomplete proteins, released by puromycin, could be secreted into the lumen of the endoplasmic reticulum. However, the study of protein secretion then lagged for nearly a decade. What rearoused interest was the discovery that the membrane-bound ribosomes do not differ from other ribosomes (as was at first assumed), but are attached to the membrane by a hydrophobic signal sequence on their nascent polypeptide. This possibility was suggested by Sabatini and Blobel in 1971; but if they had tried to test their hypothesis,

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using the then still arduous methods of protein sequencing to seek a hydrophobic initial sequence on the secreted protein, no such sequence would have been found. In a classical example of serendipity, the key was revealed in 1972 in an immunological study in which Milstein and his colleagues first achieved synthesis of an immunoglobulin chain by cell extracts (from a myeloma cell line). They unexpectedly found that synthesis in the absence of membrane (but not in its presence) yielded a product with an extra segment of ca. 3000 daltons; because this segment was N-terminal they proposed that it served as a signal, initiating entry into the membrane and then being cleaved by a membrane enzyme.

Schaechter showed that this signal segment was highly hydrophobic, and then Blobel and Dobberstein determined its sequence. The field was broadened when Blobel further showed that integral membrane proteins, as well as secreted proteins, are synthesized as precursors carrying such a cleavable sequence; he also achieved protein translocation into vesicles in extracts. Since no systematic differences have been found between the signal sequences of membrane proteins and those of secreted proteins, one of the major challenges in the field is to identify what later sequences determine this choice. A closely related problem is to determine what tickets various proteins for entry into various membranes. Here the signal sequence appears to play a role.

Bacteria, brought into the field of protein secretion belatedly, have led to two distinctive contributions. Cotranslational secretion was directly demonstrated by extracellular labeling or cleavage of growing chains – a conclusion also established in animal cells by demonstrating attachment of oligosaccharides, in the lumen of the endoplasmic reticulum, to growing chains. And extensive genetic studies, mostly by the Beckwith school, have illuminated several aspects of protein translocation. Mutations in the hydrophobic signal sequence have not only confirmed the expectation that insertion of polar residues would impair secretion, but also revealed more subtle relations between structure and function. More interestingly, unexpected fates have been observed for β-galactosidase fused with various lengths of proteins destined for secretion or for translocation to the outer membrane, and other genetic studies have also identified several *sec* genes whose mutations interfere with secretion. We might further note that the outer and the inner membrane in gram-negative bacteria provide a relatively simple example of a multiple-membrane system, whose mechanism(s) of distribution of proteins may well prove to be similar to those in their evolutionary descendants, the mitochondria and the chloroplasts.

In an important further insight into the steps that initiate export, Walter and Blobel have shown that some eukaryotic systems involve a nucleoprotein signal recognition particle on the ribosome, which binds the signal and arrests further synthesis until the ribosome interacts with the membrane. Some bacterial systems involve a simpler secretory particle on the ribosome, whose action is less clear. These findings are no doubt pertinent to cotranslational export. But after the triumph of establishing this process, the big surprise was the finding that it is not universal: some proteins are evidently secreted post-translationally, since they are made in the cell on unbound polysomes. Moreover, even proteins that are synthesized in the cell only on membrane-bound ribosomes can be

inserted post-translationally into vesicles in extracts; and it turns out that many proteins are exported without the help of a cleavable signal sequence.

We are thus faced with multiple mechanisms. The most challenging problem is the mechanism that takes up a protein on one face of a membrane and releases it on the other. Though this process utilizes energy, how it does so is completely obscure. Moreover, while a pore through a contractile protein machinery initially seemed an attractive way of accounting for this feature, it seems increasingly likely that the protein traverses the membrane in a way that allows highly hydrophobic segments, when present, to interact with and be anchored in the lipid surroundings.

But now I am poaching on the papers in this volume, which cover the current state of the field. Let me close by noting that passage across a double membrane does not necessarily involve secretion across either component. With gram-negative bacteria there is good evidence that some excreted proteins do not pass successively through inner membrane, periplasm, and outer membrane but instead flow along the junctions from the inner to the outer membrane and then are released. A similar lateral flow may well occur in the passage of cytoplasmic proteins into the matrix of the mitochondrion. To avoid a prejudicial terminology, it might be useful to restrict the use of the term secretion to those instances where it is clear that the protein crosses a membrane and then is released to the aqueous phase on the other side.

I would also like to add a speculative suggestion: that the predominance of aqueous environments in living cells, and in the world that we inhabit, has probably exaggerated our impression of nonspecificity in hydrophobic reactions. In the interior of the membrane, the lipids, and hydrophobic surfaces of proteins, may well interact with much specificity, while intruding polar regions of proteins and polysaccharides would aggregate nonspecifically to escape the hydrophobic environment. This area of biochemistry is now at a stage akin to that of protein chemistry when I was a student in the laboratory of E.J. Cohn in the late 1930s. It was then believed that the behavior of different proteins could be best explained in terms of their electrical properties. Specific sequences, three-dimensional structures, and surface patterns were then out of reach and, indeed, beyond imagination; but they are accessible today. Similarly, specificity in the interactions of lipids with each other, and with proteins, is beginning to be observed. There can be little doubt that studies of the functional consequences of these interactions, in terms of mechanisms of secretion and of ticketing for specific destinations, will not only solve these major problems but will also contribute much to our understanding of the fine structure of membranes.

Genetic Studies on Protein Export in Bacteria

J. BECKWITH and S. FERRO-NOVICK [1]

1 Introduction

The study of protein export has proceeded in parallel in eukaryotic and prokaryotic systems over the past 10 years. Much of the information on eukaryotic protein secretion has come from the detailed analysis of an in vitro system which appears faithfully to mimic the in vivo pathway. In prokaryotes much of the progress has come from in vivo analysis including genetic studies. The genetic studies have focussed both on the components of specific proteins which determine their localization and on the nature of cellular components which are part of the export process. There now exists a large collection of mutations in the signal sequences of bacterial envelope proteins. These have been accumu-

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lated using both in vivo selections and in vitro constructions. These mutations have confirmed many aspects of the original signal sequence hypothesis and have provided some insight into the important features of such sequences. Studies on signal sequence mutations have been recently reviewed (MICHAELIS and BECKWITH 1982; BENSON et al. 1985) and will not be covered in this article.

Another approach to analyzing the role of the amino acid sequence of a protein in its export is the use of gene fusions. Such fusions have been constructed, again using both in vivo and in vitro techniques. These fusions code for hybrid proteins composed either of portions of two exported proteins or of portions of an exported protein and a cytoplasmic protein. The properties of gene fusion strains of this sort have provided information on several aspects of the export process. In addition, the phenotype of certain of these gene fusion strains has allowed genetic selections for mutants affecting secretion. The mutations thus obtained have either altered the export of specific proteins or appear to affect cellular components of a secretory apparatus. In addition to the use of gene fusions, other approaches have been employed to obtain mutants with pleiotropic effects on secretion. Studies on these mutants and characterization of the genes affected by them has led to new information on the protein export process in bacteria.

In this review we wish to summarize studies on the properties of gene fusions and cloned heterologous genes in bacteria which have shed light on the process of protein localization. In addition, we will describe the work done to this point on genes of *E. coli* which may be involved in the export process. Finally, in the process of summarizing these areas, we will refer occasionally to the similarities between the early steps in protein secretion in eukaryotic cells (transfer of proteins into the lumen of the rough endoplasmic reticulum) and the export of proteins across the cytoplasmic membrane of bacteria. Are these processes identical? Do they use the same general mechanism? Will genetic studies in bacteria contribute to an understanding of the secretory process in eukaryotic cells? These are questions that are still unresolved but merit a further analysis.

2 Gene Fusions and Recombinant DNA Clones in the Study of Protein Export

2.1 Introduction

The construction of gene or operon fusions has had wide utility in analyzing a variety of biological phenomena (SCHWARTZ 1985; SILHAVY and BECKWITH 1985; GUARENTE 1985). The protein export process is no exception. Gene fusions coding for hybrid proteins have been particularly useful in these studies. In this review, we will discuss those cases of gene fusion in which the amino-terminal portion of the hybrid protein includes the signal sequence from an exported protein. Gene fusions of several classes have been constructed – those which contain fusions of the signal sequence of one prokaryotic protein to

the mature portion of another; those which contain the fusion of the signal sequence of a prokaryotic protein to the mature portion of a eukaryotic secreted protein; and those which contain the fusion of a signal sequence to a protein which is normally cytoplasmic. In conjunction with these studies, we will also discuss those cases where the gene for an exported protein from one organism has been cloned into a different organism. These will include eukaryotic genes cloned into bacteria and genes from one prokaryote cloned into another. The cellular location and form (e.g., precursor or processed) of the hybrid proteins or cloned gene products have been determined.

While the use of gene fusions and cloned genes has unquestionably answered a number of questions about the protein export process, there is one general limitation to these studies. The techniques available for determining the cellular location of a protein are fraught with difficulties and artifacts. For instance, different techniques for preparation of the periplasmic protein fraction yield somewhat different patterns of proteins (BEACHAM 1979). It is possible that one of the approaches causes leakage of some cytoplasmic proteins. Also, there can be problems in determining the cellular location of abnormal proteins. Such proteins include those coded for by gene fusions, fragments of proteins produced by deletion or chain-terminating mutants, and proteins coded for by genes from foreign organisms. In some cases, these proteins may form aggregates or have other properties which cause them to partition with membranes in various fractionation procedures, when their actual location is in one of the soluble compartments – the periplasm or the cytoplasm. The protein may then be mistakenly identified as a membrane protein. The case of an amber fragment of the periplasmic protein, β-lactamase, illustrates certain of these problems. KOSHLAND and BOTSTEIN (1982) found that a procedure which releases the periplasmic fraction did not release this particular amino-terminal fragment of β-lactamase. The procedure disrupts the outer membrane of the bacteria, but leaves intact spheroplasts. The fragment was found in the spheroplast fraction. When the spheroplasts were broken, the fragment was released into the soluble fraction. The simplest interpretation of this result was that the amber fragment was cytoplasmic. However, subsequent studies on the accessibility of this fragment to proteolysis in intact spheroplasts suggested that, in fact, the protein was loosely adhering to the outer surface of the spheroplast (KOSHLAND et al. 1982). These cautions should be kept in mind when reading this review of gene fusion studies and, in some cases, we will point out where such factors should be taken into account. What this brief summary of fractionation problems in bacteria points to is the need for improved or alternative techniques for accurately determining the location of proteins in bacteria.

2.2 Fusions Between Genes for Exported Prokaryotic Proteins

Certain major questions concerning protein export in bacteria can be approached by replacing the signal sequence of one prokaryotic protein with that of another. The first and simplest question is whether there is any match between the signal sequence and mature sequence of a particular protein; or can one

exchange signal sequences between proteins at will without altering the ability of the cell to export a particular protein. This question has been answered in several studies. In one case, the signal sequence of the outer membrane porin PhoE was substituted with that of a similar protein OmpF, or vice versa (TOMMASSEN et al. 1983, 1984). In both cases, the proteins were localized in their active form to the outer membrane. Similarly, the signal sequence of the periplasmic TEM β-lactamase can be substituted for that of the periplasmic alkaline phosphatase and the latter enzyme is still found in the periplasm (HOFFMAN and WRIGHT 1985; MANOIL and BECKWITH 1985).

Are signal sequences merely signals for the initiation of the export process, or do they contain information for the ultimate location of a protein? For example, does an outer membrane protein require information in its signal sequence to reach its destination, or can a signal sequence for a periplasmic protein suffice? TOMMASSEN and coworkers found that when the PhoE protein had its signal sequence replaced with that of β-lactamase (TOMMASSEN et al. 1983), it still was properly exported to the outer membrane. This result indicates that the localization to the outer membrane involves amino acid sequences or structures within the mature portion of the PhoE protein. In a complementary experiment, it was shown that the signal sequence of the outer membrane protein OmpA can be substituted for that of β-lactamase, and the β-lactamase is still found in the periplasm (GHRAYEB et al. 1984). When more than the signal sequence of an outer membrane protein is added to a protein from another compartment, under some conditions the hybrid protein *will* be exported to the outer membrane. INOUYE and coworkers constructed a fusion in which the signal sequence plus nine amino acids of the mature portion of the outer membrane lipoprotein were fused to the normally periplasmic β-lactamase (GHRAYEB and INOUYE 1984). In this case, the β-lactamase was found in the outer membrane, presumably anchored by the amino-terminal sequence of lipoprotein which contains an acyl linkage to lipid.

Another issue of localization deals with certain inner membrane proteins. Many, if not most, proteins of the bacterial cytoplasmic membrane are not synthesized with cleavable amino-terminal signal sequences. These proteins include peripheral membrane proteins which are effectively cytoplasmic proteins bound to the membrane by their interaction with integral membrane proteins, and those which contain two or more hydrophobic membrane-spanning segments which anchor them tightly in the membrane. It is still not clear whether the incorporation of the latter class of protein into the membrane follows the same pathway as that of proteins with cleavable signal sequences. A third class of inner membrane proteins includes those with cleavable signal sequences which span the membrane only once. It has been presumed that these proteins are exported like periplasmic proteins, but remain anchored near their carboxy termini to the cytoplasmic membrane by that one segment. Evidence for this proposal comes from the studies of BOEKE and MODEL (1982), who have shown that deletion of the hydrophobic region of the protein results in a periplasmic location for the truncated polypeptide. Thus, the signal sequence for an integral (trans-membrane) protein can also function to export a protein to the periplasm.

Gene fusions have also been used to determine whether a protein designed for export in one organism can also cross the membrane barrier in another. Among bacteria, this question has been studied in several cases. The signal sequence from the α-amylase of the gram-positive *Bacillus amyloliquefaciens* has been fused to the mature portion of the TEM β-lactamase of gram-negative enteric bacteria (PALVA et al. 1982; ULMANEN et al. 1985). The α-amylase is normally secreted into the growth medium, while β-lactamase is normally periplasmic. When this gene fusion was introduced into *Bacillus subtilis*, 90% of the β-lactamase was found in the growth medium. Since there is no outer membrane in gram-positive bacteria and, therefore, no periplasmic space, it appears that β-lactamase can cross the cytoplasmic membrane of these bacteria. Similarly, the DNA for the signal sequence of the *E. coli* outer membrane protein, OmpA, was fused to the gene for the staphylococcal nuclease A of the gram-positive *S. aureus* and introduced into *E. coli* (TAKAHARA et al. 1985). Nearly all the nuclease activity was found in the supernatant after washing spheroplasts generated by osmotic shock. The authors propose that the enzyme is periplasmic, although some caution must be exercised in this conclusion, since the protein is not found in the original osmotic shock fluid.

In another case, the gene for the *Bacillus licheniformis* penicillinase, a major fraction of which is secreted into the medium in its native organism, was introduced into *E. coli*. The enzyme produced by the cloned genes was found bound to the *E. coli* outer membrane (LAI et al. 1981; HAYASHI and WU 1983; SARVAS and PALVA 1983). The reason for this location is presumably the existence of an amino acid sequence in penicillinase, quite similar to that found in the lipoprotein described above, which causes it to be modified and attached to lipid in the outer membrane.

These results together show that gram-positive signal sequences are recognized by the gram-negative bacteria as export signals and the protein is transported across the cytoplasmic membrane.

2.3 Expression of Eukaryotic Secreted Proteins in Bacteria

Evidence also exists that the gram-negative bacteria can recognize certain eukaryotic signal sequences and secrete and correctly process the proteins to which they are attached. In one case, the gene for rat preproinsulin was expressed in *E. coli* (TALMADGE et al. 1980a, b; CHAN et al. 1981). The protein was exported across the cytoplasmic membrane and correctly processed to yield periplasmic proinsulin. Similarly, the gene for human growth hormone was introduced into *Pseudomonas aeruginosa*, again yielding the correctly processed polypeptide in the periplasm (GRAY et al. 1984). Finally, the immunoglobulin light chain with its signal sequence coding region intact was cloned into *E. coli* and a portion of the immunoglobulin found in the periplasmic space (ZEMEL-DREASEN and ZAMIR 1984). The amount apparently exported was only around 25% of the total. In this paper, we note that the authors did not do controls to insure that the procedure for releasing periplasmic contents was not also resulting in some cell lysis. However, the fact that the protein found in the "periplasmic" fraction was processed supports their analysis.