In Vivo and In Vitro Synthesis of Escherichia coli Maltose-Binding Protein under Regulatory Control of the lacUV5 Promoter-Operator

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It has not been possible to obtain in vitro expression of the positively regulated malE gene encoding the periplasmic maltose-binding protein (MBP) of Escherichia coli. To facilitate in vitro malE expression, we constructed plasmids that place the malE gene under transcriptional control of the lacUV5 promoter-operator. These plasmids could be grouped into three classes, based upon their ability to complement in vivo a chromosomal malE deletion in the presence or absence of isopropyl thiogalactoside. In the one class I plasmid analyzed, the lacUV5-malE junction was just 3′ to the malE ATG initiation codon, and this plasmid did not complement the malE deletion. Class II and class III plasmids retained various amounts of the malE promoter. MBP synthesis was solely under control of the lacUV5 promoter in the class II plasmids, and MBP synthesis was under control of both the lacUV5 and malE promoters in the class III plasmids. A malE mutation that renders the MBP signal peptide export defective was genetically recombined onto one of the class II plasmids. The in vivo synthesis and export of plasmid-encoded MBP were studied in the presence and absence of isopropyl thiogalactoside and maltose and in a strain harboring a prlA mutation that suppresses the malE signal sequence mutation and is thought to alter the export machinery of cells. In addition, both class II and class III plasmids programmed the synthesis of precursor MBP in an in vitro-coupled transcription-translation system. When precursor MBP was synthesized in vitro in the presence of E. coli membrane vesicles, a significant portion of wild-type precursor MBP, but not export-defective precursor MBP, was converted to a form that migrated on sodium dodecyl sulfate-polyacrylamide gels identically to mature MBP synthesized in vivo.

The maltose-binding protein (MBP) of Escherichia coli, the malE gene product, is an extracytoplasmic protein. Although it is synthesized on ribosomes located within the confines of the cytoplasmic membrane, its final cellular location is the periplasm. The synthesis and export of the MBP have been extensively investigated in several laboratories. The early steps in MBP export appear to be analogous to those of other exported proteins in both procaryotic and eucaryotic systems. The MBP is synthesized on polyribosomes bound to the cytoplasmic membrane (28, 30, 31). It is initially synthesized as a higher-molecular-weight precursor which has an amino-terminal signal peptide of 26 residues (6) and is thought to be chiefly responsible for initiating export of the protein while it is still a nascent chain attached to a ribosome (11, 27). Translocation of the MBP across the cytoplasmic membrane and proteolytic cleavage of the signal peptide have been demonstrated to occur both cotranslationally and posttranslationally, but only after at least 80% of the nascent chain has been synthesized (16, 27). These findings indicate that elongation and translocation are not tightly coupled events.

Much of the information concerning the mechanism of MBP synthesis and secretion has resulted from analyses of E. coli mutants with mutations affecting these processes (recently reviewed in reference 3). Biochemical approaches have also furthered our understanding, but have been more limited (recently reviewed in reference 29). Attempts to develop an in vitro procaryotic system capable of efficiently synthesizing and exporting MBP have been hindered by two major obstacles. First, the absence of specific organelles utilized for protein export in E. coli and other procaryotes has slowed the development of a purified protein-exporting system. However, workers in several laboratories have recently reported considerable success in this area (9, 24, 25), indicating that similar studies with MBP may now be feasible. Second, past attempts to synthesize MBP in an in vitro, coupled transcription-translation system by using a wild-type malE DNA template have been unsuccessful. It was believed that expression of the malEFG operon was not initiated in S30 extracts due to inadequate levels of functional MalT protein, the positive effector of the mal regulon (10).

In an earlier study, Marchal et al. (21) were able to study the in vitro synthesis and maturation of the LamB protein, which is encoded by a second gene in the mal regulon (lamB), by placing it under transcriptional control of the lacUV5 promoter-operator. Expression from this promoter is regulated by the LacI repressor, is inducible with isopropyl thiogalactoside (IPTG), and is independent of cyclic AMP-mediated catabolite repression (18). In addition, this promoter is an active promoter in vitro. We describe here the construction of lacUV5-malE plasmids. We analyzed the in vivo synthesis of plasmid-encoded MBP in several different E. coli strains and under different growth conditions. In addition, these plasmids were used to direct the synthesis of precursor MBP (pMBP) in vitro. When pMBP was synthesized in the presence of E. coli membrane vesicles, a significant portion of wild-type pMBP, but not export-defective pMBP, was converted to the apparently mature form.
MATERIALS AND METHODS

Bacterial strains and plasmids. Most of the bacterial strains used in this study are isogenic derivatives of E. coli strain FCB26 (MC4100 [8] lac"^" lac"^"Tr"^"; obtained from S. Benson, Princeton University). Strain BAR1091 is strain ECFB526 containing the malEΔ312 mutation, an inframe, nonpoler deletion that removes DNA sequences encoding residue 15 of the MBP signal peptide through residue 159 of the mature MBP (mMBP) (V. A. Bankaitis and P. J. Bassford, Jr., unpublished data). Strains BAR1092 and BAR1134 are strains BAR1091 and ECFB526, respectively, harboring the prlA402 mutation (1). The malEΔ312 and prlA402 alleles were introduced into strain ECFB526 by P1 transduction. Strain KK2186, which was used to propagate bacteriophage M13, also was obtained from S. Benson. Plasmid pGL101 (18) was used as the source of the lacUV5 promoter and was provided by G. Bogosian, University of North Carolina. Plasmid pCL was obtained from C. Lee (Harvard Medical School) via H. Shuman (Columbia University). The S30 extracts were prepared from either E. coli strain CF300 (DNase-), which was provided by M. Cashel (National Institutes of Health), or strain MRE600 (RNase I-), which was provided by H. Inouye (Harvard Medical School). The S100 extracts, purified ribosomes, and membrane vesicles were prepared from strain MRE600.

Reagents. Minimal medium M63 supplemented with glycerol (0.4%) and thiamine (2 μg/ml) and maltose-tetrazolium indicator agar were prepared as described previously (23). When used, ampicillin was added to liquid medium at a concentration of 20 mg/ml and to plate medium at a concentration of 100 mg/liter. Maltose, glycerol, and methionine were purchased as reagent grade chemicals from Fisher Scientific Co. Rabbit anti-MBP has been described previously (13). Restriction enzymes, T4 DNA ligase, and Bal 31 exonuclease were purchased from Bethesda Research Laboratories, Inc. [35S]Methionine (10 μCi/ml) was purchased from Amersham Corp. [35P]ATP (800 Ci/mmole) was obtained from New England Nuclear Corp.

Plasmid isolation and genetic techniques. Large-scale plasmid preparations were made as described previously (34). Plasmids used for in vitro translation studies were banded twice in CsCl, ethanol precipitated, and suspended in 10 mM Tris (pH 8.0)-0.1 mM EDTA. Mini-scale plasmid preparations were obtained as previously described (34). For DNA manipulation and cloning experiments, a 10 to 100-ml scaled-up version of the mini plasmid preparation was used. The DNA was incubated with heat-treated RNase, phenol extracted, ethanol precipitated, and suspended in 1 mM Tris (pH 8.0)-0.1 mM EDTA. The other standard DNA and genetic techniques used have been described previously (19, 23, 34).

Construction of plasmids carrying malE under control of the lacUV5 promoter-operator. Plasmid pCL was digested with EcoRI, and then 8-pmol ends of the linearized plasmid were treated with 0.6 U of Bal 31 exonuclease for 28 min at 30°C. We calculated that this removed approximately 400 base pairs (bp) from each free end. Bal 31 activity was stopped by extracting the reaction mixture with phenol, followed by ethanol precipitation. The treated plasmid was next restricted with PstI and ligated to a purified PstI-PvuII fragment carrying the lacUV5 promoter. The ligated DNA was used to transform strain BAR1091. Transformants were selected on maltose-tetrazolium-ampicillin plates. These transformants were screened for an IPTG-inducible Mal" phenotype by streaking them onto maltose-tetrazolium agar and looking for transformants that formed white colonies when 1 mM IPTG was incorporated into the medium. The lacUV5 promoter fragment was obtained by digesting plasmid pGL101 simultaneously with PstI and PvuII. The DNA fragments were resolved on a 5% polyacrylamide gel, and the 851-bp lacUV5 fragment was excised and eluted from the gel as previously described (2).

Sequencing of the lacUV5-malE fusion joint. The lacUV5-malE junction is included on an EcoRI-BglII fragment that is less than 1 kilobase long. This fragment was isolated by restricting the appropriate plasmids with EcoRI and then with BglII. These fragments were inserted between the unique EcoRI and BamHI sites of bacteriophage M13mp8 RF (22), and the DNA sequence in the region of the lacUV5-malE junction was determined by using the dyeodeoxy sequencing method of Sanger et al. (32). A 77-bp TaqI-TaqI fragment (plasmids pBAR2, pBAR4, and pBAR5) and a 39-bp AvaI-HinII fragment (plasmids pBAR1, pBAR3, pBAR26, and pBAR43) were used as primers for the sequencing reactions (2).

Genetic recombination of the malE18-1 signal sequence mutation onto plasmid pBAR43. A strain carrying plasmid pBAR43 was lysogenized with λap malB13 (cI857 st7) (20) onto which the malE18-1 mutation had been recombined as previously described (2), and heat-induced phage lysates were prepared. A very small fraction of the phage carried an integrated copy of plasmid pBAR43 as a result of a single crossover event between homologous malB sequences. Cells of strain BAR1091 were infected with this lysate and plated onto maltose-tetrazolium-ampicillin agar at 42°C. Transduction to Amp" and survival at 42°C required that the integrated plasmid be excised from the phage DNA, most probably by homologous recombination, and establish itself in the recipient cell; the phage DNA was subsequently cured. Plasmid integration into the phage genome in the donor strain and subsequent excision in the recipient strain could occur by different crossover events and may have resulted in the acquisition of the malE18-1 mutation by the plasmid. Surviving colonies were screened to determine their IPTG-inducible Mal" phenotypes as described above. One colony exhibiting a Mal" phenotype in the presence of IPTG was used for genetic transformation of the recipient strain.

Pulse radiolabeling. Cells were grown in glycerol minimal medium at 30°C with aeration. MBP synthesis was induced by adding 5 mM IPTG or 0.4% maltose or both to a mid-log-phase culture (optical density at 600 nm, 0.3 to 0.35). After induction for 45 min, 1 ml of the culture was removed and mixed with 5 μCi of [35S]Methionine for 1 min. Incorporation of label was terminated by adding 0.5 ml of 15% trichloroacetic acid and immersing the preparation in an ice water bath. The trichloroacetic acid precipitates were pelleted, washed once with acetone, air dried, suspended in 100 μl of 10 mM Tris (pH 7.5)-1 mM EDTA-1% sodium dodecyl sulfate (SDS), and solubilized by heating in a boiling water bath for 3 min. These labeled antigen extracts were used in the radioimmunoprecipitation reactions described below.

Pulse-chase experiments. Cells were grown and induced as described above. At 45 min postinduction, 1.5 ml of a culture was removed and mixed with 7.5 μCi of [35S]Methionine for 1 min. Incorporation of label was terminated by adding 1.5 ml of glycerol minimal medium containing 0.8% unlabeled methionine. Immediately after addition of the chase solu-
tion, 1 ml of the culture was removed, mixed with 0.5 ml of 15% trichloroacetic acid, and placed in an ice water bath. At 10 min after the chase solution was added, a second 1-ml portion of culture was removed and treated as described above. Antigen extracts were prepared from the trichloroacetic acid precipitates as described above.

Radioimmunoprecipitation and SDS-polyacrylamide gel electrophoresis. MBP species were immune precipitated from radiolabeled antigen extracts as previously described (13) by using rabbit anti-MBP serum, and the precipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography as previously described (2, 13).

In vitro protein synthesis and processing. Plasmid-encoded proteins were synthesized in vitro by using coupled transcription-translation systems. The S30 fraction was prepared from strain CF300 as previously described (23), except that CaCl2 was omitted and polyethylene glycol 8000 (16 mg/ml) and spermidine (2 mM) were added. For translocation experiments, the S30 fraction and crude membranes were prepared from strain MRE600 by the procedure of Müller and Blobel (25); the S100 fraction was prepared from the S30 fraction by centrifugation at 120,000 × g for 15 min. Purified ribosomes were prepared from the S30 fraction by centrifugation and washing with Triton X-100 (manuscript in preparation). The DNA concentration was approximately 2 μg/25-μl reaction mixture.

RESULTS

Placement of the malE gene under transcriptional control of the lacUV5 promoter-operator. Plasmid pCL carries a segment of the E. coli malB region, including the complete malB regulatory interval and an intact malE gene. The nucleotide sequence of the malB regulatory interval (7, 12) revealed no convenient restriction endonuclease sites that would permit us to simply excise the unwanted promoter for the malE gene (malEp) and substitute the lacUV5 promoter-operator. Therefore, as shown in Fig. 1, we attempted to remove malEp from in front of the malE gene by using exonuclease Bal31. After Bal31 treatment, the lacUV5 promoter-operator region was moved in front of the malE gene on a PstI-PvuII fragment. After ligation, the plasmid mixture was transformed into strain BAR1091, selecting for Amp'. Fifty Amp' transductants were purified and tested for the ability of their plasmids to complement, in the presence and absence of IPTG, the internal malEΔ312 deletion carried on the bacterial chromosome. Three different classes of plasmids were recognized on the basis of the observed Mal phenotypes. Strains harboring a class I plasmid were Mal− in the presence or absence of IPTG; strains harboring a class II plasmid were Mal− or weakly Mal+ in the absence of IPTG but strongly Mal+ in the presence of IPTG; and strains harboring a class III plasmid were strongly Mal+ in the presence or absence of IPTG. It should be noted that strains carrying a class III plasmid exhibited a significantly stronger Mal+ phenotype than did a strain harboring the parental malE+ plasmid pCL. A total of seven plasmids, including representatives of each class, were selected for further study.

Junction between the lacUV5 promoter-operator and the malE gene. For each of seven different plasmids, the exact site at which the lacUV5 promoter-operator fragment had been joined to the malE gene was determined by DNA sequencing, as described in Materials and Methods (Fig. 2). The fusion joint for class I plasmid pBAR3 was found to lie just within the malE structural gene. The lacUV5 promoter-operator in class II plasmids pBAR26, pBAR43, and pBAR1 was fused 1, 10, and 38 bp, respectively, upstream from the malE ATG initiation codon. All three plasmids were missing the −10 and −35 regions of malEp identified by Bedouelle and Hofnung (7). For plasmids pBAR26 and pBAR43, the malE Shine-Dalgarno sequence also had been removed. These sequences, which are presumably required for malE expression, can be provided by the lacUV5 insertion, the PvuII site being 5 bp downstream from the lac Shine-Dalgarno sequence (18). Plasmid pBAR2 also was designated a class II plasmid. However, strain BAR1091 harbor-
FIG. 2. lacUV5-malE fusion joints for seven different pBAR plasmids. The nucleotide sequence for the malE sense strand is shown, beginning with the nucleotide of regions Note that Figure 2. lacUV5-malE fusion joints for seven different pBAR plasmids. The nucleotide sequence for the malE sense strand is shown, beginning with the nucleotide of regions Note that Figure 2. lacUV5-malE fusion joints for seven different pBAR plasmids. The nucleotide sequence for the malE sense strand is shown, beginning with the nucleotide of regions Note that Figure 2. lacUV5-malE fusion joints for seven different pBAR plasmids. The nucleotide sequence for the malE sense strand is shown, beginning with the nucleotide of regions Note that Figure 2. lacUV5-malE fusion joints for seven different pBAR plasmids. The nucleotide sequence for the malE sense strand is shown, beginning with the nucleotide of regions Note that Figure 2. lacUV5-malE fusion joints for seven different pBAR plasmids. The nucleotide sequence for the malE sense strand is shown, beginning with the nucleotide of regions Note that the nucleotide sequence of the malK coding sequence and extending by 84 nucleotides into the malE structural gene (7, 12). The EcoRI restriction site in malK is located 134 nucleotides upstream from the sequence given. The narrow lines underline the −10 and −35 sequences for both the malE and malK genes. The Shine-Dalgarno sequence (S-D) for the malE gene is also underlined. The thick lines underline DNA sequences believed to represent partial portions of the activation sites for the MalT protein (5). Transcription initiates at the positions of the vertical bars; the attached arrows indicate the direction of transcription. The amino acid sequence for the MBP signal peptide and the first two residues of the mature protein (6) is shown below its coding region. The nucleotide substitution and amino acid alteration resulting from the malE84-1 mutation (6) are indicated by an arrow. The lacUV5-malE fusion joints and their respective plasmid designations are indicated by the arrows above the nucleotide sequence. DNA to the left of the arrows was removed and replaced with the lacUV5 promoter-operator fragment.

In vivo synthesis of plasmid-encoded MBP in the presence and absence of inducers. Not unexpectedly, we were unable to identify a MBP species encoded by class I plasmid pBAR3 (data not shown). The in vivo synthesis of MBP encoded by plasmid pCL and several representative class II and III plasmids described above was studied in the presence and absence of maltose and IPTG. Cells were radiolabeled with [35S]methionine for 1 min, and the MBP species were immunoprecipitated and analyzed by SDSPAGE and autoradiography, as described in Materials and Methods (Fig. 3). For parental strain ECB526 having a single chromosomal malE+ gene, only a small amount of MBP was synthesized in the absence of maltose. There was an approximately 10-fold increase in MBP synthesis when maltose was added to the growth medium. In a strain in which malE+ was carried on multicyclic plasmid pCL, a relatively large amount of MBP was synthesized in the absence of maltose. In this instance, only a two- to threefold increase in MBP synthesis was observed in the presence of maltose. As expected, the addition of IPTG had no effect on the amount of MBP synthesized by either strain, and MBP synthesis was not detectable when either of these strains harbored a malT deletion mutation.

In contrast to these results, cells carrying class II plasmid pBAR1 or pBAR4 synthesized detectable levels of MBP only if IPTG was added to the culture. The presence of maltose or the absence of MalT protein had no effect on MBP synthesis. The total amount of MBP synthesized in response to IPTG was not significantly greater than the amount observed in a haploid malE+ strain grown with maltose. The results with class III plasmid pBAR5 were somewhat different. Low but detectable levels of MBP were synthesized in the absence of either inducer. The addition of either maltose or IPTG increased the amount of MBP synthesized, the former having a slightly greater effect. However, total MBP synthesis in the presence of maltose was less than the synthesis by maltose-induced strains harboring plasmid pCL. The combination of both maltose and IPTG did not appear to increase the level of MBP synthesis above the level observed with maltose alone.

Under the radiolabeling conditions used in these experiments, some MBP was precipitated in its precursor form. Also, it is important to note that plasmid-bearing strains carried the internal, in-frame malE312 deletion on the chromosome. The malE312 gene product was immunoprecipitated from antigen extracts by anti-MBP serum. Synthesis of this truncated MBP species was maltose inducible. However, when they were grown in the presence of maltose, only strains carrying the class II and class III plasmids synthesized high levels of this protein. Barely detectable levels were synthesized in the strain carrying plasmid pCL.

Genetic recombination of the malE84-1 mutation onto plasmid pBAR43 and suppression by prA402. The malE84-1 signal sequence mutation was genetically recombined into class II plasmid pBAR43 as described in Materials and Methods, generating plasmid pBAR43-11. Strain BAR1091 harboring this plasmid remained phenotypically Mal+ in the presence of IPTG. The malE84-1 mutation introduces a charged residue into the hydrophobic core of the MBP signal peptide (6) (Fig. 2). Less than 2% of the malE84-1 product synthesized is secreted to the periplasm and processed; the remainder accumulates as unprocessed pMBP in the cytoplasm (4, 13). Workers have described mutations at several genetic loci that restore export of MBP having a defective signal peptide (1, 13). One such suppressor mutation, desig-
radiolabeled with [35S]methionine for 1 min and either not chased (Fig. 4A) or chased in the presence of unlabeled methionine for 10 min (Fig. 4B), as described in Materials and Methods. For each of the strains synthesizing wild-type MBP, under either malEp or lacUV5 regulation, no pMBP was detected after the 10-min chase. This was observed with strains harboring either a prlA* allele (data not shown) or a prlA402 allele. For a prlA* strain synthesizing export-defective MBP encoded by plasmid pBAR43-11, no mMBP was detected after the 1-min pulse-label, and only barely detectable levels of mature MBP were detected after the 10-min chase period. Large amounts of pMBP were observed at both time points. In contrast, the presence of the prlA402 allele resulted in maturation of more than 60% of the total MBP by the end of the 10-min chase. Only a very small fraction was mature after the 1-min labeling period.

**In vitro synthesis and processing of MBP.** Various plasmids were tested for the ability to program MBP synthesis in vitro by using an S30 coupled transcription-translation system (Fig. 5). Using either class II or class III plasmid DNA resulted in the synthesis of large amounts of MBP. The great majority of MBP synthesized was found in its precursor form. However, a faint band that migrated identical to bona fide mMBP also was observed. In contrast to these results, only a very faint band of pMBP was detected among the in vitro products of plasmid pCL, which were carrying malE* under malEp control. The addition of maltose to the reaction mixture had no effect on MBP synthesis (data not shown). When plasmid pBAR43-11 was used to program protein synthesis, large amounts of pMBP were produced, but in this case no mMBP was evident. This pMBP species with an altered signal peptide migrated slightly slower on SDS-PAGE gels than the wild-type pMBP did. This small anomaly in the migration rate also was observed for pMBP synthesized in vivo (Fig. 4). The proteins migrating in the region of pMBP and mMBP were precipitable with anti-MBP serum (data not shown), confirming their identity as MBP species. Synthesis of β-lactamase, which was encoded by the bla gene of plasmid pCL and the different pBAR plasmids, served as an internal control for the ability of the plasmids to function in the in vitro reaction. Note that the level of β-lactamase synthesis programmed by each plasmid was relatively constant. Thus, the differences in the level of synthesis of MBP presumably reflected the efficiency of transcription of the malE gene initiated from different promoters.

We thought that the small amount of processing of wild-type pMBP observed in the in vitro reactions was most likely due to the presence of membrane vesicles in the S30 mixture. Thus, in vitro synthesis of MBP was investigated by using an S100 mixture and purified ribosomes (Fig. 6). In this case, no mMBP band could be easily discerned when either plasmid pBAR43 or pBAR43-11 was used. (The somewhat indistinct bands migrating just below pMBP in this experiment probably represented incomplete translation products.) Finally, when membrane vesicles were included with the S100 mixture and purified ribosomes, a substantial amount of what appeared to be authentic mMBP, based upon its gel mobility, was produced from pBAR43. The amount of mMBP produced in this reaction was calculated by scanning the autoradiogram shown in Fig. 6. Allowing for the fact that the MBP signal peptide includes one-third of the total methionine residues present in pMBP (12), approximately 30% of the pMBP synthesized was processed to the mature form by the membrane vesicles. Once again, only pMBP was produced from plasmid pBAR43-11. The reaction mixtures

![FIG. 3. In vivo synthesis of MBP in the presence or absence of maltose and IPTG. Cells were grown in glycerol minimal medium and either were not induced (A) or were induced with IPTG (B and E), maltose (C), or maltose and IPTG (D). The radiolabeled MBP was immunoprecipitated and analyzed by SDS-PAGE and autoradiography, as described in Materials and Methods. Lane 1, Precipitate obtained from strain ECB526; lane 2 through 5, precipitates obtained from strain BAR1091 carrying the plasmids pCL (lane 2), pBAR43 (lane 3), pBAR1 (lane 4), and pBAR5 (lane 5). (E) MBP species synthesized when the host strains harbored a malT deletion. The positions of pMBP, mMBP, and the malEΔ312 gene product are indicated. The gels shown in panels A, B, C, and D were 12.5% acrylamide gels; the gel shown in panel E was a 10% acrylamide gel. Only the relevant portion of each autoradiograph is shown.

nated prlA402, recently was reported to be extremely efficient at restoring export of the malE18-1 product (1). When a prlA402 derivative of strain BAR1091 was transformed with plasmid pBAR43-11, it was found to be phenotypically Mal* when it was induced with IPTG.

MBP was precipitated from strain BAR1091 harboring plasmid pBAR43-11 and from a prlA402 derivative of strain BAR1091 harboring various plasmids. In this experiment, MBP was precipitated from induced cells that had been
were centrifuged and separated into membrane (pellet) and soluble fractions. The mMBP made from pBAR43 was found to be entirely membrane associated, while the majority of pMBP made from either pBAR43 or pBAR43-11 remained soluble (data not shown).

**DISCUSSION**

We constructed a series of plasmids in which the *malE* gene was placed under transcriptional control of the *lacUV5* promoter-operator. These plasmids were grouped into three classes based upon the phenotypes which they conferred upon a *malE* deletion strain in the presence or absence of IPTG. These groupings correlated well with the amount of the *mab* regulatory interval separating the *lacUV5* promoter-operator and the *malE* ATG initiation codon. In the one class I plasmid analyzed, the *lacUV5* sequence actually was fused to a site just within the *malE* structural gene. This plasmid did not complement a chromosomal *malE* deletion, and we were unable to precipitate an IPTG-inducible MBP species from a strain harboring this plasmid. For class II

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**FIG. 4.** Synthesis and processing of plasmid-encoded MBP in *prrA402* strains. Cells were grown and radiolabeled with [35S]methionine as described in Materials and Methods. MBP species were immunoprecipitated and analyzed by SDS-PAGE, using a 10% polyacrylamide gel, and autoradiography. (A) Precipitates obtained after the 1-min pulse. (B) Precipitates obtained after the 10-min chase period. Lane 1, Precipitate obtained from strain BAR1134 (strain ECB526 *prrA402*); lanes 2 through 5, precipitates obtained from strain BAR1092 (strain ECB526 *malEΔ312 prrA402*) carrying plasmids pCL (lane 2), pBAR5 (lane 3), pBAR3 (lane 4), and pBAR43-11 (lane 5); lane 6, precipitate obtained from strain BAR1091 (strain ECB526 *malEΔ312 prrA*') carrying plasmid pBAR43-11. The positions of pMBP and mMBP are indicated. Only the relevant portion of each autoradiograph is shown.

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**FIG. 5.** In vitro synthesis of MBP by using an S30 coupled transcription-translation system. An S30 mixture containing [35S]methionine was programmed with various plasmids as described in Materials and Methods. Reaction mixtures were incubated for 30 min at 37°C. The total array of radiolabeled protein products was analyzed by SDS-PAGE, using a 10% polyacrylamide gel and autoradiography. Samples were prepared for SDS-PAGE by mixing 5 μl of the reaction mixture with 20 μl of 1.25× sample buffer; 12.5 μl was loaded per lane. Lane 1, Marker pMBP and mMBP synthesized in vivo; lanes 2 through 12, radiolabeled protein products synthesized in vitro when the S30 mixture was programmed with plasmids pBAR1 (lane 2), pBAR2 (lane 3), pBAR26 (lane 4), pBAR43 (lane 5), pBAR43-11 (lane 6), pBAR5 (lane 7), pBAR3 (lane 8), pCL (lane 9), pBR322 (lane 10), and ColE1 (lane 11) and with no plasmid DNA (lane 12). The positions of pMBP, mMBP, and β-lactamase (Bla) are indicated.
plasmids pBAR1, pBAR26, and pBAR43, expression of malE was regulated solely by the lacUV5 promoter-operator. These three plasmids contained an intact malE gene, but not the malEp -10 or -35 sequences that were identified previously (7). Class II plasmid pBAR2 retained 108 bp upstream from the malE initiating ATG codon, leaving intact the -10 and -35 sequences, as well as a site thought to be a part of the malEp activation site for the MalT protein (5, 7). The extent of malEp carried by plasmid pBAR2 appears to be sufficient to permit weak MalT-dependent expression of malE in the absence of IPTG. However, our data indicate that maximal MalT-dependent expression of the malEFG operon requires additional upstream sequences. Note that class III plasmids carry approximately 200 bp upstream from the malE initiating ATG codon, and strains harboring these plasmids are phenotypically Mal + in the absence of IPTG. Class III plasmid-encoded MBP mRNA synthesis can be initiated from either malEp or the lacUV5 promoter. However, even these class III plasmids may be missing some information required for MalT induction at 100% efficiency. Note that MalT-dependent synthesis of MBP was somewhat reduced in a strain harboring plasmid pBAR5 compared with a strain harboring plasmid pCL (Fig. 3A and C).

It was interesting to find that strain BAR1091 harboring a class III plasmid exhibited a stronger Mal + phenotype in the absence of IPTG than the same strain harboring the parental plasmid, pCL. This can be explained by considering the amount of IPTG and the other conditions present in the different plasmids. Plasmid pCL carries an intact malB regulatory interval and, therefore, the MalT binding sites for both the malEFG and malK-lamB operons. The class III plasmids lack at least one site thought to be required for the interaction of the MalT protein with malKp (5, 7) (Fig. 2). Synthesis of the MalT protein is not autoregulated. This protein appears to be synthesized at relatively constant levels under most growth conditions, although its synthesis is subject to catabolite repression (10). For a strain harboring multicopy plasmid pCL with its two MalT interaction sites, it seems likely that almost all of the MalT protein in the cell binds to plasmid DNA. The result is much lower than normal expression of the chromosomal malA and malB operons required for growth on maltose as a carbon source. However, class III plasmids lack the malKp-MalT interaction site and, therefore, do not have as severe a titration effect on MalT protein as plasmid pCL does. Thus, in strains harboring class III plasmids, there is still sufficient MalT protein available to efficiently induce chromosomal mal operons, and such strains are fully Mal +. This is further supported by our finding that strains harboring either a class II plasmid or a class III plasmid synthesized high levels of the chromosomal malE312 product when they were induced with maltose. In contrast, strains harboring plasmid pCL synthesized very low levels, indicating that insufficient MalT protein was available to induce the chromosomal malEFG operon.

In this study, we were somewhat surprised to find that the presence in E. coli of the malE gene on a multicopy plasmid under the regulatory control of malEp or the lacUV5 promoter-operator or both did not result in a substantial overproduction of MBP under conditions that should have provided full induction. The malE gene dosage effect was easily observed in a strain harboring plasmid pCL under noninducing conditions, when the basal level of MBP synthesis was approximately 10-fold higher than the level seen in an isogenic strain having a single chromosomal copy of the malE gene. Whereas maltose induction of the latter strain resulted in a 10-fold increase in MBP synthesis, which was expected (17), induction of the plasmid-bearing strain resulted in only a 2- to 3-fold increase in MBP synthesis. Likewise, IPTG induction of strains harboring class II plasmids or maltose and IPTG induction of strains harboring class III plasmids resulted in a rate of MBP synthesis that was not significantly different than that seen in the single-copy strain. One possibility is that there is an upper limit on the amount of MBP that can be synthesized at any one time. There is good evidence that synthesis and secretion of MBP are obligately coupled events (15, 26). It could be that the maximum amount of MBP that can be synthesized at any one time is limited by the capacity of the secretory machinery of a cell. This possibility is being investigated further.

We were able to genetically recombine the malE18-1 signal sequence mutation onto class II plasmid pBAR43 with little difficulty, despite the fact that there was a relatively small region of homologous DNA lying between this mutation and the lacUV5-malE fusion joint. This indicates that we should be able to conveniently place a variety of additional malE alleles encoding export-defective MBP (summarized in reference 3) under lacUV5 control by using an identical approach. In response to IPTG, strain BAR1091 harboring plasmid pBAR43-11 synthesized large amounts of pMBP, very little of which was processed to the mature form. It was of interest to find that malE18-1, which is exclusively under lacUV5 control, can be efficiently suppressed by the prlA402 mutation. The mechanism by which signal sequence mutations are suppressed by different prlA alleles has not been determined yet. It is believed that suppression most likely involves a direct interaction of the prlA product with the signal peptide as it emerges from the ribosome (13, 14). It has been suggested that this interaction
might also be involved in the coupling of MBP synthesis and secretion (3, 33). Our finding indicates that a specific mRNA sequence upstream from the malE ATG initiation codon most likely is not required for this interaction. We also found that prlA402-mediated maturation of the plasmid-encoded malE18-I product occurs considerably slower than the maturation observed in a malE' strain. In a separate study, the kinetics of MBP maturation in different prlA strains was studied in considerable detail (J. P. Ryan and P. J. Bassford, Jr., J. Biol. Chem., in press).

A major goal of our research has been to develop a totally in vitro system with which to study MBP synthesis, secretion, and processing. Previous efforts to synthesize MBP in a coupled in vitro E. coli transcription-translation system have been unsuccessful, presumably due to an inability to activate malEp. Using an S30 extract programmed with plasmid pCL DNA, we found that the addition of maltose to the in vitro reaction mixture did not induce MBP synthesis. Such a result strongly suggests that the absence of functional MalT protein is the underlying reason for inefficient malE' expression in vitro. Unlike malEp, the lacUV5 promoter is very efficient in vitro and, when placed 5' to an intact malE gene, directs high levels of MBP synthesis. In vitro MBP synthesis was achieved by using each of the class II and class III plasmids tested. The MBP synthesized was found predominantly as pMBP. A very small amount of what we believe to be authentic process MBP was detected among the in vitro protein products. Such processing may have resulted from the presence of small membrane vesicles present in the S30 mixture. No processing was observed when we used purified ribosomes and an S100 mixture from which all membranes should have been removed. Only pMBP was observed in the protein products when plasmid pBAR43-11 encoding export-defective MBP was used to program in vitro protein synthesis in either system.

Müller and Blobel (25) have recently described experimental conditions under which three different E. coli exported proteins synthesized in vitro were imported into inverted membrane vesicles with up to 25% efficiency and processed to their mature forms. Segregation into membrane vesicles was demonstrated by the presence of the precursor forms to externally added protease K. We prepared membrane vesicles in the same manner and added these to our S100-ribosome mixture synthesizing either wild-type or export-defective MBP. We found that approximately 30% of the wild-type MBP synthesized was processed to a form that migrates on SD-S-PAGE gels identically to bona fide mMBP. Since mMBP is highly protease resistant (27), we were unable to confirm import of the processed MBP into membrane vesicles by simply scoring resistance to externally added proteolytic enzymes. However, all of the processed MBP pelleted with the membrane vesicles, strongly suggesting that import had occurred. In contrast to these results, no processing of export-defective MBP was observed in this system. If import into membrane vesicles was not required for processing, then presumably the malE18-I product also would have been processed in vitro, since this protein matures in vivo when the block in export is suppressed by altering the protein export machinery (1, 13).

In summary, we believe that we have developed a system in which the synthesis, secretion, and processing of MBP can be analyzed in vitro. We are presently attempting to maximize the efficiency of in vitro MBP secretion and to develop more direct methods of demonstrating MBP import into inverted membrane vesicles. Since so much is known concerning the synthesis and secretion of MBP, such a system will hopefully permit us to dissect the E. coli protein export pathway and further clarify the mode of action of the products of various genes which have been implicated as having important roles in this process.

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LITERATURE CITED


