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MINI-REVIEW

Export of the Periplasmic Maltose-Binding Protein of *Escherichia coli*

Philip J. Bassford, Jr.[1]

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Abstract

The export of the maltose-binding protein (MBP), the *malE* gene product, to the periplasm of *Eschericha coli* cells has been extensively investigated. The isolation of strains synthesizing MalE-LacZ hybrid proteins led to a novel genetic selection for mutants that accumulate export-defective precursor MBP (preMBP) in the cytoplasm. The export defects were subsequently shown to result from alterations in the MBP signal peptide. Analysis of these and a variety of mutants obtained in other ways has provided considerable insight into the requirements for an optimally functional MBP signal peptide. This structure has been shown to have multiple roles in the export process, including promoting entry of preMBP into the export pathway and initiating MBP translocation across the cytoplasmic membrane. The latter has been shown to be a late event relative to synthesis and can occur entirely posttranslationally, even many minutes after the completion of synthesis. Translocation requires that the MBP polypeptide exist in an export-competent conformation that most likely represents an unfolded state that is not inhibitory to membrane transit. The signal peptide contributes to the export competence of preMBP by slowing the rate at which the attached mature moiety folds. In addition, preMBP folding is thought to be further retarded by the binding of a cytoplasmic protein, SecB, to the mature moiety of nascent preMBP. In cells lacking this antifolding factor, MBP export represents a race between delivery of newly synthesized, export-competent preMBP to the translocation machinery in the cytoplasmic membrane and folding of preMBP into an export-incompetent conformation. SecB is one of three *E. coli* proteins classified as "molecular chaperones" by their ability to stabilize precursor proteins for membrane translocation.

Key Words: Chaperones; cotranslational export; export competence; *in vitro* translocation; maltose-binding protein; membrane-bound polysomes; posttranslational export; protein folding; SecB; signal peptide.

[1]Department of Microbiology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599-7290.

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Introduction

The maltose-binding protein (MBP[2]) of *Escherichia coli*, the product of the *malE* gene, is secreted through the cytoplasmic membrane to the periplasm where it functions in both maltose uptake and chemotaxis. It is initially synthesized as a precursor protein that includes a 26 amino acid signal peptide and a 40.6-kDa mature moiety (370 residues). As a model for protein translocation across a biological membrane, the study of MBP export in *E. coli* is particularly attractive for the following reasons:

(1) The genetics of the *E. coli mal* regulon, of which the *malE* gene is a component, are very well understood.

(2) MBP is an essential component of the maltose transport system. Thus, secretion of this protein into the periplasm is essential for cells to utilize maltose as a carbon source. This greatly facilitates the use of genetic selections for analyzing MBP export and, in addition, the efficiency of MBP export can often be monitored by the growth of cells on maltose minimal medium or by colony color on maltose indicator agar.

(3) MBP export involves translocation across a single membrane. There is only one processing step, and the protein is not further localized or modified.

(4) Unlike many exported proteins of *E. coli*, precursor MBP (preMBP) is unusually stable in the cytoplasm when secretion is blocked.

(5) Mature MBP (mMBP) is a soluble protein that can be produced and secreted in large amounts without affecting cell viability and is easily purified by affinity chromatography.

(6) Mature MBP folds into a highly stable, tertiary structure that is unusually protease-resistant. In fact, acquisition of protease resistance can be used as an indicator of MBP folding. In studies involving the latter, the lack of Cys residues in the protein eliminates the possible complication of intramolecular disulfide bond formation.

MBP export has been investigated in a number of laboratories but most extensively in those of Bassford and Randall. This review will largely concentrate on those studies concerned with the export properties of the preMBP molecule itself, as well as the interaction of preMBP with the secretion machinery, particularly SecB.

[2]Abbreviations: MBP, maltose-binding protein and RBP, ribose-binding protein (the prefix "pre" specifically indicates the precursor form of the protein, whereas "m" indicates the mature form); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SRP, signal recognition particle.

MalE-LacZ Hybrid Proteins

Initial studies of MBP export were greatly facilitated by the isolation of *E. coli* strains in which the *malE* gene was genetically fused to the *lacZ* gene encoding the cytoplasmic enzyme β-galactosidase (reviewed in detail by Bankaitis *et al.*, 1985). In certain instances, a *malE-lacZ* hybrid gene resulted that encodes a hybrid protein having, at its amino terminus, an amino-terminal portion of the MBP and, at its carboxyl terminus, enzymatically active β-galactosidase (Bassford *et al.*, 1979). It was originally anticipated that those hybrid proteins possessing an intact MBP signal peptide at the amino terminus would be secreted into the periplasm. Such a result would have provided strong support for the hypothesized role of the signal peptide in initiating protein export.

Five different MalE-LacZ hybrid protein-producing strains were characterized. The hybrid proteins differ in the amount of MBP attached to β-galactosidase. The smallest protein retains only the first 14 residues of the MBP signal peptide (designated class I); the largest one lacks only a small carboxyl-terminal portion of the MBP (designated class V). Secretion of the hybrid protein into the periplasm could not be demonstrated for any of the fusion strains. However, those hybrid proteins that include an intact MBP signal peptide (classes II–V) are inserted into the cytoplasmic membrane to various degrees, strongly suggesting that the export of the hybrid proteins is at least initiated.

The *malE-lacZ* fusion strains encoding class II–class V hybrid proteins exhibit an unusual phenotype. Cells that are otherwise *mal*$^+$ but which synthesize these MalE-LacZ hybrid proteins are sensitive to maltose, the inducer of the *mal* regulon. This maltose-sensitive (Mal^s) phenotype is a direct consequence of the induction of high-level hybrid protein synthesis, which is under control of the *malE* promoter. Following induction, the hybrid protein accumulates in the cytoplasmic membrane, resulting in a progressive, coordinate inhibition of export of virtually all periplasmic and outer membrane proteins (Bassford *et al.*, 1979; Ito *et al.*, 1981), and even some cytoplasmic membrane proteins (Herrero *et al.*, 1982). This was demonstrated by the observation that the proportion of the precursor form to the mature form for various exported proteins (e.g., wild-type MBP, alkaline phosphatase, LamB, OmpA) pulse-radiolabeled and analyzed at various times postinduction continually increases with time. Several hours after induction of hybrid protein synthesis, these exported proteins are encountered almost exclusively in their precursor forms. Under these conditions, the cells form long filaments and eventually lyse. It was proposed that this Mal^s phenotype results from the progressive occupation by the hybrid protein of specific sites in the cytoplasmic membrane required for general protein export

(Bassford *et al.*, 1979; Ito *et al.*, 1981). More recent studies by Bieker and Silhavy (1989) conclusively demonstrated that PrlA (SecY) is the component of the protein export pathway that is sequestered by LacZ hybrid proteins in the membrane.

Studies by Rasmussen *et al.* (1984) showed that the MBP portion of MalE-LacZ hybrid proteins with an intact signal peptide is transferred across the cytoplasmic membrane into the periplasm. This was indicated by the findings that the signal peptide is removed from those hybrid protein molecules synthesized immediately following induction, and that the processed forms are susceptible to protease added to spheroplasts. It was suggested that secretion of the hybrid proteins commences in the same manner as MBP itself. However, at some point in the translocation process, the sequence or structure of the β-galactosidase tail aborts the export process, leaving the hybrid protein embedded in the cytoplasmic membrane. It is not known why β-galactosidase is incompatible with membrane translocation. Several studies indicate that there are multiple regions within the polypeptide that each contribute to this problem (Bassford *et al.*, 1979; Schwartz *et al.*, 1981; Lee *et al.*, 1989). Lee *et al.* (1989) have hypothesized that rapid folding of small domains of β-galactosidase may be resposible for the failure to translocate this protein. Regardless of the explanation, note that it was the early studies with both MalE-LacZ and LamB-LacZ fusions that were the first to clearly demonstrate that the presence of an amino-terminal signal peptide is not necessarily sufficient to assure the secretion of a normally cytoplasmic protein (Silhavy *et al.*, 1977; Bassford and Beckwith, 1979; Bassford *et al.*, 1979; Moreno *et al.*, 1980).

The unusual Mal^s phenotype of certain *malE-lacZ* fusion strains suggested a selection for the isolation of mutants defective in MBP export. It was predicted that mutants that survive induction with maltose (Mal^r) and continue to synthesize the hybrid protein (Lac^+) will no longer attempt to export the hybrid protein from the cytoplasm. Indeed, Mal^r Lac^+ mutants were obtained and in each case the hybrid protein produced by these cells fractionates primarily with the cytoplasm. The mutations responsible for the Mal^r phenotype were mapped to the very early *malE* portion of the hybrid gene. It was subsequently demonstrated that the export-defective nature of the hybrid proteins is due to mutational alterations of the MBP signal peptide (see below). At the time, it was somewhat disappointing that mutants with alterations in the protein export machinery of the cell were not also obtained by this selection. However, another unusual property exhibited by these same *malE-lacZ* fusion strains was later exploited to isolate mutations that led to the identification of both the *secA* (Oliver and Beckwith, 1981) and *secB* genes (Kumamoto and Beckwith, 1983) of *E. coli* (see articles by Oliver and Kumamoto, this volume).

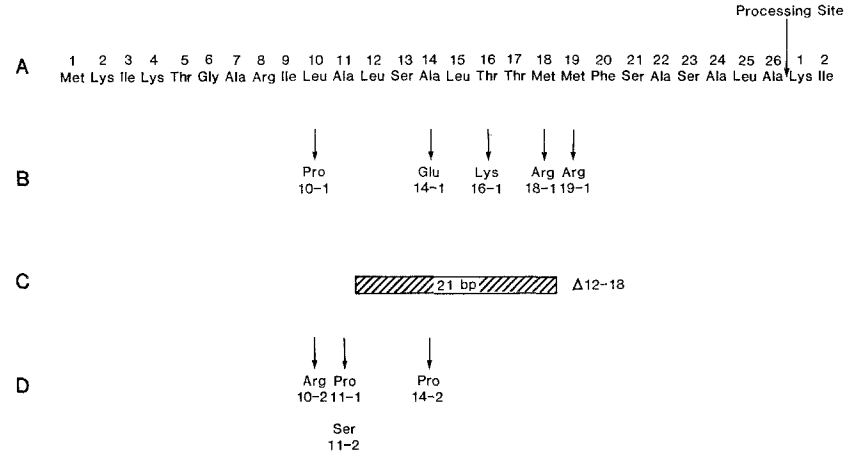

Fig. 1. Primary amino acid sequence of the wild-type MBP signal peptide and alterations resulting from various *malE* signal sequence mutations. (A) The wild-type MBP signal peptide and the first two residues of mMBP. The cleavage site for processing by signal peptidase I is indicated. (B) MBP signal peptide alterations resulting from strong *malE* point mutations obtained in the original selection (Bassford and Beckwith, 1979; Bedouelle *et al.*, 1980). (C) The *malEΔ12–18* mutation removes residues 12–18 from the hydrophobic core (Bankaitis *et al.*, 1984). (D) Signal peptide alterations resulting from a selection for weak signal sequence mutations (Bankaitis *et al.*, 1985). Single amino acid alterations in the wild-type sequence are indicated by arrows. Residues removed as a result of a deletion mutation are indicated by a shaded bar. The corresponding *malE* allelic designations are also given. (From Ryan *et al.*, 1986b.)

The MBP Signal Peptide

Although the MBP signal peptide displays little primary sequence homology with other signal peptides, it exhibits the conserved features typical of signal peptides of both prokaryotic and eukaryotic origin (Perlman and Halvorson, 1983; von Heijne, 1985; Sjöström *et al.*, 1987; see articles by Gierasch and Inouye *et al.*, this volume). It is 26 amino acids long and has three recognizable regions (Fig. 1A). The first eight residues constitute the hydrophilic segment (designated the *n*-region; von Heijne, 1985, 1986). This region carries a net positive charge due to the presence of three basic residues. The hydrophilic segment is followed by the hydrophobic core (residues 9–20; *h*-region), a region devoid of charged residues and predicted to assume an α-helical conformation (Bedouelle and Hofnung, 1981). The overall hydrophobic character of this region has been shown to be a major determinant of signal peptide function. Finally, the six carboxyl-terminal residues of the MBP signal peptide (residues -6 to -1 relative to the cleavage site) represent the recognition sequence for the processing enzyme, signal peptidase I. This region (*c*-region) is the most highly conserved among various signal

peptides, since the cleavage site must be recognized by the processing enzyme (Perlman and Halvorson, 1983; von Heijne, 1983). A β-turn structure is thought to occur at the boundary between the hydrophobic core and the signal peptidase recognition sequence (Perlman and Halvorson, 1983). According to the loop model (Inouye and Halegoua, 1980), the positively charged amino terminus interacts with the inner surface of the cytoplasmic membrane, and the hydrophobic core inserts into and spans the membrane as a loop or reverse hairpin structure, exposing the cleavage site on the external face at some point during the translocation process. Many details of this model remain a matter of conjecture; still, the experimental evidence supports this orientation of the signal peptide during translocation (Duffaud *et al.*, 1985; Dalby and Wickner, 1985; Fikes and Bassford, 1987; Millan *et al.*, 1989; Summers *et al.*, 1989).

The Hydrophobic Core

Mutational alterations in the MBP signal peptide were obtained using the selection procedure described above (reviewed in detail by Bankaitis *et al.*, 1985). Although such signal sequence mutations were originally isolated in a *malE-lacZ* hybrid gene, such mutations were easily recombined into an intact *malE* gene where their effect on MBP export was subsequently determined (Bassford and Beckwith, 1979). The original selection yielded five unique single amino acid substitutions in the MBP signal peptide (Fig. 1B), each of which alters the hydrophobic core and results in a significant export defect (Bedouelle *et al.*, 1980). The substitution of proline for leucine at residue 10 is predicted to significantly disrupt the secondary structure through the core region (Bedouelle and Hofnung, 1981). The other four mutations introduce a charged residue into the core, strongly supporting the idea that the hydrophobic nature of the core is essential for the role of the signal peptide in initiating export. In *E. coli* cells synthesizing these various mutant MBP species, the MBP accumulates in the cytoplasm as unprocessed preMBP to various extents (Fig. 2). MBP18-1 exhibits the strongest export defect; only about 4% of the total MBP synthesized is exported and processed to the mature form.

Subsequent studies employing a similar selection yielded additional alterations in the signal peptide, including several in-frame deletion mutations that extend into the mature coding sequence (not shown), and one that removes seven residues from the core (designated *malEΔ12–18*) (Figs. 1C,2). The latter represents, in terms of its effect on MBP export, the strongest alteration in the wild-type signal peptide obtained by genetic selection. However, this extremely truncated signal peptide still facilitates export of approximately 1% of the total MBP synthesized, which is sufficient to permit

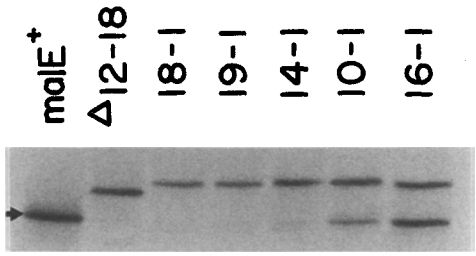

Fig. 2. SDS-PAGE of wild-type and export defective MBP. Immune precipitation of radiolabeled MBP from solubilized extracts of *E. coli* cells and analysis by SDS-PAGE and autoradiography was performed as described by Bankaitis *et al.* (1984). Note that, under the radiolabeling conditions employed, only mMBP is recovered from wild-type (*malE*$^+$) cells. MBP precipitated from *malEΔ12–18* cells is found almost exclusively in its truncated precursor form. Other MBP species (see Fig. 1B) are found in varying ratios of intact preMBP and mMBP. The arrow indicates the position of mMBP.

growth of *E. coli* cells on maltose, albeit with a very slow doubling time (Bankaitis *et al.*, 1984).

The signal peptide alterations shown in Fig. 1D were obtained using a selection procedure specifically designed to yield mutants demonstrating only minor export defects (Bankaitis *et al.*, 1985). Interestingly, such alterations are also confined to the hydrophobic core region, as are all the previous mutants obtained (except those resulting from large deletions). The characterization of these mutants revealed that the introduction of a charged residue early in the core at position 10 is not nearly as disruptive as placing a charged residue further into this region. Likewise, the introduction of a Pro residue at either position 11 or 14 is not as detrimental to signal peptide function as the Pro at position 10. In the latter case, the helix-breaking property of the Pro is probably considerably amplified by its close proximity to the Gly at residue 6, which is also a strong helix breaker (Fikes *et al.*, 1987). A similar situation was described in a study of LamB signal peptide alterations (Emr and Silhavy, 1983).

It was interesting that most of the mutants described above were obtained multiple times among a large number of independently isolated mutants that were analyzed. Alterations confined to either the hydrophilic segment or the cleavage site regions were not obtained, nor were substitutions at several positions within the hydrophobic core, including the Leu residue at position 15. Recently, oligonucleotide mutagenesis was employed to substitute Arg for Leu at this position (designated MBP15-1) (S. Van Meter, S. M. Strobel, and P. J. Bassford, Jr., unpublished results). This mutant was constructed for several reasons. Leu is extremely hydrophobic, has a high propensity for α-helix formation (Arfmann *et al.*, 1977), and on a statistical basis is the most frequently occurring amino acid in the hydrophobic cores

of prokaryotic signal peptides (von Heijne, 1986). Also, it requires a minimum of two nucleotide changes to convert this Leu codon (TTA) to one encoding a charged residue. To date, all of the substitution mutations in the MBP signal peptide coding region obtained by genetic selections have resulted from the change of a single nucleotide. MBP15-1 was found to be totally export-defective; cells synthesizing this MBP species are unable to utilize maltose as a carbon source. This may represent the only example of a single amino acid substitution within a signal peptide that renders the corresponding precursor protein totally export-defective (see Ferenci and Silhavy, 1987).

Additional information concerning the hydrophobic core was obtained by isolating phenotypically Mal^+ revertants of various *malE* signal sequence mutations. Beginning with five different mutants, Bankaitis *et al.* (1984) and Ryan *et al.* (1986a) obtained a total of 29 unique intragenic reversion mutations. Most of the revertants harbor second-site suppressor mutations that suppress to some degree the effects of the original lesion. For the six unique revertants of the deletion mutation *malEΔ12–18* (Bankaitis *et al.*, 1984), this is necessarily the case. Except for two instances (see below), each of the reversion mutations results in an increase in the overall hydrophobicity of the core region. Hydrophobicity is restored by several different mechanisms, including one in which the core is lengthened at the expense of the hydrophilic segment by converting the Arg at residue 8 to a neutral or nonpolar residue. It was also apparent from the analysis of these revertants that the hydrophobic core can be functionally extended into the cleavage site region, even to include the first several residues of the mMBP, without any obvious effect on signal peptide processing (see below). Because of the variety of ways in which hydrophobicity can be restored to the core, and the clear correlation between the efficiency of MBP export and the hydrophobic nature of the core region, it was concluded that the overall hydrophobicity of this structure, rather than the absolute length of its uninterrupted core, is a major determinant of signal peptide export competency. Studies by other investigators with mutationally altered (Michaelis *et al.*, 1986), partially synthetic (Kendall *et al.*, 1986; Kendall and Kaiser, 1988), and randomly selected (Kaiser *et al.*, 1987) signal peptides have provided additional support to this concept.

Two of the intragenic suppressor mutations obtained in these selections are single amino acid substitutions at residue 19 of the mature MBP. In the case of a suppressor of *malEΔ12–18*, the improvement in MBP export is barely discernible (Bankaitis *et al.*, 1984). However, in the case of an intragenic revertant of *malE*19–1, MBP export is substantially improved (Ryan *et al.*, 1986a). It was not obvious how these changes, fairly far removed from the signal peptide, serve to partially suppress an export defect resulting from an altered hydrophobic core. The isolation of such mutations suggested

that the early region of mMBP may contain important information for initiating protein export. In the case of LamB, several studies have indicated that the early mature region of this protein may have a role in export (Benson *et al.*, 1984; Rasmussen and Silhavy, 1987). However, the deletion of residues 17–21 from the mature moiety of otherwise wild-type preMBP was found to have no effect on MBP export (J. W. Puziss and P. J. Bassford, Jr., unpublished results). In a more recent study, a signal sequence suppressor mutation was characterized that altered residue 283 of the mMBP (Cover *et al.*, 1987). Suppression in this instance resulted from the effect of this alteration on the folding properties of preMBP (see below). It now seems probable that the alterations at residue 19 of the mMBP mediate their suppressive effects through a similar mechanism, although this has not yet been specifically demonstrated.

Extragenic suppressor mutations were also obtained among Mal^+ revertants of various MBP signal sequence mutants (Bankaitis and Bassford, 1985; Ryan and Bassford, 1985). The isolation of such mutations has proven particularly useful for genetically dissecting the *E. coli* protein export pathway. Silhavy and coworkers have extensively characterized numerous extragenic suppressors of LamB signal sequence mutations. These studies led to the identification of several new genes, including at least two genes, *prlA* (*secY*) and *prlG* (*secE*), encoding essential components of the *E. coli* protein export machinery (Emr *et al.*, 1981; Stader *et al.*, 1989). *prlA* mutations were found to suppress signal sequence mutations in other genes as well, including *malE* (Emr and Bassford, 1982) and *phoA* (Michaelis *et al*,. 1986). Among extragenic revertants of *malE* signal sequence mutations a number of new alleles of *prlA* were obtained, including several that were particularly powerful suppressors (Bankaitis and Bassford, 1985). A new class of suppressor mutations was also obtained that were designated *prlD* (Bankaitis and Bassford, 1985; Ryan and Bassford, 1985). Although originally thought to identify a new gene, Fikes and Bassford (1989) subsequently demonstrated that these represent novel alleles of the *secA* locus previously characterized by Oliver and Beckwith (1982). The finding that mutational alterations in key components of the export pathway can partially restore export of proteins with defective signal peptides suggests that the PrlA, PrlG, and SecA proteins may directly interact with the signal peptide in the protein export process. Except for SecA (see below), this has not been directly demonstrated. For more information concerning *prl* mutations, see the article by Bieker *et al.* (this volume).

The R2 Signal Peptide

One of the Mal^+ revertants of *malEΔ12–18* substitutes Leu for Arg at residue 8 of the MBP signal peptide (designated *malEΔ12–18R2* and

henceforth referred to as R2) (Bankaitis *et al.*, 1984). The MBP signal peptide synthesized by the R2 mutant is 7 residues shorter than the wild-type and has one fewer basic residue in the hydrophilic (Fig. 3). Still, it facilitates MBP secretion and processing at a rate and efficiency that is comparable to those of the wild-type structure (Bankaitis *et al.*, 1984; Fikes *et al.*, 1987; Puziss *et al.*, 1989). It was postulated that the functional hydrophobic core of this signal peptide extends from the Ala at residue 7 through to the cleavage site. To test this possibility, mutational alterations in the R2 signal peptide were selected by the same method that previously yielded alterations in the wild-type signal peptide (Fikes *et al.*, 1987). Seven unique single amino acid substitutions in the R2 signal peptide were obtained (Fig. 3). As was previously the case for the wild-type MBP signal peptide, the introduction of either charged residues or proline residues into the R2 signal peptide adversely affects export function. Furthermore, alterations that are expected to disrupt the R2 core structure are found at positions -3 and -2 relative to the cleavage site, residues normally constituting the processing site region of the MBP signal peptide. Since the unaltered R2 signal peptide is processed rapidly and efficiently to yield mMBP, almost certainly at the normal cleavage site (Fikes and Bassford, 1987), these results strongly indicate that the processing site can overlap the hydrophobic core and does not necessarily constitute a totally distinct region of the signal peptide.

One of the mutants obtained in this study, designated CC15, proved to be of considerable interest. In this instance, Asp is substituted for Ala at residue -3 relative to the cleavage site. For prokaryotic signal peptides, Ala-X-Ala is the most frequently observed sequence at positions -3 to -1 and is thought to represent the recognition site for signal peptidase I (see

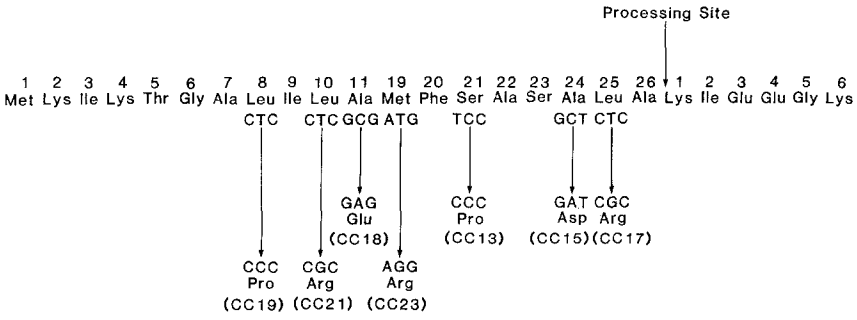

Fig. 3. Mutational alterations in the R2 signal peptide. The primary amino acid sequence of the truncated R2 signal peptide, including the R2-encoded alteration at position 8, and the first six residues of mMBP are shown. Seven unique amino acid alterations resulting in export defects are indicated. The changes in the nucleotide sequence and the corresponding designations are also given. (From Fikes *et al.*, 1987.)

below). Although inefficiently exported due to the alteration in the signal peptide, preMBP secreted by CC15 cells is not processed; rather, unprocessed preMBP is released into the periplasm in a soluble form and is functional in facilitating maltose uptake (Fikes and Basford, 1987). This finding prompted the construction by oligonucleotide mutagenesis of another mutant MBP species, designated MBP24-1, in which Asp is substituted for Ala at position 24 (i.e., −3) of an otherwise wild-type signal peptide. This alteration has no adverse effect on MBP translocation across the cytoplasmic membrane, indicating that, in the wild-type MBP signal peptide, the hydrophobic core and the signal peptidase recognition sequence are probably distinct, nonoverlapping regions.

As is the case with CC15 preMBP, the substitution of Asp for Ala at residue −3 effectively prevents processing of preMBP24-1 by signal peptidase I. However, in marked contrast to the former, the latter remains tethered to the cytoplasmic membrane by its unprocessed signal peptide having a full-length hydrophobic core. It was somewhat surprising that the tethered preMBP24-1 efficiently participates in maltose uptake, as does the soluble preMBP produced by CC15 cells (Fikes and Bassford, 1987; Dean *et al.*, 1989). The finding that the signal peptide of MBP24-1 serves as a membrane anchor is most consistent with the orientation of the signal peptide relative to the membrane as proposed in the loop model (Inouye and Halegoua, 1980). On the other hand, the severely truncated hydrophobic core retained by CC15 preMBP is capable of inefficiently promoting MBP export, but is less than the minimal structure thought to be capable of serving as an anchor domain (Davis and Model, 1985).

The Processing Site

Comparative analysis of numerous prokaryotic signal peptides has identified a strongly conserved feature regarding the residues at positions −3 and −1 relative to the cleavage site (see article by Dev and Ray, this volume). It was noted that amino acids with small, neutral side chains predominated at these two positions (Perlman and Halvorson, 1983; von Heijne, 1983). von Heijne (1986) compared 36 prokaryotic signal peptides processed by signal peptidase I, and only Ala, Gly, Leu, Ser, Thr, and Val were encountered at the −3 position. Somewhat more restrictive, position −1 was found to harbor only Ala, Gly, Ser, and Thr. As previously mentioned, Ala-X-Ala is the most frequently observed sequence preceding the cleavage site. Positions −3 and −1 have been proposed to constitute a recognition site for the processing enzyme, signal peptidase I. These observations were also used to formulate the A-X-B model (Perlman and Halvorson, 1983) and the (−3, −1) rule (von Heijne, 1983) for predicting signal peptide cleavage sites. The

latter was further modified by von Heijne (1986) to incorporate a larger statistical basis and a weight matrix approach.

The studies described above with MBP24-1 identified an excellent system to conduct mutational analysis of cleavage site structural requirements without affecting protein translocation. Subsequently, oligonucleotide-directed mutagenesis was utilized to generate fourteen different amino acid substitutions at -3 and thirteen different substitutions at -1 of the MBP signal peptide (Fikes *et al.*, 1990). Overall, the results obtained agree fairly well with statistically derived models of signal peptidase I specificity, with the following three exceptions: (i) Although not previously encountered at -3, Ile at this position is compatible with efficient preMBP processing. Leu and Val were previously recognized to permit efficient processing at this position in the signal peptide (von Heijne, 1986). (ii) Thr at -1 results in inefficient processing of preMBP. Kuhn and Wickner (1985) had previously reported that Thr at -1 of M13 procoat is a poor substrate for signal peptidase I, both *in vivo* and *in vitro*. (iii) Cys was found to permit efficient processing when present at either -3 or -1. Although Cys is commonly found at either -3 or -1 of eukaryotic signal peptides (von Heijne, 1986), Cys is probably excluded from the processing site of those prokaryotic signal peptides cleaved by signal peptidase I to help distinguish precursor nonlipoproteins from precursor lipoproteins which are modified and subsequently processed by signal peptidase II (Wu and Tokunaga, 1986). Precursor lipoproteins require a Cys residue in the $+1$ position.

In this same study, it was somewhat surprising to find that substitutions at -1 which block processing at the normal cleavage site redirect processing, with varying efficiencies, to an alternate site in the signal peptide represented by the Ala-X-Ala sequence at positions -5 to -3 (see Fig. 1A). The substitution of Asp for Ala at -5 prevents processing at this alternate site but has only a very small effect on processing at the normal site. Processing at the alternate site can be quite efficient. For example, with Asp at -1, which totally blocks processing at the normal site, greater than 90% of the preMBP synthesized is processed at the alternate site. On the other hand, with Tyr at -1 blocking processing at the normal site, very little processing at the alternate site is detected. At present, it is not understood why different substitutions at -1, each of which effectively blocks the normal site, can result in wide variations in processing efficiency at the alternate site. However, even when processing at the alternate site is highly efficient, the processing kinetics are much slower than those exhibited by preMBP species processed at the normal site. This may be due to the closer proximity of the alternate site to the β-turn structure separating the core and the signal peptidase recognition sequence. Presumably, in the wild-type signal peptide extremely rapid processing

at the normal cleavage site effectively precludes the slower, alternate processing event.

The Hydrophilic Segment

A specific role for the hydrophilic segment in protein export was first suggested in the loop model (Inouye and Halegoua, 1980). The basic residues of the hydrophilic segment were proposed to initiate an ionic interaction between the signal peptide and the negatively charged inner face of the cytoplasmic membrane. More recent studies have suggested that the role of the hydrophilic segment is primarily to orient the hydrophobic core with respect to the direction in which it spans the membrane. Thus, positively charged amino acids immediately preceding a hydrophobic segment help to ensure that it functions as a signal sequence, whereas when positively charged residues immediately follow a hydrophobic segment, the latter will most likely serve as a membrane anchor with its amino terminus on the external face of the membrane (Li *et al.*, 1988; von Heijne *et al.*, 1988; Szczesna-Skopupa *et al.*, 1988; Millan *et al.*, 1989; Summers *et al.*, 1989; Summers and Knowles, 1989; Boyd and Beckwith, 1989). Several studies have shown that eliminating the net positive charge at the amino terminus of signal peptides of several proteins decreases export efficiency, particularly if the end result is a signal peptide with a net negative charge (Inouye *et al.*, 1982; Vlasuk *et al.*, 1983; Iino *et al.*, 1987; Bosch *et al.*, 1989). Such changes in some but not all instances have also been shown to result in a decrease in synthesis.

Puziss *et al.* (1989) used oligonucleotide-directed mutagenesis to substitute either neutral or acidic residues for the basic residues at the amino-terminus of both the wild-type and R2 signal peptides. As with other proteins studied, a net positive charge is not absolutely required for MBP export to the periplasm. However, export is most rapid and efficient when the signal peptide retains at least a single basic residue and a net charge of $+1$. The nature of the adjacent hydrophobic core helps to determine the effect of charge changes on MBP export efficiency. Thus, the export competency of the R2 signal peptide with its truncated hydrophobic core is more sensitive to changes in the hydrophilic segment, such that a mutant with a net charge of -3 is totally export-defective. On the other hand, greater than 60% of preMBP with a full-length hydrophobic core and a net charge of -3 is exported. The finding that a full-length hydrophobic core can compensate for changes in the hydrophilic segment suggests that these two regions of the signal peptide do not have totally distinct functions.

It was of particular interest to find that virtually 100% of preMBP with a full-length hydrophobic core and a net charge of -2 is eventually exported to the periplasm and processed, albeit with significantly slower kinetics than

wild-type preMBP (see below). Thus, the lack of basic residues preceding the hydrophobic core is not sufficient in itself to convert this signal peptide to a membrane anchor, as might have been suggested from earlier studies. Specific amino acid changes in the region immediately following the core, particularly the addition of one or more Arg residues (Summers *et al.*, 1989), may be required to complete the conversion of the MBP signal peptide to a membrane anchor that no longer promotes the export of the trailing mature MBP moiety.

Puziss *et al.* (1989) also found that the export defect resulting from alterations in the hydrophilic segment of the MBP signal peptide is not noticeably improved in cells harboring *prlA* suppressor mutations but is markedly improved in cells harboring a *prlD* mutation. The failure of *prlA* alleles, all of which were originally obtained as suppressors of hydrophobic core mutations, to suppress similar export defects in two other proteins has been reported by Iino and Sako (1988) and Bosch *et al.* (1989). The markedly different pattern of suppression observed with MBP species altered in the hydrophilic segment may be related to the nature of the respective *prl* gene products, both of which are essential components of the export pathway. PrlA (SecY) is an extremely hydrophobic, integral cytoplasmic membrane protein (Ito, 1984), whereas SecA (PrlD) is a mostly hydrophilic, peripheral membrane protein (Oliver and Beckwith, 1981; Schmidt *et al.*, 1988) that has recently been shown to functionally bind signal peptides (Cunningham and Wickner, 1989). Puziss *et al.* (1989) speculated that PrlA interacts more strongly with the signal peptide hydrophobic core and SecA interacts more strongly with the hydrophilic segment in facilitating protein export.

For both the wild-type and R2 signal peptides, the total amount of MBP synthesized progressively decreases as the net charge of the hydrophilic segment is systematically decreased to -3. Similar results have been reported for Lpp (Inouye *et al.*, 1982; Vlasuk *et al.*, 1983) and LamB (Hall *et al.*, 1983). The reduction in synthesis of the latter two proteins has been interpreted as possible evidence for a mechanism that obligately couples protein export and translation, perhaps in a manner similar to that described for signal recognition particle (SRP) in eukaryotic systems (reviewed by Walter and Lingappa, 1986). However, the results of Puziss *et al.* (1989) strongly suggest that translational coupling is not responsible for the observed decrease in MBP synthesis. It seems likely that mutations very early in the coding region of the *malE* message that are responsible for the alterations in the hydrophilic segment may induce changes in the mRNA primary sequence or secondary structure that somehow reduce the efficiency of translation initiation. Most recently, additional experimental support for the latter interpretation has been obtained (J. W. Puziss, R. J. Harvey, and P. J. Bassford, Jr., manuscript in preparation).

Redundant Information Contained Within the MBP Signal Peptide

Extensive analysis of the MBP signal peptide has revealed that very few mutational alterations in this structure, even including small deletions such as *malEΔ12–18*, totally abolish its ability to promote MBP export and processing. The studies described above demonstrate that the export competence of the MBP signal peptide is not noticeably different whether the hydrophilic segment has one, two, or the three basic residues of the wild-type structure. The hydrophobic core of the MBP signal appears to be longer than absolutely required, particularly if the signal peptidase recognition site is included, and an alternate cleavage site is present within the signal that can be used if cleavage at the normal site is blocked. As discussed by Fikes *et al.* (1990), this built-in redundancy of export information established for the MBP signal peptide may be generally true for most other *E. coli* signal peptides. Such redundancy may exist to ensure that single mutational events are unlikely to totally inactivate the signal peptide. On the other hand, there may be circumstances faced by the cell beyond the laboratory setting where such seemingly redundant information may be crucial to maintain protein translocation and signal peptide cleavage as rapid and highly efficient cellular processes.

Biochemical Studies of MBP Export

Precursor MBP Is Primarily Synthesized on Membrane-Bound Polysomes

Early studies with *E. coli* alkaline phosphatase indicated that this periplasmic protein is primarily synthesized on membrane-bound polysomes (Cancedda and Schlesinger, 1974), strongly suggesting that protein export in bacteria is a cotranslational process similar to that put forth for protein secretion in higher organisms by the signal hypothesis (Blobel and Dobberstein, 1975). Randall and Hardy (1977) first demonstrated that MBP is preferentially synthesized on membrane-bound polysomes. Since these same workers showed that ribosomes enriched in the membrane-bound fraction appeared to be no different from those engaged in the synthesis of cytoplasmic proteins (Randall and Hardy, 1975), it seemed likely that the MBP nascent chain is chiefly responsible for determining the membrane association of ribosomes engaged in its synthesis. Randall *et al.* (1978) isolated membrane-bound polysomes from *E. coli* cells, and the translation of nascent chains initiated *in vivo* was completed *in vitro*. Under these circumstances, MBP and two other exported proteins were shown to be synthesized as larger-molecular-weight precursor proteins. It was hypothesized that exported proteins in prokaryotes are initially synthesized with signal peptides similar to those previously described for several eukaryotic secreted proteins. The

determination of the *malE* gene sequence (Bedouelle *et al.*, 1980: Duplay *et al.*, 1984) and the isolation and characterization of MBP signal sequence mutants (Bassford and Beckwith, 1979) that accumulated preMBP in the cytoplasm subsequently firmly established that MBP is initially synthesized with an amino-terminal signal peptide that plays a key role in the secretion process.

Rasmussen and Bassford (1985) investigated the site of synthesis of wild-type and export-defective MBP in *E. coli* cells. This study confirmed that wild-type preMBP is synthesized primarily on membrane-bound polysomes. Thus, these results further indicated that it is primarily the nascent chain that attaches ribosomes to the membrane. In this same study, it was shown that the presence of a strong *prlA* suppressor allele partially restores synthesis of export-defective preMBP on membrane-bound polysomes whereas, in the absence of functional SecA, wild-type preMBP is synthesized primarily on free polysomes. It was concluded that it is the interaction of the nascent chain, most likely the signal peptide, with components of the export machinery that leads to the synthesis of preMBP at the membrane surface.

Thom and Randall (1988) investigated the interaction of newly synthesized preMBP and nearly completed nascent preMBP chains with the cytoplasmic membrane, using the technique of flotation gradient centrifugation. Their studies indicated that the MBP export process can be divided into four discrete steps. The first of these was operationally defined as entry into the export pathway, which includes all the steps occurring in the cytoplasm, as well as the initial association of the MBP polypeptide with the cytoplasmic membrane. Next is the translocation event itself, which includes transfer of the nascent or fully elongated precursor from the site of initial interaction with the membrane to the translocation site. The latter was found to be associated with a membrane fraction of density 1.21 g/ml, somewhat heavier than that of bulk cytoplasmic membrane (1.16 g/ml), suggesting that translocation sites may be associated with zones of adhesion between the cytoplasmic and outer membranes (Bayer *et al.*, 1982). The third step in this process is the proteolytic removal of the signal peptide, which is followed by the release step involving folding of the mature MBP and its dissociation from the periplasmic side of the membrane. With regard to the latter, Ito and Beckwith (1981) had previously suggested that release of the newly translocated and processed polypeptide from the cytoplasmic membrane requires proper folding of the mMBP into a water-soluble conformation.

Thom and Randall (1988) also demonstrated that MBP synthesized without a signal peptide (designated MBPΔ2-26; see below) fails to enter the export pathway. Somewhat surprisingly, MBP18-1 that is synthesized with a defective signal peptide (see Fig. 1B) appears to proceed through the pathway as far as delivery to the translocation site. It appears that the mutant signal

peptide is specifically unable to mediate the actual membrane translocation event. It was concluded that the signal peptide is involved in both of the first two steps of protein export (and, obviously, the processing step, as well), and that the MBP18-1 signal peptide is sufficiently competent to facilitate entry of preMBP into the export pathway. Other investigators have also proposed multiple roles for the signal peptide in export (Ryan and Bassford, 1985; Stader *et al.*, 1986; Kaiser *et al.*, 1987). Although this finding may appear inconsistent with the earlier results of Rasmussen and Bassford (1985) discussed above, it seems likely that the association of MBP18-1 polysomes with membranes may be considerably weaker and, thus, more easily lost when polysomes are prepared by the methods employed in that particular study. The use of flotation gradient centrifugation by Thom and Randall was undoubtedly crucial to their experiments.

The finding that MBP18-1 having a charged residue inserted into the hydrophobic core is specifically blocked at the translocation step suggests that hydrophobic interactions are involved in this process. Dierstein and Wickner (1985) demonstrated that purified preMBP but not mMBP binds to detergent; several export-defective preMBP species, including MBP18-1, were unable to bind detergent. It was suggested that the hydrophobic partition of the signal peptide into the membrane is responsible for initiating membrane translocation. Likewise, from studies of the interaction of synthetic wild-type and mutant LamB signal peptides with various lipid systems, Briggs *et al.* (1985) reached similar conclusions.

MBP Translocation Is Independent of Chain Elongation

The signal hypothesis proposed that the translocation of proteins across membranes is directly coupled to translation. This concept was strongly supported by the identification of SRP in eukaryotic systems that binds to the nascent polypeptide chain and prevents further translation until the ribosome associates with the membrane. The finding that *E. coli* exported proteins are synthesized with amino-terminal signal peptides on membrane-bound polysomes led many investigators to extend the principles of the signal hypothesis to prokaryotic systems (reviewed by Silhavy *et al.*, 1983). However, it soon became apparent that protein translocation in prokaryotes is not obligatorily cotranslational. Early studies by Ito *et al.* (1979) demonstrated that the bacteriophage M13 coat protein is posttranslationally inserted into the cytoplasmic membrane of *E. coli* cells. Shortly afterwards, Koshland and Botstein (1980) reported that TEM β-lactamase is exported to the periplasm of *E. coli* cells in a totally posttranslational manner. Likewise, in eukaryotic cells, it is now recognized that the translocation of proteins into mitochondria and chloroplasts is a posttranslational event, and posttranslational

translocation has even been demonstrated for some proteins targeted for the endoplasmic reticulum (see Eilers and Schatz, 1988; Rothman, 1989).

The studies of Randall and coworkers have shed considerable light on the mode of MBP translocation. Josefsson and Randall (1981a) immune-precipitated MBP polypeptides from extracts prepared from cells that had been briefly pulse-radiolabeled with [^{35}S]methionine and were able to transiently detect fully elongated preMBP, indicating that processing can occur entirely posttranslationally. In addition, an analysis by two-dimensional SDS-PAGE revealed that the signal peptide can be proteolytically removed from the amino-terminus of some incompletely synthesized nascent chains. Approximately one-third of the MBP molecules are cotranslationally processed. Since maturation requires translocation of at least the cleavage site across the cytoplasmic membrane (Date *et al.*, 1980), this result demonstrated that translocation of MBP can be initiated cotranslationally. However, cotranslational processing is a late event relative to synthesis, since signal peptide cleavage is only detected for polypeptides elongated to approximately 80% of their full length or more. A subsequent analysis of a number of *E. coli* exported proteins demonstrated that processing can exhibit either or both temporal modes, but cotranslational processing invariably is a late event. It was stated that if translocation of these polypeptides, like processing, also proves to be a late cotranslational or entirely posttranslational event, then clearly translocation cannot be directly coupled to chain elongation (Josefsson and Randall, 1981b).

Randall (1983) used the accessibility of MBP nascent chains to externally added proteinase K in *E. coli* spheroplasts as the criterion for membrane translocation. Nascent chains that retain the signal peptide are protease-resistant, whereas processed nascent chains are always degraded. Only when the membrane is disrupted prior to protease addition are the precursor forms of nascent chains accessible to protease degradation. Likewise, ribose-binding protein (RBP), which exhibits a strictly posttranslational mode of processing, only becomes protease-accessible after chain elongation is complete. From these studies, it was concluded that maturation occurs coincident with translocation (and, thus, is a good indicator of translocation), and that both translocation and processing are late events in the synthesis of both MBP and RBP and probably other precursor proteins, as well. It was further postulated that entire domains of polypeptides are translocated after their synthesis, as originally proposed in the membrane trigger hypothesis of Wickner (1979). These findings are not necessarily inconsistent with earlier studies indicating that exported proteins are synthesized on membrane-bound polysomes. As noted previously, the events that bring the nascent chain into association with the membrane appear to be independent of translocation. In addition, in her study Randall (1983) suggested that folding of the polypeptide at the

membrane surface could be required for it to achieve a translocation-competent conformation (see below).

Since protein synthesis cannot be the driving force for protein translocation, what does provide the energy for this process? It has been suggested that conformational changes in the polypeptide could be sufficient as the source of energy for both insertion into and transfer across membranes (Engelman and Steitz, 1981). However, experiments using *in vivo* and *in vitro* systems have clearly demonstrated that both ATP and the proton-motive force are required for protein translocation in bacteria (reviewed by Randall, 1987; see articles by Fandl and Tai and Mizushima and Tokuda, this issue).

Kinetic Studies of MBP Export in Signal Sequence Mutants

Josefsson and Randall (1981a,b) demonstrated that MBP export is a very rapid process, even for that fraction of the total MBP synthesized that is translocated and processed in a posttranslational fashion. Intact preMBP could only be detected using a very short radiolabeling period. Bassford and coworkers (Bankaitis *et al.*, 1984; Bankaitis and Bassford, 1984; Ryan and Bassford, 1985; Ryan *et al.*, 1986a) also employed pulse-chase studies to investigate the kinetics of MBP export. When cells were pulse-radiolabeled with [^{35}S]methionine and incubated (chased) for various time periods in the presence of excess unlabeled methionine, preMBP was transiently detected in small amounts only at early chase points. Even after no chase period, the great majority of MBP precipitated is processed (Fig. 4). The same analysis has been applied to a variety of MBP species having mutational alterations in the hydrophobic core or hydrophilic segment. (Recall that most of these

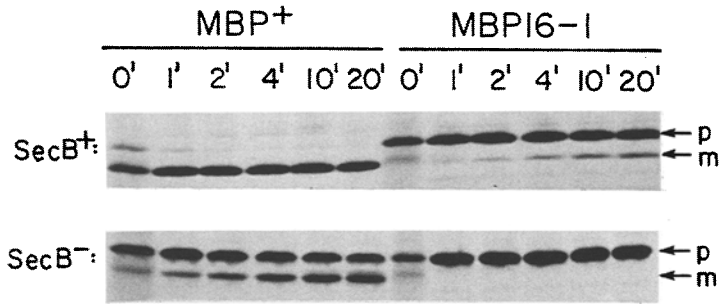

Fig. 4. Kinetics of MBP export in SecB$^+$ and SecB$^-$ cells. Cells synthesizing either wild-type MBP (MBP$^+$) or export-defective MBP16-1 were pulse-radiolabeled with [^{35}S]methionine for 15 sec and chased with excess unlabeled methionine. At the indicated time points (min), samples were removed, the chase was terminated, and the MBP immune-precipitated and analyzed by SDS-PAGE and autoradiography. The positions of preMBP (p) and mMBP (m) are indicated by arrows. See the text for a discussion of the SecB requirement for MBP export. (From Collier and Bassford, 1989).

species are not totally export-defective.) In many instances, only preMBP can be precipitated at the earliest time point, but a slow conversion of preMBP at later time points is clearly discerned. It was found that, the stronger the export defect, the longer into the chase that it takes for mMBP to be detected.

Fractionation of cells radiolabeled and chased for various time periods convincingly demonstrated that preMBP which is detectable at various chase points resides in the cytoplasm, whereas mMBP is in the periplasm. Thus, preMBP processing once again proved to be a good indicator for translocation. For some mutants, essentially 100% of the preMBP seen at the earliest chase point is eventually converted to mMBP (Ryan *et al.*, 1986a). For other mutants, generally those exhibiting a stronger export defect, the preMBP/mMBP ratio is seen to slowly decrease for some period of time and then stabilize (e.g., MBP16-1; see Fig. 4). The preMBP detected beyond this point appears to be permanently trapped in the cytoplasm (Ryan and Bassford, 1985). These studies indicated that preMBP can remain in the cytoplasm in a state competent for membrane translocation for many minutes after its synthesis. Eventually, however, export competence appears to be permanently lost.

Ryan and Bassford (1985) investigated the export kinetics of various preMBP species in cells harboring *prlA* and *prlD* suppressor alleles. In most instances, *prl*-mediated export of MBP species with defective signal peptides markedly increases both the fraction of MBP that is translocated and processed and the rate at which this is accomplished. The presence of both a *prlA* and *prlD* mutation in the same strain can act synergistically in suppressing MBP export defects, suggesting that the PrlA (SecY) and SecA (PrlD) proteins may directly interact in facilitating MBP export. Still, MBP export kinetics in any of these strains are much slower relative to that of wild-type MBP, and membrane translocation remains a posttranslational process. Interestingly, the kinetics of wild-type MBP export appear to be no different in cells harboring either prl^+ or mutant *prl* alleles.

On the basis of their kinetic studies, Ryan and Bassford (1985) proposed a model in which the nascent preMBP assembles into a complex with several components of the export pathway, either in the cytoplasm or on the cytoplasmic membrane, which subsequently serves to promote MBP translocation across the cytoplasmic membrane. For MBP with a wild-type signal peptide, translocation is rapidly effected either cotranslationally or posttranslationally. For MBP species with a defective signal peptide, it was proposed that the efficiency of complex formation is reduced relative to the strength of the export defect. In those instances where an export complex does not form cotranslationally, the preMBP assumes an export-incompetent conformation and can never be translocated from the cytoplasm. In those instances where a complex is formed, subsequent steps in export are still inefficient due to the

presence of the defective signal peptide and, thus, the export kinetics are by necessity slower than those observed for wild-type MBP. Alteration of the PrlA and/or SecA proteins can improve both the efficiency of complex formation and the subsequent rate of MBP export to various extents, although the defective signal peptide will always slow the translocation step. However, in both *prl*$^+$ and *prl* suppressor strains, the complex has a limited half-life and may disassemble prior to initiating MBP translocation. Again, such a model suggests multiple roles for the signal peptide in the export process. Also note that, according to this model, there are no mechanistic differences between co- and posttranslational translocation. Rather, both export modes are dependent on the early interaction of the precursor protein with soluble factors.

The suggestion that the defective signal peptide interacts inefficiently with at least certain components of the translocation machinery was supported by an interesting observation. The addition of chloramphenicol early during the chase period to inhibit new protein synthesis was found to dramatically improve both the proportion of mutant preMBP that is eventually exported and the rate at which this is accomplished (Ryan and Bassford, 1985). Presumably, since proteins with fully competent signal peptides are no longer being synthesized and therefore competing with preMBP for access to the translocation machinery, there is greater opportunity for the mutant preMBP to productively interact with that same machinery during the limited period that it remains export-competent.

Precursor MBP Folding and Export Competence

The finding that MBP translocation is an event that is not initiated until either late in synthesis or after synthesis has been completed has implications for the export process beyond those concerned with the driving force for translocation. One of these concerns the structural state of the polypeptide in the cytoplasm prior to translocation. Presumably, a fully elongated preMBP polypeptide, or perhaps even a nascent chain that is 80% completed, should be capable of folding. As noted previously, Randall (1983) suggested that synthesis of the preMBP on membrane-bound polysomes might be beneficial in terms of the ability of the entire polypeptide or individual domains to assume some conformation crucial for translocation. The studies of Ryan and Bassford (1985) showed that preMBP under some circumstances can remain in an export-competent state in the cytoplasm for many minutes prior to translocation. What about the structural state of preMBP under these conditions? Ferenci and Randall (1979) reported that preMBP can bind substrate, indicating that the unprocessed precursor can assume a conformation similar to that of mMBP. In fact, just as periplasmic

mMBP is highly resistant to protease digestion, preMBP also is capable of achieving a stable, tertiary conformation in which only the signal peptide is susceptible to protease digestion (Randall, 1983; Dierstein and Wickner, 1985).

In light of the fact that intact preMBP can be released from the ribosome prior to translocation, several questions need to be considered: First, can preMBP assume a stable, tertiary structure in the cytoplasm and, if so, can it subsequently be translocated across the cytoplasmic membrane or does folding render it permanently export-incompetent? Second, is the observed loss of export competence by preMBP in the cytoplasm a result of folding of the protein into a conformation that is not compatible with translocation? Finally, if folding does negatively impact on the export competence of preMBP, are there factors that modulate folding of preMBP in the cytoplasm or, alternatively, exhibit some ability to unfold preMBP prior to translocation?

Randall and Hardy (1986) used sensitivity to proteinase K degradation to determine the structural state of export-competent and export-incompetent preMBP *in vivo*. Wild-type preMBP whose export is specifically blocked at the translocation step by uncoupler is maintained in a state sensitive to proteinase K degradation. In contrast, wild-type preMBP whose export is presumably blocked at an earlier step in the export pathway by concomitant synthesis of a MalE-LacZ hybrid protein (see above) is rapidly converted to a proteinase K-resistant conformation, indicating that the preMBP under these conditions folds into a stable, tertiary structure. Precursor MBP is also rapidly converted from a proteinase K-sensitive to resistant state in cells producing MBP species with defective signal peptides. In the latter case, a strong correlation was established between the kinetics of mutant preMBP export and the kinetics of preMBP folding; the cessation in MBP export coincides with the point at which the intracellular population of preMBP is completely folded.

Based on their results, Randall and Hardy (1986) proposed the following: (i) For newly synthesized preMBP, a kinetic competition exists between productive entry into the export pathway and folding of the precursor in the cytoplasm into a stable, export-incompetent conformation. (ii) The interaction with either the membrane or, more likely, one or more protein components of the export pathway helps to maintain preMBP in an export-competent state. (iii) It is probably the lack of stable, tertiary structure, rather than the attainment of a specific conformation, that determines the export competence of the precursor. This last point is most consistent with the picture that has emerged from a variety of experimental systems over the last several years. It is now widely believed that the translocation of proteins across virtually any biological membrane (bacterial, endoplasmic reticulum, mitochondrial,

etc.) requires that the polypeptide exhibit a "translocation-competent" conformation representing a largely unfolded or loosely folded state (for recent reviews, see Eilers and Schatz, 1988; Meyer, 1988; Verner and Schatz, 1988; Bernstein, 1989; Rothman, 1989).

Further studies indicating that the early interaction with the export machinery serves, at least in part, to maintain preMBP in an export-competent conformation will be considered below. In addition, Randall and coworkers (reviewed by Randall and Hardy, 1989) have demonstrated that the presence of the signal peptide itself has a significant effect on the folding of the attached mature moiety of preMBP. Using fluorescence spectroscopy, relaxation times for folding and unfolding transitions were determined for both purified preMBP and mMBP by diluting these proteins either out of or into denaturant (guanidinium hydrochloride). The presence of the signal peptide has little effect on MBP unfolding but significantly slows the rate at which MBP refolds following denaturation (Park *et al.*, 1988). A similar difference in the rate of refolding of purified preRBP compared to mRBP was also shown in this same study. It was suggested that this interference with folding of the mature moiety by the signal peptide may be crucial in allowing precursor proteins to enter the export pathway. Strong support for this concept has been provided by both *in vitro* and *in vivo* experiments in which an MBP species (MBPΔ2-26) is synthesized without a signal peptide (see below).

Finally, with regard to preMBP folding and export competence, additional information was provided from a somewhat unexpected source. Beginning with cells synthesizing export-defective MBP14-1 (see Fig. 2B), a phenotypically Mal^+ revertant was obtained in which the responsible mutation (designated *malE2261*) substitutes Asp for Tyr at position 283 of the mature MBP moiety. Cover *et al.* (1987) found that the double mutant species, MBP14-1,2261, although exported very poorly in comparison to wild-type MBP, is exported at an efficiency approximately twice that of MBP14-1. The export of MBP2261 synthesized with an unaltered signal peptide is indistinguishable from that of wild-type MBP. Although mMBP2261 in the periplasm facilitates maltose uptake, its significantly reduced affinity for substrate, marked instability *in vivo*, and aberrant migration on SDS-PAGE indicate that the mutational alteration significantly affects the structure of the protein.

The mechanism by which a mutational alteration late in the precursor polypeptide partially suppresses the export defect resulting from an alteration in the signal peptide is not readily obvious. The possibility of a direct interaction between the signal peptide and a distant region of the mature moiety seems unlikely. Instead, it was suggested that the alteration at residue 283 of mMBP slows the folding of preMBP in the cytoplasm, thus extending the period that the precursor remains competent for export. This,

in turn, provides additional time for the defective signal peptide to productively interact with the export machinery and promote translocation of MBP to the periplasm. The export kinetics determined for MBP14-1 and MBP14-1,2261 were consistent with this hypothesis (Cover *et al.*, 1987). Subsequently, Liu *et al.* (1988) purified preMBP14-1, preMBP14-1,2261, and mMBP2261 and once again used fluorescence spectroscopy to measure relaxation times for refolding and unfolding of these proteins. The results clearly demonstrated that the change in the mature mMBP sequence dramatically decreases the rate of preMBP folding. As will become apparent shortly, MBP2261 has proven to be a particularly useful mutant protein.

In Vitro *Studies of MBP Synthesis and Translocation*

Rasmussen *et al.* (1985) placed the *malE* gene under regulatory control of the *lacUV5* promoter-operator to facilitate MBP expression in an *in vitro*, coupled transcription–translation system. When wild-type MBP is synthesized *in vitro* using an S-100 fraction and purified ribosomes, only precursor is detected by SDS-PAGE. When membrane vesicles are included in the reaction mixture, approximately 30% of MBP synthesized migrates on SDS-PAGE identically to mMBP. No mMBP is detected when export-defective MBP18-1 is synthesized *in vitro*, either in the presence or absence of membranes. Although resistance to proteolytic degradation is usually employed to confirm protein import into membrane vesicles *in vitro* (Müller and Blobel, 1984; Rhoads *et al.*, 1984; Bacallao *et al.*, 1986), this is not possible with wild-type mMBP which, as noted previously, is highly protease-resistant. However, the mMBP produced *in vitro* pellets with the membranes, whereas the majority of the preMBP remains in the supernatant. Thus, preMBP appears to be imported into vesicles and processed at an efficiency similar to that reported for most other *E. coli* proteins analyzed *in vitro* (Müller and Blobel, 1984; Chen *et al.*, 1985).

Weiss *et al.* (1989) further investigated MBP translocation *in vitro*. In order to be able to monitor MBP import into vesicles by resistance to proteinase K, this analysis included the use of protease-sensitive MBP species. The latter was accomplished by recombining into the *malE* genes of interest a small, internal, in-frame deletion designated *malEΔ116* that removes residues 142–150 from the mature MBP moiety. The loss of these residues renders the MBP exquisitely sensitive to proteinase K digestion. The export kinetics of MBPΔ116 *in vivo* are indistinguishable from wild-type MBP, indicating that MBPΔ116 is fully export-competent. The amount of precursor processing discerned when either wild-type preMBP or preMBPΔ116 are synthesized *in vitro* is essentially identical. However, proteinase K-resistance revealed that some small but still significant proportion of the total preMBP synthesized is consistently imported

into vesicles without processing. Again, this has also been the case with other proteins similarly analyzed (Müller and Blobel, 1984; Rhoads *et al.*, 1984; Yamane *et al.*, 1987).

It was found that approximately 40% of preMBP synthesized with a wild-type signal peptide is imported into vesicles. In marked contrast, MBP species with alterations in the signal peptide hydrophobic core are imported into vesicles with an efficiency that is much lower than predicted from *in vivo* studies. For example, MBP with the R2 signal peptide (see above) appears to be fully export-competent *in vivo* but its ability to be translocated *in vitro* is only 25% of wild-type MBP. Likewise, MBP11-2 which exhibits only a minor kinetic defect *in vivo* is translocated *in vitro* with an efficiency only 10% of wild-type. Import of MBP species with stronger export defects (MBP16-1, MBP19-1) is not detectable. The inability of these altered MBP species to be translocated *in vitro* was suggested to reflect a defective interaction of the signal peptide with some component, possibly a soluble one, early in the export pathway. This was supported by the finding that the use of vesicles prepared from cells harboring strong *prlA* suppressor alleles does not appreciably increase import efficiency (Weiss *et al.*, 1989).

Wild-type preMBP can be posttranslationally imported into vesicles, although at an efficiency much reduced from that obtained if vesicles are present cotranslationally. It is very difficult to demonstrate posttranslational import of wild-type preMBP when vesicles are present cotranslationally, indicating that import of translocation-competent preMBP is effected very rapidly. In addition, if vesicles are added to the reaction mixture at various times posttranslationally, wild-type preMBP rapidly loses its ability to be translocated. Similar results have been demonstrated for other *E. coli* proteins *in vitro* (Goodman *et al.*, 1981; Chen *et al.*, 1985). Folding of the preMBP into a translocation-incompetent conformation is at least partially responsible, since several MBP species that are defective in folding, including MBP2261 (see above) and MBPΔ116, maintain competence for posttranslational import for significantly longer time periods. However, with time these latter MBP species also lose their translocation competence. From their results, Weiss *et al.* (1989) proposed essentially the same model for protein export that was previously offered by Ryan and Bassford (1985) (see above).

The folding of preMBP synthesized *in vitro* in the absence of membranes was also investigated, again using acquisition of protease resistance as an assay. Wild-type preMBP slowly assumes a protease-resistant conformation under these experimental conditions. Somewhat surprisingly, it takes approximately 60 min for 100% of the preMBP to reach this state which is, admittedly, a very stringent assay for protein folding (Weiss *et al.*, 1989).

This is clearly a much longer time for folding to occur than that exhibited by purified preMBP (Park *et al.*, 1988; see above), indicating that the interaction of preMBP with soluble components of the *in vitro* reaction mix probably contribute to the folding kinetics (see below). In addition, the fact that competence for posttranslational import into vesicles is completely lost in a considerably shorter period indicates that intermediates in preMBP folding that remain protease-sensitive have already lost translocation competence. Folding of preMBP19-1 is somewhat accelerated over wild-type preMBP, indicating that at lease some factors interact specifically with the signal peptide to slow folding. This analysis also included an MBP species, designated MBPΔ2-26, that is deleted for the entire signal peptide except for the initiating Met residue; the mature moiety remains completely intact. It is very difficult to even detect unfolded MBPΔ2-26 by protease sensitivity, further indicating that the presence of the signal peptide dramatically influences folding.

The Role of SecB in MBP Export

SecB is a nonessential, tetrameric, cytoplasmic protein composed of identical 16.4-kDa subunits that is required for the efficient export of a subset of *E. coli* envelope proteins, including MBP. Mutations in the *secB* gene were first described by Kumamoto and Beckwith (1983, 1985). Recent studies, primarily by Bassford and coworkers (Collier *et al.*, 1988; Weiss *et al.*, 1989; Collier and Bassford, 1989), have strongly indicated that SecB promotes MBP export by serving as an antifolding factor that specifically binds to the mature MBP moiety and retards folding of preMBP in the cytoplasm into a translocation-incompetent form. Thus, SecB is thought to be one of a class of cytoplasmic proteins, termed "chaperones" (see Ellis and Hemmingsen, 1989), that can serve to maintain the export competence of precursor polypeptides in *E. coli*. This proposed role for SecB is entirely consistent with the work described in the preceding section concerning preMBP folding and export competence, and has been strongly supported by several studies in other laboratories (Kumamoto and Gannon, 1988; Gannon and Kumamoto, 1989; Lecker *et al.*, 1989; Liu *et al.*, 1989). However, it should be noted that there is some controversy concerning the role of SecB. Watanabe and Blobel (1989c) have recently presented evidence indicating that SecB binds to the emerging signal peptide of newly synthesized preMBP. They proposed that SecB functions as the prokaryotic equivalent of SRP, targeting preMBP to the export machinery in the cytoplasmic membrane. This section will deal primarily with the work of Bassford and coworkers on SecB. For additional information, see the article by Kumamoto, this issue.

Synthesis of Export-Defective preMBP Interferes with Normal Protein Export

Bankaitis *et al.* (1984) first observed that the MBP export kinetics discerned for cells harboring intragenic suppressor mutations of *malEΔ12-18* (see above) are significantly slower when the cells also harbor the *lamBS60* signal sequence mutation. It was suggested that export-defective LamB encoded by *lamBS60* at least transiently enters the protein export pathway and competes with wild-type and mutant MBP species for components of the export machinery. Bankaitis and Bassford (1984) subsequently investigated this finding in more detail. They demonstrated that the synthesis of either export-defective MBP or LamB interferes with the normal export of wild-type MBP, LamB, and OmpA proteins. The effect is quite subtle and does not confer any obvious phenotypic abnormalities to the host cell. Instead, interference in normal protein export is primarily manifested as a kinetic delay in the export of these proteins, although 40% of the total wild-type preMBP synthesized is rendered permanently export-incompetent and, thus, accumulates in the cytoplasm.

In order for this interference phenomenon to be detected, three requirements were identified: (i) interfering MBP and LamB species must exhibit a strong export defect; (ii) such proteins need to be expressed at a fairly high level; and (iii) the interfering protein must be actively synthesized at the time interference is measured. The latter suggested that it is not the accumulation of export-defective protein in the cytoplasm *per se* that is responsible for interfering with normal protein export; rather, that export-defective proteins mediate interference as nascent polypeptides. This and other evidence suggested that interference occurs at an early step in the export process. Bankaitis and Bassford (1984) concluded that export-defective proteins, even those missing a major portion of their signal peptide, are still capable of engaging the export machinery. However, the inability of such proteins to exit the export pathway via the normal route results in a titration effect on some component of this machinery, thus limiting the availability of this component to participate in normal protein export.

Collier *et al.* (1988) demonstrated that MBPΔ323, a totally export-defective MBP species missing the last 20 residues of the signal peptide and the first 89 residues of mMBP, is a particularly strong interfering species. Since only the first six residues of the signal peptide hydrophilic segment are retained by MBPΔ323, this finding strongly suggested that the region of the MBP responsible for mediating interference is contained within the mature moiety. By analyzing the interfering capabilities of a number of truncated and hybrid proteins, this same study showed that the region between residues 151 and 186 of mMBP is sufficient to mediate interference in normal protein

export. Thus, it appears that preMBP exhibits a secretion determinant that resides very near the middle of the mature protein sequence.

Interference Results from the Depletion of SecB

Although Bankaitis and Bassford (1984) concluded that interference was manifested at the level of general protein export, the studies of Collier *et al.* (1988) revealed that the export of many *E. coli* envelope proteins is not obviously affected by high-level synthesis of MBPΔ323. These included RBP, alkaline phosphatase, TEM β-lactamase, lipoprotein, and phage M13 coat protein. A general pattern was noted that those proteins whose export is SecB-dependent are also subject to interference (e.g., MBP, LamB, OmpA), whereas the export of SecB-independent proteins is not subject to interference (e.g., RBP, alkaline phosphatase). It was then found that interference in normal protein export by synthesis of MBPΔ323 is totally suppressed in cells overproducing SecB due to the presence of the *secB*$^+$ gene on a multicopy plasmid. In addition, wild-type MBP export in SecB$^-$ cells was found to be virtually indistinguishable from that obtained in SecB$^+$ cells synthesizing MBPΔ323.

On the basis of these and other results, Collier *et al.* (1988) concluded that the interference phenomenon results from the prolonged interaction of SecB with interfering protein species in the cytoplasm. This, in turn, depletes cells of SecB, resulting in the loss of SecB- dependent protein export. Since the region of export-defective MBP that mediates interference is contained within the mature moiety, it was proposed that SecB directly binds to this region of the preMBP in facilitating its role in normal MBP export. Although the similarities between SecB$^+$ cells synthesizing MBPΔ323 and SecB$^-$ cells are striking, in terms of the export kinetics determined for both SecB-dependent and SecB-independent proteins, there is one difference that has not yet been satisfactorily explained. Whereas cells harboring null mutations in the *secB* gene are able to grow on minimal media but not complex media (Kumamoto and Beckwith, 1985), cells synthesizing high levels of MBPΔ323 grow well on both types of media. Thus, cells depleted of SecB as in the latter case are not physiologically equivalent to cells that are incapable of making SecB.

SecB Retards the Folding of preMBP

An important clue as to the role of SecB in preMBP export was provided by the finding that the export of certain preMBP species is significantly less SecB-dependent than wild-type preMBP export. MBPΔ116 (see above), MBPΔ57–145 (deleted for residues 57–145 of the mature moiety), and

MBP2261 (see above) are each synthesized with a wild-type signal peptide and exhibit export kinetics identical to wild-type preMBP in $SecB^+$ cells. However, in $SecB^-$ cells each of these proteins is exported much more efficiently and at a significantly faster rate than wild-type MBP (Collier *et al.*, 1988). These three mutant MBP species are similar in their inability to fold into a stable, tertiary conformation. This has previously been discussed for MBPΔ116 and MBP2261 and should be obvious for MBPΔ57-145 in which nearly a quarter of the mature moiety has been deleted. The markedly reduced SecB-dependence of these proteins suggests that at least one important function of SecB is to prevent or at least retard the folding of cytoplasmic preMBP into an export-incompetent conformation. The value of such an antifolding factor was previously suggested by the studies of Randall and Hardy (1986) correlating the folding of preMBP into a stable, tertiary structure with the loss of export competence (see above). Thus, if the ability of a protein to attain a stable conformation is adversely affected by mutational alteration, the requirement for an antifolding factor should be substantially reduced.

Collier *et al.* (1988) considered the possibility that an MBP species that is exported in a slow, entirely posttranslational manner would be particularly dependent on SecB. This proved to be the case. Although greater than 90% of MBP19-1-R8 synthesized in $SecB^+$ cells is eventually translocated and processed, in $SecB^-$ cells the export of this preMBP species is totally blocked. This could be explained if this protein, in the absence of SecB, is assuming an export-incompetent conformation before translocation can be effected. The influence of SecB availability on the folding of wild-type preMBP synthesized *in vitro* into a stable structure was also tested. Using acquisition of proteinase K-resistance as the assay, preMBP folding occurs most rapidly in the absence of SecB, is significantly slower in the presence of haploid levels of SecB, and is virtually undetectable when excess SecB is present. Shortly thereafter, Kumamoto and Gannon (1988) reported that preMBP that accumulates in $SecB^-$ cells very rapidly acquires a protease-resistant conformation. All of these results provided strong support for the proposal that SecB serves as an antifolding factor. Collier *et al.* (1988) also demonstrated that SecB is incapable of unfolding preMBP once it has assumed a protease-resistant state, thus distinguishing SecB from unfolding factors that have been strongly implicated in the posttranslational translocation of various eukaryotic proteins (see Eilers and Schatz, 1988; Rothman, 1989).

Studies with Purified SecB

Weiss *et al.* (1988) purified SecB from overexpressing *E. coli* cells and showed it to be a very minor, cytoplasmic, multimeric protein of identical

17-kDa subunits [close to the compositional molecular weight of 16.4 kDa from the *secB* DNA sequence (Kumamoto and Nault, 1989)]. SecB is required for efficient import of MBP into membrane vesicles *in vitro*, whereas RBP import is totally SecB-independent, as it is *in vivo*. The addition of purified SecB to a SecB-deficient *in vitro* system significantly enhances MBP translocation, although not to the level obtained when cell fractions are prepared from $SecB^+$ cells. Purified SecB also retards folding of wild-type MBP synthesized *in vitro*. Finally, in this same study it was demonstrated that excess purified SecB prolongs the competence of preMBP for posttranslational translocation. As described above, wild-type preMBP synthesized *in vitro* in the absence of membranes rapidly loses competence for translocation into vesicles added after the termination of protein synthesis. These experiments were performed with extracts containing haploid levels of SecB (Weiss *et al.*, 1989). In an identical experiment, the inclusion of excess SecB, well above the haploid level, in the reaction mix during synthesis was found to significantly extend the time in which wild-type preMBP remained competent for post-translational translocation (Weiss *et al.*, 1988). These findings underscore the importance of SecB in promoting posttranslational MBP translocation.

SecB Binds Directly to preMBP

The nature of the SecB-preMBP interaction in maintaining preMBP export competence is not known. Collier *et al.* (1988) originally proposed that SecB binds directly to the mature moiety of the newly synthesized preMBP, and Gannon *et al.* (1989) clearly demonstrated that it is the mature moiety of preMBP that renders this protein SecB-dependent. Subsequently, three different studies demonstrated that SecB directly associates with several different precursor proteins, including preMBP, to form soluble complexes that presumably represent transient intermediates in the export process (Watanabe and Blobel, 1989b; Kumamoto, 1989; Lecker *et al.*, 1989). In addition, Liu *et al.* (1989) very recently demonstrated that the refolding of both mMBP2261 and wild-type mMBP is immediately and completely blocked when these proteins are diluted out of denaturant in the presence of purified SecB. Refolding of the latter can only be blocked by SecB if the reaction temperature is decreased to 5°C, reflecting the very strong propensity of this protein, lacking the signal peptide, to rapidly assume its final tertiary conformation (see above). This same study found that export-defective MBPΔ2-26 (for all practical purposes, the equivalent of mMBP) is not an interfering MBP species when expressed *in vivo*, even though it contains the SecB binding site previously identified by Collier *et al.* (1988). However, synthesis of the double mutant species, MBPΔ2-26,2261, does interfere with SecB-dependent protein export. Liu *et al.* (1989) concluded

that SecB binds to the mature moiety of preMBP, and that one important function of the signal peptide is to slow folding of preMBP and thereby expose the binding site for SecB.

Weiss and Bassford (1990) have also demonstrated a direct physical interaction of SecB with MBP. Sucrose gradient sedimentation confirmed that SecB is a tetramer that binds to preMBP in a 1:1 stoichiometric complex, as was previously reported (Watanabe and Blobel, 1989b; Lecker *et al.*, 1989). Precursor MBP synthesized *in vitro* or *in vivo* could be precipitated with anti-SecB serum. RBP synthesized *in vitro* could not be precipitated with anti-SecB serum. The association of SecB with wild-type preMBP is relatively unstable; such a complex forms only when SecB is present cotranslationally or following denaturation of previously synthesized preMBP and is detected by coimmunoprecipitation with only low efficiency. In marked contrast, MBP species that are defective in folding (MBPΔ323, MBPΔ57-145) or that exhibit slow folding (MBP2261) form much more stable complexes with SecB, either co- or posttranslationally. This study also demonstrated that complex formation is not dependent on the signal peptide, and the presence of an intact signal peptide does not strengthen the interaction of SecB with MBP species that are defective in folding. However, as also described by Liu *et al.* (1989), this interaction is dependent on the folding properties of preMBP which, in the case of the wild-type protein, are markedly influenced by the presence of the signal peptide.

Watanabe and Blobel (1989b) found that SecB represents less than 0.1% of total cytoplasmic protein. Both Liu *et al.* (1989) and Weiss and Bassford (1990) found that the interaction of SecB with MBP is very dependent on the relative concentration of the two proteins. For example, a molar ratio of at least 4:1 SecB to MBP2261 is required to totally block MBP2261 folding (Liu *et al.*, 1989). Considering the strong propensity of MBP to fold, even with its signal peptide attached, together with the low intracellular concentration of SecB, it seems clear that the proposed antifolding role of SecB is to retard rather than totally prevent the folding of preMBP in the cytoplasm. This interaction is purposely designed to be a transient one, since under normal circumstances the export of preMBP is achieved very rapidly following the completion of translation.

Alterations in the MBP Signal Peptide that Efficiently Suppress the Requirement for SecB

Wild-type MBP export is only partially blocked in $SecB^-$ cells, and such cells are phenotypically Mal^+ (see Fig. 4). In contrast, MBP species with altered signal peptides that exhibit slow export kinetics in $SecB^+$ cells are totally export-defective in $SecB^-$ cells (see above). One such MBP species is

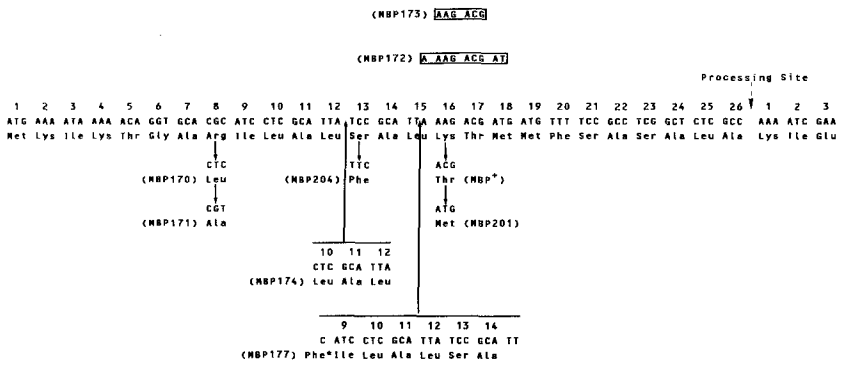

Fig. 5. Mutational alterations in the MBP16-1 signal peptide that restore export in SecB$^-$ cells. The amino acid sequence for the amino-terminal 29 residues of MBP16-1 is shown, including the entire signal peptide. The nucleotide sequence for the corresponding *malE* coding region is also shown. Single amino acid substitutions that suppress the SecB$^-$ export defect are indicated by downward-pointing arrows. An upward-pointing arrow indicates addition of residues to the signal peptide as a result of genetic duplications. Nucleotides deleted from the coding region are boxed. The corresponding designations for each mutant protein are also provided. Note that for MBP172, the deletion of nine base pairs removes residues 16–18 from the hydrophobic core. For MBP 177, Phe* denotes the addition of a novel Phe codon. (From Collier and Bassford, 1989.)

MBP16-1 (see Figs. 1B, 4). SecB$^-$ cells synthesizing MBP16-1 are unable to utilize maltose as sole carbon source. Beginning with such cells, Collier and Bassford (1989) isolated a number of phenotypically Mal$^+$ revertants. Functional SecB synthesis restored in a small proportion of these revertants. However, this selection primarily yielded mutants with alterations in the MBP16-1 signal peptide that permit SecB-independent MBP export to the periplasm to various extents. Altogether, nine unique mutational events were identified (Fig. 5). Although each of these alterations increases the overall hydrophobicity of the signal peptide, it is not possible to strictly equate changes in hydrophobicity with the degree of SecB-independent export.

Somewhat unexpectedly, two of these mutant MBP species, designated MBP172 and MBP173, were found to be exported in SecB$^-$ cells at rates and efficiencies that are markedly superior to those of wild-type MBP. These mutants result from the deletion of two or three residues in the signal peptide hydrophobic core, including in both cases the Lys at postion 16 responsible for the original export defect and the adjacent neutral Thr residue. Although wild-type MBP is not cotranslationally translocated in SecB$^-$ cells (see below), MBP172 and MBP173 exhibit significant cotranslational export in the absence of SecB. Thus, it appears that these mutant signal peptides mediate more rapid entry of preMBP into the export pathway than does the wild-type signal. In addition, Collier and Bassford (1989) observed that a preMBP species having both the MBP172 signal peptide and an additional

alteration in the mature moiety that slows folding (from MBP2261) is exported in SecB$^-$ cells nearly as well as wild-type preMBP in SecB$^+$ cells. The finding that two alterations in the preMBP, one that promotes more rapid export and one that slows folding of the mature moiety, are sufficient to virtually eliminate the SecB requirement for efficient MBP export is considered to provide very strong support for the proposed antifolding function of SecB.

The functional difference between the wild-type MBP signal peptide and those of MBP172 and MBP173 is not known. The hydropathy profiles of these three peptides reveal that the deletion of two slightly hydrophilic Thr residues from the wild-type core increases to a small extent the overall hydrophobicity of this structure. However, the signal peptide of MBP201 (see Fig. 5) also presents a more hydrophobic profile than the wild-type signal, but export of this MBP species in SecB$^-$ cells is considerably less efficient than wild-type preMBP. Regardless of the mechanism, since the MBP172 and MBP173 signal peptides more strongly promote cotranslational MBP translocation, the nature of the signal peptide must have a major role in determining whether a protein is co- or posttranslationally translocated. Freudl *et al.* (1988) reached the same conclusion for the OmpA signal peptide. In this same regard, the ability of the MBP signal peptide to convert RBP translocation from a strictly posttranslational mode (Josefsson and Randall, 1981b) to a cotranslational mode was recently investigated (D. N. Collier, S. M. Strobel, and P. J. Bassford, Jr., manuscript in preparation). Interestingly, the wild-type MBP signal peptide is unable to mediate cotranslational export of a MBP-RBP hybrid protein, but a hybrid protein composed of the MBP172 signal peptide and mRBP does exhibit significant cotranslational export.

Does SecB Actively Promote Entrance of preMBP into the Export Pathway?

Although 60% of wild-type preMBP synthesized in SecB$^-$ cells is exported (see Fig. 4), Kumamoto and Gannon (1988) demonstrated that such export is achieved in a strictly posttranslational fashion. Most of the MBP is translocated within a short time period following synthesis, while preMBP that is not translocated rapidly acquires a protease-resistant conformation. These results are consistent with the idea that wild-type MBP export in SecB$^-$ cells represents a race between delivery of the newly synthesized, export-competent polypeptide to the export machinery in the cytoplasmic membrane and folding of the preMBP into a translocation-incompetent conformation. However, if SecB only functions as an antifolding factor, why is cotranslational export defective in SecB$^-$ cells? As previously described, Randall

(1983) found that cotranslational translocation of MBP is not initiated until after 80% of the nascent chain has been synthesized (see above), which includes the SecB binding region identified by Collier *et al.* (1988). Weiss *et al.* (1988) suggested that the antifolding activity of SecB might promote cotranslational preMBP export simply by binding to the nascent chain in such a manner that ensures that the signal peptide is readily accessible to the export machinery. On the other hand, both Collier *et al.* (1988) and Kumamoto and Gannon (1988) raised the possibility that SecB actively facilitates the entry of preMBP into the export pathway, perhaps by directly interacting with a membrane receptor as well as the nascent chain. This could provide a possible explanation for a much earlier observation by Rasmussen *et al.* (1984) that a MalE-LacZ hybrid protein that retains the first 186 residues of the mMBP, now recognized to include the SecB binding site, is inserted into the cytoplasmic membrane faster and with greater efficiency than a hybrid protein that contains only the first 23 residues of the mMBP. In addition, although the export of a mutant MBP species such as MBPΔ57-145 that is severely defective in folding is much improved over wild-type MBP in $SecB^-$ cells, export is still achieved at a considerably slower rate than for wild-type MBP in $SecB^+$ cells (Collier *et al.*, 1988).

Watanabe and Blobel (1989a,b) demonstrated that SecB is a component of a previously characterized cytosolic factor necessary for preprotein import into inverted *E. coli* membrane vesicles *in vitro*. Most recently, they found that SecB competes with canine SRP in binding to the nascent MBP signal peptide and concluded that SecB functions as the prokaryotic SRP equivalent (Watanabe and Blobel, 1989c). These same workers were unable to demonstrate an interaction of SecB with either mMBP or intact wild-type preMBP, although SecB could be shown to interact with a truncated preMBP species defective in folding. A model was presented in which SecB interacts with the preMBP signal peptide cotranslationally and then targets preMBP delivery to the cytoplasmic membrane, either co- or post-translationally, by subsequently interacting with a membrane SecB receptor. An alternate, SecB-independent pathway was proposed for proteins whose export does not depend on this protein. This model does not account for the 60% of wild-type preMBP that is exported in $SecB^-$ cells, and it is difficult to reconcile with much of the data from other laboratories. However, it is consistent with the suggestion that SecB has two separate roles in the export process. As for the finding that SecB binds to the signal peptide, there is some evidence that hydrophobicity may be one important element in binding of SecB to precursor proteins (Lecker *et al.*, 1989). Thus, some interaction of SecB with the MBP signal peptide, as assayed by Watanabe and Blobel (1989c), might not be unexpected. This clearly requires additional investigation.

Other Chaperone Proteins

Chaperones have been defined as proteins whose role is to mediate the folding and/or assembly of other proteins, but which are excluded from the final structure. This definition has also been expanded to include proteins that can stabilize proteins in a conformation competent for membrane insertion or translocation, since in some instances the same proteins have been implicated in both processes. Chaperones have been identified in both prokaryotes and eukaryotes, and one group of such proteins, termed "chaperonins," appear to be highly conserved in bacteria, mitochondria, and plastids (reviewed by Ellis and Hemmingsen, 1989). In addition to SecB, two *E. coli* proteins, designated trigger factor and GroEL, have been postulated to help maintain the export competence of precursor polypeptides.

Trigger factor has been extensively characterized by Wickner and coworkers (Crooke and Wickner, 1987; Crooke *et al.*, 1988a,b; Lill *et al.*, 1988). It was first identified during studies of proOmpA translocation into *E. coli* membrane vesicles. Purified proOmpA is competent for membrane translocation for only a short time period following rapid renaturation. Trigger factor was isolated from an *E. coli* cytoplasmic fraction by its ability to stabilize purified proOmpA in a conformation competent for posttranslational import into vesicles (Crooke and Wickner, 1987). It is a 63-kDa ribosome-associated protein which forms a soluble, 1 : 1 stoichiometric complex with proOmpA. Lill *et al.* (1988) proposed a model in which trigger factor associates with proOmpA cotranslationally, and simultaneously maintains the tranlocation competence of proOmpA as it helps to deliver it to the export machinery in the cytoplasmic membrane. However, while the findings from *in vitro* experiments are most convincing, as yet there is no evidence demonstrating that trigger factor is involved in protein export *in vivo*.

GroEL is a bacterial heat shock protein of 14 identical 65-kDa subunits (Hohn *et al.*, 1979; Hendrix, 1979) which has been shown to stimulate oligomeric protein and phage assembly (Georgopoulos and Hohn, 1978; Hemmingsen *et al.*, 1988; Goloubinoff *et al.*, 1989). Bochkareva *et al.* (1988) found that GroEL binds newly synthesized pre-β-lactamase *in vitro* and stabilizes it for posttranslational import into membrane vesicles. More recently, Kusukawa *et al.* (1989) found that cells producing temperature-sensitive GroEL are deficient in β-lactamase export under nonpermissive conditions. However, the export of several other proteins is not obviously affected, indicating that this protein, like SecB, is only involved in the export of a subset of *E. coli* envelope proteins. Interestingly, it has been reported that induction of the heat-shock response in *E. coli* can suppress the growth defects of SecB$^-$ cells on rich media (Lecker *et al.*, 1989). Although this effect

was not shown to be specifically due to the induced expression of GroEL, it at least suggests some overlap in the roles of GroEL and SecB.

Lecker *et al.* (1989) found that purified trigger factor, GroEL, and SecB each have the ability to stabilize proOmpA for membrane translocation; however, SecB is clearly the most potent in this regard. SecB is known to help facilitate OmpA export *in vivo*. In addition, all three chaperones form soluble complexes with prePhoE, and even mature OmpA, once again demonstrating that the signal peptide is not essential for complex formation with SecB. A number of soluble proteins, including SecA, albumin, lysozyme, and ribonuclease, are unable to complex with any of the chaperones, indicating that there clearly is some specificity to the interaction of these chaperones with exported proteins. It was suggested that understanding the binding specificity of this interaction might reveal a fundamental distinction between cytoplasmic and noncytoplasmic proteins. Furthermore, these studies indicate that each of these chaperones may function in a similar manner to maintain the export competence of precursor proteins and, possibly, to help promote their entrance into the export pathway.

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