

## SecB-Independent Export of *Escherichia coli* Ribose-Binding Protein (RBP): Some Comparisons with Export of Maltose-Binding Protein (MBP) and Studies with RBP-MBP Hybrid Proteins

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The efficient export of the *Escherichia coli* maltose-binding protein (MBP) is known to be SecB dependent, whereas ribose-binding protein (RBP) export is SecB independent. When the MBP and RBP signal peptides were exchanged precisely at the signal peptidase processing sites, the resultant RBP-MBP and MBP-RBP hybrid proteins both were efficiently exported in SecB<sup>+</sup> cells. However, only MBP-RBP was efficiently exported in SecB<sup>-</sup> cells; RBP-MBP exhibited a significant export defect, a finding that was consistent with previous proposals that SecB specifically interacts with the mature moiety of precursor MBP to promote export. The relatively slow, totally posttranslational export mode exhibited by certain mutant RBP and MBP-RBP species in SecB<sup>+</sup> cells was not affected by the loss of SecB. In contrast, MBP and RBP-MBP species with similarly altered signal peptides were totally export defective in SecB<sup>-</sup> cells. Both export-defective MBP and RBP-MBP interfered with SecB-mediated protein export by depleting cells of functional SecB. In contrast, neither export-defective RBP nor MBP-RBP elicited such an interference effect. These and other data indicated that SecB is unable to interact with precursor RBP or that any interaction between these two proteins is considerably weaker than that of SecB with precursor MBP. In addition, no correlation could be established between a SecB requirement for export and PrlA-mediated suppression of signal peptide export defects. Finally, previous studies have established that wild-type MBP export can be accomplished cotranslationally, whereas wild-type RBP export is strictly a posttranslational process. In this study, cotranslational export was not detected for either MBP-RBP or RBP-MBP. This indicates that the export mode exhibited by a given precursor protein (cotranslational versus posttranslational) is determined by properties of both the signal peptide and the mature moiety.

SecB is a nonessential, cytoplasmic protein that is composed of four identical 16.4-kDa subunits and is required for the efficient export of a subset of *Escherichia coli* envelope proteins (8, 9, 24-28, 47, 49, 52). Such SecB-dependent proteins include maltose-binding protein (MBP), LamB, OmpA, OmpF, and OppA. On the other hand, the export of certain other proteins appears to be totally SecB independent. These include ribose-binding protein (RBP), TEM  $\beta$ -lactamase, lipoprotein, and phage M13 coat protein. The role of SecB in facilitating MBP export has been intensively investigated in several laboratories. Collier et al. (8) originally proposed that SecB interacts directly with one or more sites within the mature moiety of precursor MBP (preMBP) to maintain the polypeptide in a translocation-competent conformation, thought to be a largely unfolded or loosely folded state that is not inhibitory to membrane transit (45). A variety of genetic and biochemical experiments have provided considerable support for this model (9, 15, 27, 32, 33, 41, 50, 52), including several recent studies showing that SecB specifically binds to the mature moiety of preMBP to form a soluble complex that presumably is a transient intermediate in the export process (33, 41, 50). In cells lacking SecB, MBP export is thought to be a race between delivery of the newly synthesized, export-competent polypeptide to the export machinery in the cytoplasmic mem-

brane and folding of preMBP in the cytoplasm into an export-incompetent conformation.

Although the evidence supporting an antifolding role for SecB is compelling, it should be noted that there is some controversy concerning SecB function. Watanabe and Blobel (47, 48) were unable to demonstrate an interaction of SecB with mature MBP. Rather, these investigators proposed that SecB binds to the signal peptide of nascent preMBP and serves to target preMBP to the export machinery in the cytoplasmic membrane. By this model, SecB functions similarly to the signal recognition particle identified previously in studies of protein targeting to the rough endoplasmic reticulum of eucaryotic cells (for a review, see reference 46). Watanabe and Blobel (48) further proposed that there exists in *E. coli* cells an alternate pathway to accommodate the export of SecB-independent proteins such as RBP.

It was anticipated that a clearer understanding of the role of SecB in *E. coli* protein export might emerge from studies comparing the SecB-dependent and SecB-independent export pathways. RBP, like MBP, is a periplasmic sugar-binding protein that is initially synthesized with an amino-terminal signal peptide that exhibits the same three functionally conserved regions typical of most procaryotic signals (reviewed in reference 38) (Fig. 1). There is no obvious feature of the RBP signal peptide that readily distinguishes it from the signal peptide of MBP or other SecB-dependent proteins. In this study, the RBP and MBP signal peptides have been exchanged, and the export of the resultant hybrid proteins in both SecB<sup>+</sup> and SecB<sup>-</sup> cells has

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TABLE 1. *E. coli* strains

Strain	Genotype	Reference or source
MC4100	F <sup>-</sup> $\Delta$ lacU169 araD139 rpsL150 thi fbbB5301 deoC7 ptsF25 relA1 ( <i>malE</i> <sup>+</sup> <i>rbsB</i> <sup>+</sup> )	7
BAR1091	MC4100 <i>malE</i> $\Delta$ 312 <i>lac</i> <sup>+</sup> <i>lacI</i> <sup>q</sup> (F' <i>lacI</i> <sup>q</sup> Tn5)	42
BAR1092	BAR1091 <i>prlA</i> 402	42
DNC324	MC4100 <i>malE</i> $\Delta$ 444 <i>secB</i> ::Tn5(F' <i>lacI</i> <sup>q</sup> Tn5)	9
MRi7	MC4100 $\Delta$ <i>rbs</i> -7	34
JW46	MC4100 F' Cm <sup>r</sup> (from strain CJ236 [29])	This study
ROB1	MC4100 $\Delta$ <i>rbs</i> -7(F' <i>lacI</i> <sup>q</sup> Tn5)	This study
ROB3	ROB1 <i>prlA</i> 402	This study
SMS205	MRi7 <i>secB</i> ::Tn5(F' Cm <sup>r</sup> )	This study
SMS217	MC4100 <i>malE</i> 16-1 $\Delta$ <i>rbs</i> -7(F' <i>lacI</i> <sup>q</sup> Tn5)	This study

been investigated. The results provide additional evidence that SecB promotes MBP export via its interaction with the mature moiety of preMBP. In addition, studies of RBP export indicate not only that RBP export is SecB independent but also that there is very little, if any, interaction between these two proteins.

#### MATERIALS AND METHODS

**Bacterial strains.** The *E. coli* K-12 strains used in this study are derivatives of strain MC4100 (7) and are listed in Table 1.

**Reagents.** Minimal medium M63 supplemented with a carbon source (0.2%) and thiamine (2  $\mu$ g/ml) and maltose-tetrazolium indicator agar were prepared as described previously (37). When required, ampicillin was added to minimal and complex media at concentrations of 25 and 50  $\mu$ g/ml, respectively. To induce *malE* genes under *lacUV5* promoter-operator control, isopropylthiogalactoside was used on agar plates and in liquid media at 1 and 2 mM, respectively. [<sup>35</sup>S]methionine (Trans<sup>35</sup>S-label; 1,162 Ci/mmol) was obtained from ICN Biochemicals, Inc., Irvine, Calif. Electrophoresis reagents, restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and Klenow fragment were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Kodak XAR film was obtained from Eastman Kodak Co., Rochester, N.Y. Rabbit anti-MBP serum has been described previously (11). Rabbit anti-RBP serum was prepared in the same manner with purified RBP provided by Paul Ray of Wellcome Research Laboratories, Research Triangle Park, N.C.

**Plasmids and plasmid constructions.** The plasmids used in this study are derivatives of pJF2 (14), which carries the *malE* gene under *lacUV5* promoter-operator control and the phage M13 intergenic region. Plasmid pDNC186 carries the *rbsB* gene encoding RBP under *lacUV5* promoter-operator control and was constructed as follows. The 1.3-kb *Pst*I-*Nde*I fragment of pSE100 (which harbors the *rbsB* and *rbsK* genes on a 4-kb *Hind*III insert [34]) was replaced with a 1.8-kb *Pst*I-*Nde*I fragment of pJF2 (includes the M13 intergenic region [14]), yielding pROB1. The two *Pvu*II sites downstream of *rbsB* in pROB1 were destroyed, and a unique *Pvu*II site was introduced 24 bp upstream of the initiation codon of *rbsB*. The *Pst*I-*Pvu*II fragment of this plasmid was replaced with an 851-bp *Pst*I-*Pvu*II fragment from pGL101 (31) carrying the *lacUV5* promoter-operator, yielding plasmid pDNC186.

Mutagenesis of plasmid pDNC186 DNA with synthetic oligonucleotides encoding the *rbsB*-9-1, *rbsB*-12-1, and *rbsB*-15-1 mutations yielded pDNC188, pDNC189, and pDNC190, respectively. A unique *Nar*I restriction site was introduced at the junction of the signal peptide and mature coding regions of the *rbsB*<sup>+</sup> gene on pDNC186 and the *malE*<sup>+</sup>, *malE*10-1, and *malE*16-1 genes carried on pJF2, pJF18 (J. D. Fikes and P. J. Bassford, Jr., unpublished data), and pJF27 (51), respectively. Next, the signal peptide-coding region of *rbsB* was replaced with *Pst*I-*Nar*I fragments carrying the various *malE* signal peptide-coding regions. In vitro mutagenesis with a chimeric oligonucleotide (*malE*-*rbsB*) removed the *Nar*I site such that the resultant MBP-RBP hybrid proteins were fused precisely at the processing site. This resulted in plasmids pDNC197 (encoding MBP-RBP), pDNC198 (MBP10-1-RBP), and pDNC199 (MBP16-1-RBP). Plasmids pSKV2 encoding MBP15-1 and pSMS33 encoding MBP15-1-RBP were constructed by in vitro mutagenesis of pJF2 DNA and pDNC197 DNA, respectively.

Replacement of the *malE* signal sequence coding region of pJF2 by that of *rbsB*, with the strategy outlined above, resulted in pSMS41 encoding RBP-MBP. Mutagenesis of pSMS41 DNA with the appropriate synthetic oligonucleotides generated plasmids pSMS42 (encoding RBP9-1-MBP), pSMS43 (encoding RBP12-1-MBP), and pSMS44 (encoding RBP15-1-MBP).

Plasmid pJF32 (8) is a derivative of pJF2 harboring *malE* $\Delta$ 323 (an in-frame deletion that removes residue 7 of the MBP signal peptide through residue 89 of the mature moiety). Plasmid pJW21 (52) is a derivative of pBR322 harboring *secB*<sup>+</sup>.

**Oligonucleotide-directed mutagenesis.** The oligonucleotide-directed mutagenesis method of Zoller and Smith (54) was used, with the following changes: first, single-stranded plasmid DNA was used as a template; second, to increase the efficiency of mutagenesis as described by Kunkel et al. (29), uracil-containing templates were prepared from cells of *E. coli* CJ236 (*ung dut*). Mutagenic primers were prepared with an Applied Biosystems 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis (PAGE) as described by Hutchison et al. (18).

Plasmids packaged as M13 particles were prepared by the method of Zagursky and Berman (53), except that M13KO7 (29) was used as the helper phage. All constructs were confirmed by DNA sequencing as described by Bankier et al. (3).

**Radiolabeling, immunoprecipitation, SDS-PAGE, and autoradiography.** Cultures were grown to the midlog phase in glycerol minimal medium supplemented with ampicillin and induced for synthesis of MBP, RBP, or hybrid proteins by the addition of isopropylthiogalactoside to the culture medium. Thirty minutes later, cells were labeled with [<sup>35</sup>S]methionine for 15 s. Chase periods were initiated and terminated as described previously (43). MBP, RBP, and hybrid proteins were immunoprecipitated with solubilized cell extracts with the appropriate antisera, and immunoprecipitates were resolved by sodium dodecyl sulfate (SDS)-PAGE and autoradiography as described previously (2, 11). The amount of radioactivity present as precursor and mature forms was determined, where indicated, either (i) by excising the corresponding areas of the dried gels, rehydrating and solubilizing the gel pieces with a mixture of NCS tissue solubilizer (Amersham) and water (9:1), and counting in ScintiVerse II (Fisher Scientific), or (ii) by using an AMBIS radioanalytic imaging system (AMBIS Systems, San Diego, Calif.) Counts

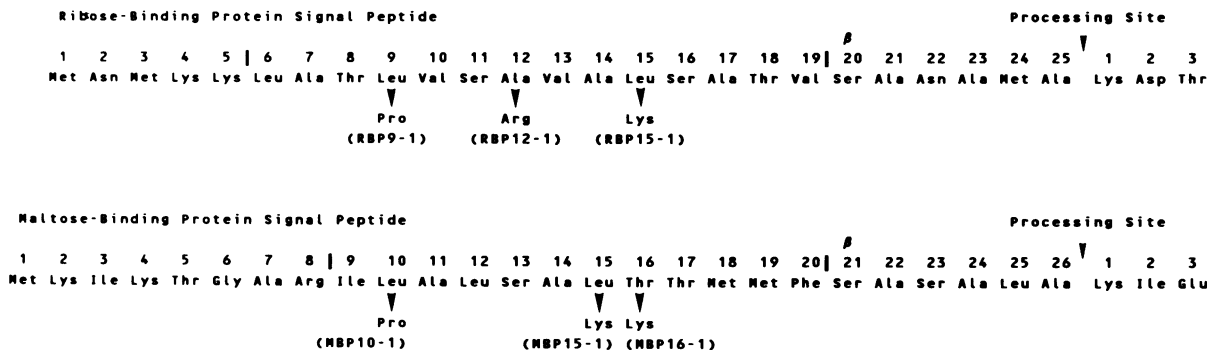


FIG. 1. RBP and MBP signal peptides. The amino-terminal 28 and 29 residues of RBP and MBP, respectively, are shown, including the entire signal peptides and the signal peptidase processing sites. The vertical bars separate the three functionally conserved regions typical of signal peptides (38): the amino-terminal hydrophilic segment, followed by the hydrophobic core and, at the carboxyl terminus, the signal peptidase recognition sequence.  $\beta$  indicates the  $\beta$ -turn that is predicted at the junction of the core and processing site regions. Plasmids encoding a variety of export-defective protein species were used in this study (see Materials and Methods). The amino acid substitutions in the core regions responsible for the export defects are indicated, along with the designations used for the corresponding mutant proteins. Note that RBP12-1, RBP15-1, and MBP15-1 are new mutant species that were constructed for this study and have not been described previously. See the text for additional details.

were adjusted for the loss of methionine residues when precursor proteins were processed to the mature form.

**Analysis of cotranslational versus posttranslational processing.** The processing of nascent chains was analyzed as previously described by Josefsson and Randall (22).

## RESULTS

**Exchange of the RBP and MBP signal peptides.** The primary amino acid sequences of the MBP and RBP signal peptides are shown in Fig. 1. Plasmids encoding RBP-MBP and MBP-RBP hybrid proteins were constructed as described in Materials and Methods. In both cases, the fusion joint of the hybrid protein was the signal peptidase cleavage site. Thus, the RBP-MBP hybrid protein was synthesized with the intact RBP signal peptide attached to the intact MBP mature moiety, and the MBP-RBP hybrid protein was synthesized with the intact MBP signal peptide preceding the intact RBP mature moiety. The syntheses of both hybrid proteins were under regulatory control of the *lacUV5* promoter-operator and inducible by isopropylthiogalactoside. Cells synthesizing these hybrid proteins at induced levels exhibited no obvious growth defects. In cells harboring an internal deletion of the *malE* gene (strain BAR1091), synthesis of the RBP-MBP hybrid protein restored a fully *Mal*<sup>+</sup> phenotype as indicated by growth on maltose minimal medium or colony color on maltose-tetrazolium indicator agar. Since *rbsB* mutants can still utilize ribose as a carbon source (34), a similar determination could not be made for cells synthesizing the MBP-RBP hybrid protein, but it was assumed that fully functional RBP was exported to the periplasm (see below).

**Export kinetics in SecB<sup>+</sup> and SecB<sup>-</sup> cells.** A pulse-chase analysis was employed to examine the export kinetics of MBP, RBP, MBP-RBP, and RBP-MBP in both SecB<sup>+</sup> cells and SecB<sup>-</sup> cells. Isopropylthiogalactoside-induced, mid-log-phase cells were pulse-radiolabeled with [<sup>35</sup>S]methionine for 15 s, an excess of unlabeled methionine was added to initiate the chase, and MBP, RBP, or the hybrid proteins were immunoprecipitated at various chase times with the appropriate antisera. The precipitates obtained were analyzed by SDS-PAGE and autoradiography. Each of these protein species was rapidly and efficiently exported in SecB<sup>+</sup> cells,

as indicated by the finding that the great majority of protein precipitated at the 1-min chase points was found in its mature form (Fig. 2).

Somewhat different results were obtained for SecB<sup>-</sup> cells (Fig. 3). As expected, there was a considerable defect in MBP export by SecB<sup>-</sup> cells. After 20 min of chase, only 60% of the total MBP synthesized had been exported and processed; the remaining precursor was permanently trapped in the cytoplasm (8, 9, 25, 27). In contrast, RBP export kinetics were not noticeably different from those seen in SecB<sup>+</sup> cells. Of the two hybrid proteins, MBP-RBP showed export kinetics that were also unchanged in SecB<sup>-</sup> cells compared with those of SecB<sup>+</sup> cells. In marked contrast, RBP-MBP export was similar to that of MBP in SecB<sup>-</sup> cells in that a major fraction of the precursor protein was rendered totally export incompetent.

**Mutational alterations in the RBP and MBP signal peptides.** The introduction of charged or helix-breaking Pro residues into the hydrophobic core of a signal peptide usually results in a significant export defect (for a recent review, see

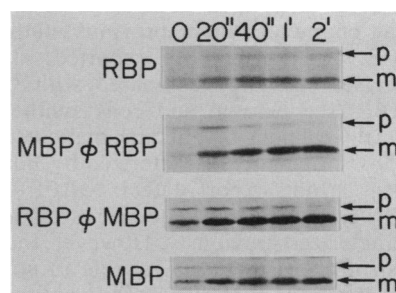


FIG. 2. Export kinetics in SecB<sup>+</sup> cells. Cells of strain BAR1091 or ROB1 (Table 1) harboring plasmids encoding the indicated proteins were pulse-labeled with [<sup>35</sup>S]methionine for 15 s and chased with excess unlabeled methionine. At the indicated time points, samples were removed, the chase was terminated, and the protein products were immunoprecipitated with appropriate antisera and analyzed by SDS-PAGE and autoradiography. The positions of the precursor (p) and mature (m) forms of each protein species are indicated by arrows at the right. (Note that signal peptide processing has been demonstrated in a variety of studies to be a reliable indicator of MBP export to the periplasm [1, 2, 11, 13, 43].)

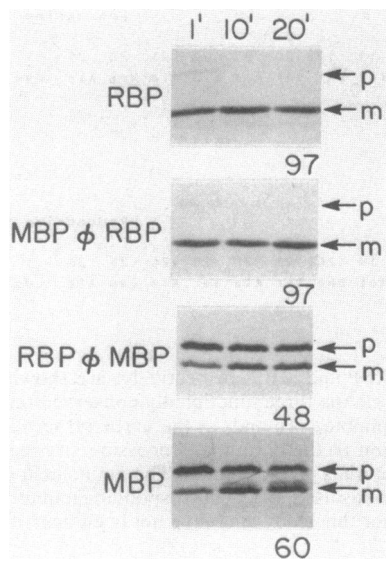


FIG. 3. Export kinetics in *SecB*<sup>-</sup> cells. The experimental conditions were as described in the legend to Fig. 2, except that *SecB*<sup>-</sup> cells of strains DNC324 or SMS205 (Table 1) were used. For each protein species, the percentage of total radiolabeled protein precipitated in the mature form at the 20-min chase point is indicated below the appropriate lane.

reference 4). Three such mutational alterations for both the MBP and RBP signal peptides are shown in Fig. 1. As previously described, the *rbsB9-1* (also designated *rbsB103* [19]) and *malE10-1* (6) mutations both result in the substitution of Pro for Leu at position -17 (relative to the signal peptidase cleavage site) of the RBP and MBP signal peptides, respectively. Oligonucleotide-directed mutagenesis was employed to introduce the *rbsB9-1* mutation into the *lacUV5-rbsB* gene of plasmid pDNC186 (see Materials and Methods). Two additional *rbsB* signal sequence mutations, designated *rbsB12-1* and *rbsB15-1*, were similarly constructed, as was *malE15-1*. Both the *rbsB15-1* and *malE15-1* mutations substitute Lys for Leu in the center of the hydrophobic core, whereas *rbsB12-1* and *malE16-1* (6) substitute a basic charged residue for a neutral residue in the hydrophobic core. The export competence of MBP10-1 and MBP16-1 was characterized in previous studies (11, 43). Both of these MBP species are exported, although at a reduced rate and efficiency compared with those of the wild-type MBP (see below), and cells synthesizing these proteins are still capable of utilizing maltose as a carbon source. RBP9-1, originally characterized by Iida et al. (19), exhibits a very strong export defect. MBP15-1, RBP12-1, and RBP15-1 were new signal sequence alterations that had not been characterized previously. However, the finding that cells synthesizing MBP15-1 were unable to utilize maltose indicated that this mutant MBP species was strongly export defective.

Plasmids encoding MBP-RBP and RBP-MBP hybrid proteins harboring each of the signal sequence alterations shown in Fig. 1 also were constructed as described in Materials and Methods. RBP15-1-MBP-synthesizing cells were unable to utilize maltose as a carbon source, indicating that the RBP15-1 signal peptide is strongly export defective. Cells synthesizing RBP9-1-MBP or RBP12-1-MBP could grow on maltose minimal medium. However, such cells formed colonies exhibiting a *Mal*<sup>-</sup> phenotype on maltose-

tetrazolium agar, indicating that the RBP9-1 and RBP12-1 signal peptides only promoted inefficient MBP export (data not presented; see below).

**Export kinetics of proteins with altered signal peptides in *SecB*<sup>+</sup> and *SecB*<sup>-</sup> cells.** The export kinetics of each mutant MBP, RBP, and hybrid protein species in *SecB*<sup>+</sup> cells was determined as described above. At each chase point, the amount of mature protein precipitated relative to total protein precipitated was determined; the data are presented graphically in Fig. 4A through D. These results confirmed that both the MBP15-1 and RBP15-1 signal peptides were strongly export defective when preceding either the MBP or RBP mature moiety. Consistent with the results of Iida et al. (19), the RBP9-1 signal peptide was found to be nearly as defective, promoting the export of less than 10% of the total RBP9-1 or RBP9-1-MBP synthesized. The RBP12-1 signal peptide was clearly much more proficient than either of the other two mutant RBP signals at mediating export of either RBP (45% processed at 20 min) or MBP (30% processed at 20 min). Export of MBP10-1 and MBP16-1 was similar to that previously reported (11, 43), and there did not appear to be major differences in the abilities of these two mutant signal peptides to effect the export of MBP versus RBP. As previously shown for various mutant MBP species (4), the export of RBP or MBP mediated by an altered signal peptide was accomplished in a relatively slow, posttranslational fashion. In most cases, very little mature protein was precipitated at the 1-min chase point; increasing amounts of processed protein were discerned at later chase times.

Although wild-type RBP and the MBP-RBP hybrid protein were exported in a *SecB*-independent fashion (Fig. 3), the possibility was considered that mutant RBP species exhibiting significantly slower export kinetics (e.g., RBP12-1; Fig. 4C) would also now exhibit significant *SecB* dependence (see Discussion). The export kinetics of RBP12-1 and MBP10-1-RBP in *SecB*<sup>+</sup> cells and *SecB*<sup>-</sup> cells were compared (Fig. 5). The export efficiency of RBP12-1 appeared to be identical in both instances. There was a slight diminution in MBP10-1-RBP export efficiency in *SecB*<sup>-</sup> cells (17% processed after a 20-min chase) compared with that of *SecB*<sup>+</sup> cells (21% processed after a 20-min chase) that proved to be reproducible in several different experiments. However, the decrease in export due to the loss of *SecB* function was not nearly as severe as for MBP10-1. Previous studies have shown that MBP species with altered signal peptides that are slowly exported in *SecB*<sup>+</sup> cells (e.g., MBP10-1, MBP16-1) are totally export defective in the absence of *SecB* (8, 9). Likewise, the three RBP-MBP hybrid proteins with altered signal peptides were found in this study to be totally export defective in *SecB*<sup>-</sup> cells, and such cells were unable to utilize maltose as a carbon source (data not presented).

**Synthesis of export-defective RBP or MBP-RBP does not interfere with MBP16-1 export.** The failure to demonstrate a *SecB* requirement for wild-type or mutant RBP export does not necessarily indicate that *SecB* is unable to interact with precursor RBP in the cytoplasm. It has been demonstrated previously that synthesis of export-defective MBP species can deplete cells of *SecB* function, presumably because the MBP that accumulates in the cytoplasm binds the small amount of available *SecB* (8, 33). One such strongly interfering species is MBPΔ323. Synthesis of MBPΔ323 in *SecB*<sup>+</sup> cells completely blocks MBP16-1 export since, as mentioned above, export of this MBP species is totally *SecB* dependent (Fig. 6F). Thus, MBP16-1 export is a good indicator of *SecB* availability. MBP16-1 export is not affected by the synthesis of either wild-type RBP (which is efficiently exported) or

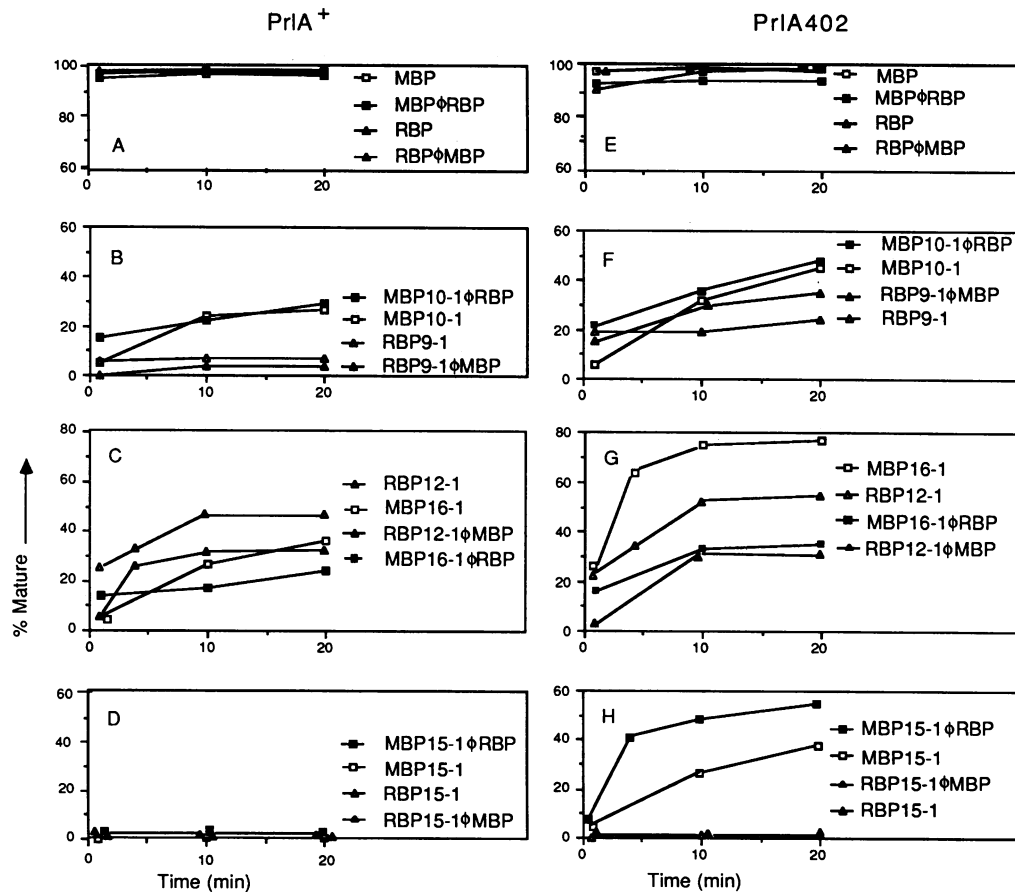


FIG. 4. Export kinetics in SecB<sup>+</sup> PrIA<sup>+</sup> and SecB<sup>+</sup> PrIA402 cells. The experimental conditions were as described in the legend to Fig. 2, except that PrIA402 cells of strain BAR1092 or ROB3 (Table 1) were used in the experiments shown in panels E through H. The results of these experiments are presented graphically. Each chase point represents the percentage of the total radiolabeled protein precipitated in its mature form (see Materials and Methods). In most cases, results for only 1-, 10-, and 20-min chase points were determined. However, in some instances (e.g., MBP16-1 in PrIA402 cells; panel G), a 4-min chase point was taken to more accurately gauge the slope of the line between the 1- and 10-min chase points. See the text for additional details.

RBP15-1 (which is totally export defective) (Fig. 6). Likewise, synthesis of export-defective hybrid proteins MBP15-1-RBP and MBP16-1-RBP failed to cause interference with MBP16-1 export. Thus, SecB did not appear to interact with

export-defective RBP or MBP-RBP hybrid proteins by this criterion. As expected, export-defective RBP-MBP hybrid proteins did exhibit interference with SecB-dependent protein export (data not presented).

**Overproduction of SecB does not affect RBP export kinetics.** The presence of the *secB*<sup>+</sup> gene on a multicopy plasmid causes a noticeable, albeit minor, delay in the export kinetics of wild-type MBP (8, 25). The cellular level of SecB is greatly increased in these cells (51), and this delay presumably results from a prolonged interaction of SecB with preMBP in the cytoplasm. If SecB also is able to interact with preRBP, one might expect to see a similar delay in RBP export kinetics in SecB-overproducing cells. However, except for a minor difference at the 0-s chase point, RBP export in cells harboring pBR322 was indistinguishable from that in cells harboring pJW21 (51), a pBR322 derivative encoding the *secB*<sup>+</sup> gene (Fig. 7). In contrast, wild-type MBP export clearly was slowed in SecB-overproducing cells (note particularly the preMBP/mature MBP ratio at the 20- and 40-s chase points).

**Export in cells harboring a strong *prlA* suppressor allele.** Various *prlA* mutations suppress a wide variety of signal sequence mutations in the *lamB*, *malE*, and *phoA* genes (1, 11, 36). The efficient export of both LamB and MBP is

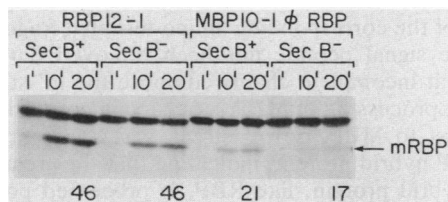


FIG. 5. Export kinetics of RBP12-1 and MBP10-1-RBP are unaffected by SecB availability. Cells of strain ROB1 (SecB<sup>+</sup>) or SMS205 (SecB<sup>-</sup>) harboring either plasmid pDNC189 encoding RBP12-1 or pDNC198 encoding MBP10-1-RBP were pulse-radiolabeled with [<sup>35</sup>S]methionine for 15 s and chased with unlabeled methionine. At the indicated time points, samples were removed, the chase was terminated, and the protein products were precipitated with anti-RBP serum and analyzed by SDS-PAGE and autoradiography. The arrow at right indicates the position of mature RBP. The percentages of total radiolabeled protein precipitated as mature RBP at the 20-min chase point are indicated below the appropriate lanes.

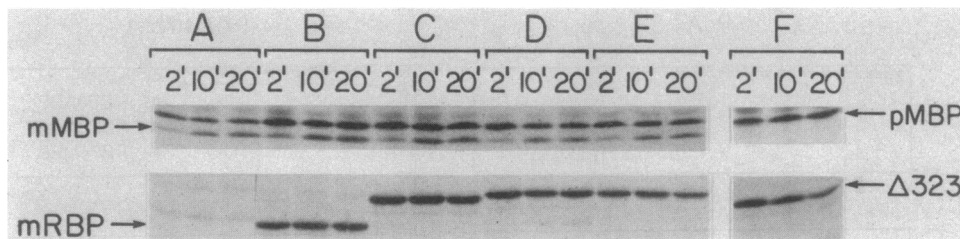


FIG. 6. Export-defective RBP and MBP-RBP species do not interfere with MBP16-1 export. Cells of strain SMS217 (*malE16-1 Δrbs7*) harboring various plasmids (see below) were pulse-radiolabeled with [ $^{35}$ S]methionine for 15 s and chased with unlabeled methionine. At the indicated time points, samples were removed, the chase was terminated, and the protein products were immunoprecipitated with the appropriate antisera and analyzed by SDS-PAGE and autoradiography. Arrows at left indicate the positions of mature MBP and RBP. Arrows at right indicate the positions of preMBP16-1 and MBPΔ323. Proteins tested for their ability to interfere with MBP16-1 export were as follows: A, none (pBR322 vector control); B, wild-type RBP (encoded by pDNC186); C, RBP15-1 (encoded by pDNC190); D, MBP16-1-RBP (encoded by pDNC199); E, MBP15-1-RBP (encoded by pSMS33); and F, MBPΔ323 (encoded by pJF32). Note that MBP16-1 maturation is totally blocked in cells concomitantly synthesizing MBPΔ323 (F).

known to be SecB dependent, and efficient PhoA export also recently was shown to exhibit considerable SecB dependence, particularly at low temperatures (30). Trun et al. (44) had demonstrated previously that SecB was required for PrlA-mediated export of LamB with various altered signal peptides. Since RBP export is SecB independent, it was considered possible that *prlA* mutations would not suppress *rbsB* signal sequence mutations due to the lack of an interaction between SecB and the RBP precursor. The export kinetics of MBP, RBP, and hybrid proteins with mutant signal peptides were determined for cells harboring the *prlA402* allele, a particularly strong *prlA* suppressor mutation (1).

Export of RBP12-1 and RBP15-1 was not significantly improved in PrlA402 cells (Fig. 4E through H). Likewise, RBP12-1-MBP and RBP15-1-MBP export was not obviously improved in PrlA402 cells. There apparently was some slight improvement in RBP15-1-MBP export that was not detected by the gel analysis, since PrlA402 cells synthesizing this MBP species could grow slowly on maltose minimal medium. Interestingly, there was a significant increase in the efficiency of export of both RBP9-1 and RBP9-1-MBP in PrlA402 cells compared with that discerned for isogenic Prl<sup>+</sup> cells. (RBP9-1 export showed a similar improvement in PrlA402 SecB<sup>-</sup> cells, whereas RBP9-1-MBP was not exported in PrlA402 SecB<sup>-</sup> cells [data not presented].) As previously demonstrated (1), the export of both MBP10-1 and MBP16-1 showed considerable improvement in PrlA402 cells. MBP10-1-RBP export also showed marked improvement in PrlA402 cells, but there was only a very modest

improvement in MBP16-1-RBP export in PrlA402 cells compared with that in Prl<sup>+</sup> cells. Finally, significant amounts of MBP15-1 and MBP15-1-RBP were exported in PrlA402 cells, whereas these two proteins were totally export incompetent in Prl<sup>+</sup> cells.

**Temporal mode of processing of RBP-MBP and MBP-RBP.** MBP translocation can be initiated cotranslationally, as demonstrated by the finding of incomplete nascent chains from which the signal peptide had been proteolytically removed (20, 21). In contrast, RBP translocation is normally a posttranslational process; nascent chains are not processed unless protein synthesis is terminated by the addition of chloramphenicol (39). The accessibility of only processed nascent chains to externally added proteinase K confirmed that processing is a valid indicator of translocation (39). To study the contribution of the MBP and RBP signal peptides to this phenomenon, the temporal mode of processing of RBP-MBP and MBP-RBP hybrid proteins in SecB<sup>+</sup> cells was determined by the technique of Josefsson and Randall (22). Cells synthesizing MBP, RBP, or one of the hybrid proteins were pulse-labeled with [ $^{35}$ S]methionine, and nascent chains were analyzed by limited proteolysis with staphylococcal V8 protease, two-dimensional SDS-PAGE, and autoradiography (Fig. 8). The horizontal streak designated p' in Fig. 8A consists of the amino-terminal proteolytic fragment of nascent MBP chains retaining the signal peptide and sufficiently elongated to carry the first V8 protease cleavage site, whereas the horizontal streak designated m' consists of the corresponding amino-terminal fragment from which the signal peptide has been removed (20-22). The finding that incomplete chains compose the m' streak indicates that processing of MBP is occurring cotranslationally. In contrast to MBP, no m' streak was apparent with the RBP-MBP hybrid protein, indicating that the great majority of this hybrid protein, like RBP, is processed posttranslationally.

The carboxy-terminal V8 proteolytic fragment was used to monitor the processing of nascent chains bearing the RBP mature moiety (Fig. 8C and D). V8 proteolysis of unprocessed, incomplete polypeptides yielded a diagonal streak that fused with the spot derived from the carboxy terminus of the full-length precursor (again designated p'). Cotranslational processing would yield a diagonal streak parallel to the p' streak that fuses with the spot derived from the carboxy terminus of matured RBP (designated m') (39). Note that neither wild-type RBP nor MBP-RBP produced a streak

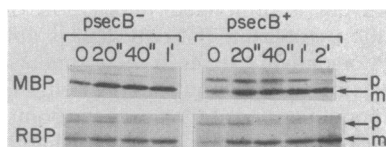


FIG. 7. Overproduction of SecB does not affect wild-type RBP export kinetics. Cells of strain JW46 (*malE<sup>+</sup> rbsB<sup>+</sup>*) harboring either pBR322 (psecB<sup>-</sup>; vector control) or pJW21 (psecB<sup>+</sup>) were pulse-radiolabeled with [ $^{35}$ S]methionine for 15 s and then chased with excess unlabeled methionine. At the indicated time points, samples were removed, the chase was terminated, and the protein products were immune precipitated with appropriate antisera and analyzed by SDS-PAGE and autoradiography. The positions of precursor (p) and mature (m) forms of both wild-type MBP and RBP are indicated by arrows at the right.



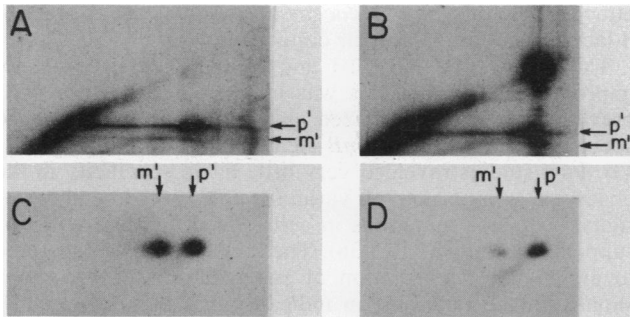


FIG. 8. Two-dimensional analysis of nascent chain processing for MBP, RBP, and hybrid proteins in SecB<sup>+</sup> cells. Cells of strain BAR1091 harboring plasmids encoding wild-type MBP (pJF2; panel A) or RBP-MBP (pSMS41; panel B) and cells of strain ROB1 harboring plasmids encoding MBP-RBP (pDNC197; panel C) or wild-type RBP (pDNC186; panel D) were pulse-radiolabeled with [<sup>35</sup>S]methionine for 15 or 17 s, respectively. MBP and RBP polypeptides, including incomplete nascent chains, were immunoprecipitated and separated by 10% SDS-PAGE. The polypeptides contained in gel slices were subjected to limited proteolysis by V8 protease, and the products were resolved by a second-dimension 15% SDS-PAGE. In panels A and B, p' indicates the amino-terminal fragment derived from MBP molecules still bearing a signal peptide, whereas m' indicates the amino-terminal fragment derived from processed MBP molecules. Note the streak extending leftward into the gel from p' in panels A and B. This indicates immunoprecipitation of unprocessed MBP nascent chains. Likewise, the streak extending leftward from m' in panel A indicates immunoprecipitation of processed nascent chains. The lack of a corresponding m' streak in panel B demonstrates that processed RBP-MBP nascent chains were not precipitated to any significant extent. In panels C and D, p' indicates the carboxy-terminal fragment derived from RBP molecules still bearing a signal peptide, whereas m' indicates the carboxy-terminal fragment from processed RBP molecules. The streak extending from p' toward the bottom left corner of the gel in panels C and D indicates immunoprecipitation of unprocessed nascent chains. The lack of a corresponding streak from m' demonstrates that processed nascent chains were not immunoprecipitated to any significant extent for either MBP-RBP or RBP. Only the relevant portion of each gel is shown. For useful discussions of this technique and interpretation of peptide patterns, see references 20 through 22 and 39.

fusing with m', indicating that these proteins were processed in a posttranslational fashion.

## DISCUSSION

MBP export is considered to be SecB dependent; the kinetics of MBP export in SecB<sup>-</sup> cells are altered significantly compared to those determined for SecB<sup>+</sup> cells, and a significant fraction (approximately 40%) of the total MBP synthesized is permanently trapped in the cytoplasm (8, 9, 24, 25). In contrast, SecB availability does not affect either the efficiency or rate of RBP export (8, 9, 24). Thus, RBP export is considered to be SecB independent. In this study, RBP-MBP and MBP-RBP hybrid proteins were constructed, and their dependence on SecB for export was investigated. The results clearly revealed that the requirement for SecB resided with the mature moiety. RBP synthesized with an MBP signal peptide exhibited no detectable dependence on SecB for export, whereas MBP synthesized with an RBP signal peptide was exported in a SecB-dependent fashion similar to wild-type preMBP. These results are consistent with several studies indicating that SecB specifically interacts with the mature moiety of preMBP (8, 33, 41, 50).

Gannon et al. (15) took a similar approach with MBP-PhoA and PhoA-MBP hybrid proteins and reached similar conclusions. However, none of the hybrid proteins studied represented fusions of the intact signal peptide with the intact mature moiety; in all cases, the fusion joint was within the respective mature moiety. Such hybrid proteins are likely to exhibit altered folding properties. Considering the proposed role of SecB as an antifolding factor, this could have had some bearing on the results obtained. In addition, although PhoA was considered to be a prototypical SecB-independent protein, a more recent study by Kusakawa et al. (30) revealed that PhoA export exhibits considerable SecB dependence, particularly at temperatures below 37°C. Studies with RBP-MBP and MBP-RBP hybrid proteins bypass both of these criticisms. There is one additional reason that makes RBP a somewhat better candidate for this chimeric protein approach. PhoA folding is dependent on the formation of intrachain disulfide bonds, which may occur less efficiently in the cytoplasmic environment (35). On the other hand, RBP is similar to MBP in that it lacks Cys residues (10, 17).

The findings of this study also directly address the results of Watanabe and Blobel (48). These workers concluded that SecB specifically recognizes the signal peptide of SecB-dependent proteins, and that the role of SecB is to target the precursor to a putative SecB receptor in the cytoplasmic membrane. Their model did not account for the more than 60% of MBP that is exported in SecB<sup>-</sup> cells. In addition, it is difficult to reconcile this model, in which SecB specifically interacts with the signal peptide, with the findings that RBP-MBP export is SecB dependent and that MBP-RBP export is SecB independent. Several recent biochemical studies have shown that the MBP signal peptide is not required for the interaction of MBP with SecB, nor does its presence appear to enhance such an interaction (33, 41, 50). At this point, the weight of the evidence seems to favor the original proposal by Collier et al. (8) that SecB specifically interacts with the mature moiety of preMBP to promote its export from the cytoplasm. On the other hand, a hybrid protein with the signal peptide and first 11 residues of OmpF (SecB dependent) fused to the carboxy-terminal 51 residues of Lpp (SecB independent) was strongly dependent on SecB for export (49). This result indicates that the OmpF signal peptide, the early OmpF mature region, or both determined the SecB dependence of this OmpF-Lpp hybrid protein. One possibility is that SecB does different things for different proteins. This clearly requires additional investigation.

Although RBP export is SecB independent, does this mean that SecB does not interact with this protein? Weiss and Bassford (50) found that anti-SecB serum could specifically precipitate a complex of SecB with wild-type preMBP, but not wild-type preRBP, when these two precursor proteins were synthesized *in vitro* with extracts prepared from either haploid *secB*<sup>+</sup> cells or SecB-overproducing cells. These results suggested that there is some specificity to the interaction of SecB with precursor proteins. On the other hand, Kumamoto (23) reported that some preRBP synthesized *in vivo* bound to an anti-SecB affinity column.

In this study, a possible interaction between SecB and preRBP *in vivo* was investigated by using several different approaches. First, RBP12-1 export in SecB<sup>-</sup> cells was examined. This RBP species with a defective signal peptide was exported in SecB<sup>+</sup> cells in a relatively slow, posttranslational manner. Previous studies had demonstrated that MBP species (e.g., MBP10-1, MBP16-1) that exhibit relatively slow, posttranslational export kinetics in SecB<sup>+</sup> cells

(Fig. 4C) are totally export defective in SecB<sup>-</sup> cells (8, 9). In this situation, it is thought that preMBP is particularly dependent on the antifolding activity of SecB to maintain it in an export-competent conformation for an extended period. However, no such SecB requirement could be established for RBP12-1 (or MBP10-1-RBP) export.

Second, the synthesis of export-defective RBP or MBP-RBP did not interfere significantly with SecB-dependent protein export in SecB<sup>+</sup> cells, whereas export-defective MBP or RBP-MBP did elicit such an interference effect. Previous studies have shown that synthesis of export-defective MBP species depletes cells of functional SecB, presumably because the small amount of available SecB is bound to the preMBP that accumulates in the cytoplasm (8, 33). In fact, the finding that the region of the preMBP responsible for this interference phenomenon resides in the mature moiety provided the initial indication that SecB specifically interacts with this portion of preMBP (8).

Third, RBP export kinetics were not significantly affected in cells overproducing SecB, whereas MBP export kinetics are affected. The latter presumably results from a direct interaction between SecB and preMBP. These results, taken together with the recent results of Weiss and Bassford (50) discussed above, indicate that it is highly probably either that SecB does not interact with preRBP or that any interaction between these two proteins is considerably weaker than that of SecB with preMBP. Thus, it appears that there is specificity to the interaction of SecB with precursor proteins, as proposed by Collier et al. (8). These findings do not rule out the possibility that RBP export is facilitated by another cytoplasmic factor with an equivalent function. The finding that RBP12-1 exists in an export-competent conformation for some period before translocation strongly suggests that this is the case. At least two other *E. coli* proteins may be able to provide such chaperone functions (32).

Trun et al. (44) concluded that SecB function was required for PrlA-mediated suppression of various *lamB* signal sequence mutations. These workers suggested that the combination of PrlA and SecB interacting with two different regions of the precursor molecule might help direct the altered pre-LamB into the export pathway with a specificity that neither PrlA nor SecB can achieve alone. RBP export is SecB independent; furthermore, SecB does not appear to significantly interact with preRBP. Thus, it was of interest to determine whether *prlA* mutations could suppress *rbsB* signal sequence mutations. The export of RBP12-1 and RBP15-1 did not noticeably improve in PrlA402 cells compared with that in isogenic Prl<sup>+</sup> cells. However, neither did export of RBP12-1-MBP or RBP15-1-MBP, hybrid proteins with which SecB would be expected to interact. On the other hand, both RBP9-1 and RBP9-1-MBP export exhibited significant improvement in PrlA402 cells, and the improvement in RBP9-1 export was not diminished in SecB<sup>-</sup> PrlA402 cells. These results indicated that the failure of PrlA402 to mediate the export of the other mutant RBP species was primarily related to the nature of the signal sequence defect as opposed to the nature of the adjacent mature moiety; this study revealed no direct correlation between SecB dependence and PrlA402-mediated protein export. It was interesting that MBP16-1 export was greatly improved in PrlA402 cells, whereas MBP16-1-RBP exhibited only minor improvement. This indicates that, at least in some instances, the mature moiety influences the interaction of the signal peptide with the export machinery. Recent studies with *E. coli* cells expressing hybrid proteins with the OmpA signal peptide

attached to either *Staphylococcus aureus* nuclease A or TEM  $\beta$ -lactamase led to a similar conclusion (16).

The finding that RBP12-1 and RBP15-1 export was not improved in PrlA402 cells was unexpected, since *prlA402* previously was characterized as suppressing a very broad spectrum of *malE* and *lamB* signal sequence mutations (1, 43). Past studies revealed very little allele specificity in the observed suppression of various *malE*, *lamB*, and *phoA* signal sequence mutations in cells harboring different *prlA* suppressor alleles (1, 11, 36). These results prompted some to argue that the isolation of such suppressor mutations should not be taken as an indicator that the PrlA (SecY) protein directly interacts with the signal peptide of precursor proteins (reviewed in reference 40 and by Bieker et al. [K. L. Bieker, G. J. Phillips, and T. J. Silhavy, *J. Bioenerg. Biomembr.*, in press]). Most recently, extragenic suppressors of the *rbsB15-1* signal sequence mutation have been obtained that map to the *prlA* region of the *E. coli* chromosome (S. M. Strobel, unpublished data). These new mutations appear to be much more limited in the spectrum of *rbsB* and *malE* signal sequence mutations that they suppress. Further characterization of these putative *prlA* mutations may provide a much stronger argument for allele-specific interactions between mutant PrlA proteins and mutant signal peptides of various precursor proteins.

It was unique to find that a single amino acid substitution in the signal peptide of either MBP (MBP15-1) or RBP (RBP15-1) resulted in a totally export-defective protein. To date, a large number of MBP signal sequence alterations have been obtained, either as a result of genetic selections or by in vitro mutagenesis (4). Heretofore, none of these mutant preMBP species, including a deletion derivative missing seven residues from the hydrophobic core (2), was sufficiently export defective such that cells expressing these proteins were incapable of utilizing maltose. The only preMBP species previously obtained that rendered cells absolutely Mal<sup>-</sup> were several with multiple changes in the hydrophobic core (13). Ferenci and Silhavy (12) noted, from a number of genetic analyses of signal peptides, that a single amino acid substitution in the signal peptide that completely abolished export function had never been described. It is interesting that MBP15-1 was not obtained previously with stringent genetic selections for *malE* signal sequence mutations (5). However, unlike most other positions in the MBP hydrophobic core, it required a minimum of two nucleotide changes to convert the Leu codon at position 15 (TTA) to one encoding a charged amino acid. The same holds true for RBP15-1, in which a charged amino acid is also substituted for a Leu residue at position 15. The nature of the residues at these positions, centrally located with respect to the hydrophobic cores of both signal peptides, might be particularly crucial for signal peptide function.

Finally, the temporal mode of RBP-MBP and MBP-RBP processing in SecB<sup>+</sup> cells was investigated. Earlier studies established that RBP translocation is normally a posttranslational event (39), and it appeared that RBP-MBP translocation was also accomplished in a strictly posttranslational fashion. Although the MBP signal peptide can promote cotranslational MBP translocation to a significant extent (21, 22, 39), cotranslational MBP-RBP translocation was not detectable. This latter finding indicated that cotranslational translocation of MBP is not solely determined by the nature of the MBP signal peptide but rather depends on properties of both the signal peptide and the mature moiety. In fact, it could be that the respective signal peptides and mature moieties of both preMBP and preRBP are really matched



sets, and that some subtle export characteristics are lost or altered when these two signal peptides are exchanged. This is being investigated further.

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