JOURNAL OF BACTERIOLOGY, Sept. 1989, p. 4752–4760
0021-9193/89/094752-09$02.00/0

Vol. 171, No. 9

Magnesium Transport in *Salmonella typhimurium*: Expression of Cloned Genes for Three Distinct Mg^{2+} Transport Systems

MARSHALL D. SNAVELY,[1] JANE B. FLORER,[1] CHARLES G. MILLER,[2] AND MICHAEL E. MAGUIRE[1]*

Department of Pharmacology[1] and Department of Molecular Biology and Microbiology,[2] School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

Received 10 November 1988/Accepted 20 May 1989

In *Salmonella typhimurium*, the *corA*, *mgtA*, and *mgtB* loci are involved in active transport of Mg^{2+} (S. P. Hmiel, M. D. Snavely, C. G. Miller, and M. E. Maguire, J. Bacteriol. 168:1444–1450, 1988; S. P. Hmiel, M. D. Snavely, J. B. Florer, M. E. Maguire, and C. G. Miller, J. Bacteriol. 171:4742–4751, 1989). In this study, the gene products coded for by the *corA*, *mgtA*, and *mgtB* genes were identified by using plasmid expression in *Escherichia coli* maxicells. Complementation was assessed by introducing plasmids into a Mg^{2+}-dependent *corA mgtA mgtB* strain and determining the ability of the plasmid to restore growth on medium without a Mg^{2+} supplement. Complementing plasmids containing *corA* expressed a 42-kilodalton (kDa) protein. This protein was not expressed by plasmids containing insertions or deletions that eliminated complementation. A plasmid containing *mgtA* expressed 37- and 91-kDa gene products. Data obtained with subclones and insertions in this plasmid indicated that plasmids expressing only the 91-kDa polypeptide complemented; plasmids that did not express this protein did not complement regardless of whether they expressed the 37-kDa protein. Plasmids carrying *mgtB* expressed a single protein of 102 kDa whose presence or absence correlated with the ability of the plasmid to complement the Mg^{2+}-dependent triple mutant. Fractionation of labeled maxicells demonstrated that the 42-kDa *corA*, the 91-kDa *mgtA*, and the 102-kDa *mgtB* gene products are all tightly associated with the membrane, a location consistent with involvement in a transport process. These data provide further support for the existence of three distinct systems for Mg^{2+} transport in *S. typhimurium*.

Many studies have demonstrated the importance of Mg^{2+} in supporting cell growth (2, 7, 26–28). In medium deficient in this cation, cells fail to grow because of a number of specific effects of Mg^{2+} on cellular physiology. Mg^{2+} is required for the maintenance of membrane integrity and ribosomal association (7), is a required cofactor for a number of important enzymes (2, 7), and may have important regulatory functions (2, 26, 27). In *Escherichia coli*, the total intracellular Mg^{2+} concentration is maintained within narrow limits when the extracellular Mg^{2+} concentration is varied over a range of 10^5-fold (28). To maintain such stringent regulation, one or more transport systems capable of mediating transmembrane Mg^{2+} flux must exist.

Uptake of $^{28}Mg^{2+}$ by *E. coli* was demonstrated nearly two decades ago (9, 13, 22). Uptake was inhibited in the cold and by metabolic poisons, indicating that Mg^{2+} transport is an active process. From genetic studies, Nelson and Kennedy (13) postulated the existence of two distinct systems for Mg^{2+} uptake in *E. coli*. One system transports Co^{2+} as well as Mg^{2+}, and mutations in this system were selected by isolating cells resistant to the cytotoxic effect of Co^{2+}. The Mg^{2+} uptake remaining in Co^{2+}-resistant mutants was repressible by growth in media containing high concentrations of Mg^{2+}. Park et al. (17) then extended these findings by describing two genetic loci in *E. coli* related to Mg^{2+} transport, *corA* and *mgt*. Strains harboring mutations in both loci were reported to exhibit no high-affinity, energy-dependent Mg^{2+} transport and to require high extracellular Mg^{2+} concentrations for growth.

In *Salmonella typhimurium*, we have identified three loci, *corA*, *mgtA*, and *mgtB*, that are involved in the transport of Mg^{2+} across the cell membrane (4, 5). Strains harboring mutations at all three loci lack detectable Mg^{2+} transport and require millimolar concentrations of extracellular Mg^{2+} for growth. The three genes have been cloned by complementation of this growth phenotype (4, 5) and represent three distinct transport systems (23). To characterize these Mg^{2+} transport systems, we have identified potential gene products coded for by the *corA*, *mgtA*, and *mgtB* genes and determined the subcellular location of each.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. typhimurium* MM206 (*corA27 mgtA5 mgtB11*::Mu dJ *zjh-1628*::Tn*10*Δcam Δ*leuBCD485*) requires high concentrations of Mg^{2+} for growth and lacks detectable Mg^{2+} uptake (4); it was used for complementation studies. *E. coli* CSR603 was obtained from Robert Hogg and used to produce maxicells (19). *E. coli* RM8 (DB4548 in reference 11) and *S. typhimurium* DB4926 (16) were obtained from Russell Maurer. RM8 was used to modify plasmids prepared from *S. typhimurium* strains for movement in CSR603. DB4926 is a *his* derivative of TS736 (16) and was used as an intermediate to move plasmids between *E. coli* and *S. typhimurium* strains. All plasmids used in this study are derivatives of pBR328 except for pJF20, pMS2573, and pMS2574, which are pT7 derivatives (25). Plasmid genotypes are listed in Table 1; details of their construction are given in this report or previous publications (4, 5). Other strains used are described in the accompanying report (4).

Media. For experiments using maxicells, K medium, Hershey medium, and Hershey salts were prepared as described by Sancar et al. (18). Other media and their supplementation with $MgSO_4$ have been previously described (4, 5).

Assay of Mg^{2+} dependence. As described in the accompanying report (4), low-Mg^{2+} medium refers to LB without

* Corresponding author.

TABLE 1. Plasmid genotypes

Plasmid	Relevant genotype	Source or reference
pD1-2	*corA*$^+$ (deletion)	This study
pD1-7	*corA* (deletion)	This study
pD2-5	*corA*$^+$ (deletion)	This study
pJF20	*mgtA*$^+$ (T7 promoter, deletion)	This study
pJF21	*mgtA*$^+$ (deletion)	This study
pJF22	*mgtA*$^+$ (deletion)	This study
pJF23	*mgtA*$^+$ (deletion)	This study
pJF26	*mgtA26*::Mu dJ	This study
pJF27	*mgtA27*::Mu dJ	This study
pJF28	*mgtA28*::Mu dJ	This study
pJF31	*mgtA31*::Mu dJ	This study
pJF32	*mgtA32*::Mu dJ	This study
pMM10	*mgtB10*::Mu dJ	4
pMM11	*mgtB11*::Mu dJ	4
pMM12	*mgtB12*::Mu dJ	4
pMS4	*corA*$^+$ (*E. coli*)	5
pMS41	*corA*$^+$	This study
pMS47	*corA47*::Mu dK	This study
pMS48	*corA48*::Mu dK	This study
pMS49	*corA49*::Mu dK	This study
pMS2573	*mgtA*$^+$ (T7 promoter)	This study
pMS2574	*mgtA*$^+$ (T7 promoter)	This study
pSPH25	*corA*$^+$	5
pSPH35	*corA*$^+$ (deletion)	5
pSPH36	*corA* (deletion)	5
pSPH37	*mgtA*$^+$	4
pSPH39	*mgtB*$^+$	4
pSPH42	*corA*$^+$ (deletion)	4

added $MgSO_4$, and high-Mg^{2+} medium refers to LB plus 10 mM $MgSO_4$. The concentration of Mg^{2+} in LB is sufficient for growth of strains carrying wild-type alleles of any one of *corA*, *mgtA*, or *mgtB* but insufficient to support growth of the *corA mgtA mgtB* triply mutant strain, MM206. Complementation was assayed by transducing plasmids into MM206 with selection for plasmid-encoded antibiotic resistance in the presence of 10 mM Mg^{2+} and scoring for the ability of antibiotic-resistant transductants to grow on LB without added Mg^{2+}.

Manipulation of DNA. All enzymes used in DNA manipulations were purchased from Boehringer Mannheim Biochemicals, New England BioLabs, Inc., or Bethesda Research Laboratories, Inc., and were used as specified by the manufacturers. Crude plasmid DNA was prepared by alkaline lysis and manipulated by standard techniques (11). Highly purified plasmid DNA was prepared by the method of Sorge and Hughes (24). Plasmids were transformed into *E. coli* strains or DB4926 by the $CaCl_2$ procedure (11). Plasmids were moved from DB4926 into or between *S. typhimurium* strains by transduction, using the generalized transducing phage P22 (*HT*12/4 *int*-3 [20]). After digestion by restriction endonucleases, DNA fragments were separated by electrophoresis through agarose gels and visualized by staining with ethidium bromide. To obtain deletions in the cloned piece of DNA carrying the *corA* gene, the insert from pSPH25 was subcloned into pBluescript (Stratagene). Deletions into the resultant plasmid, pMS41, were generated by using exonuclease III (Exo III/Mung Bean Nuclease Deletion Kit; Strategene) according to the instructions of the manufacturer.

Expression of proteins. Plasmid-encoded proteins were expressed in *E. coli* maxicells (18), with the following alterations of the published procedure: stationary cells from an overnight culture of CSR603 were inoculated at a 1:100 dilution into K medium containing antibiotic, grown to an optical density at 600 nm of 0.4 to 0.5, diluted 1:50 into the same medium, and grown again to an optical density at 600 nm of 0.4 to 0.5; 11 ml of culture was placed in a 13-mm-diameter petri dish and irradiated with UV light for 12 s at 10 ergs/cm^2; cycloserine was used at a final concentration of 200 μg/ml; and cells labeled with [^{35}S]methionine were pelleted and suspended without washing in 200 μl of 20 mM Tris (pH 7.5)–0.85% NaCl. Each 200-μl sample was mixed with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer (8), boiled for 5 min, and stored at −20°C. To visualize plasmid-encoded proteins, 30-μl portions of each sample were subjected to SDS-polyacrylamide gel electrophoresis, followed by autoradiography. Molecular weights of proteins were determined by using low-molecular-weight standards from Bio-Rad Laboratories.

Insertion of the Mu d(*lac*) element into *corA*. The Mu d(*lac*) elements Mu dI1734 and Mu dII1734 (1), referred to as Mu dJ and Mu dK, respectively, were inserted into the *corA* or *mgtA* gene present on plasmids by the method described in the accompanying paper for construction of gene fusions using Mu dJ (4). Plasmids containing a Mu dJ insertion were screened as previously described (4). Plasmids containing the Mu dK insertion were transduced into the *corA* strain, MM54, selecting for kanamycin resistance (Kan^r). Strain MM54 is resistant to Co^{2+} in a disk sensitivity test (4, 5). When pSPH25 is introduced into this strain, it becomes Co^{2+} sensitive. Plasmids carrying insertions into *corA* were identified as resistant to Co^{2+}, and the location of the inserted Mu dK was determined by restriction mapping. Three plasmids with Mu dK insertions at distinct locations were obtained (pMS47 to pMS49). One of these, pMS48, was used for subsequent studies requiring insertional inactivation of the *corA* gene.

Since the *lacZ* gene present in Mu dK lacks both a promoter and translation start signals, a fusion protein between the amino-terminal portion of the interrupted gene and β-galactosidase is formed when the Mu dK insertion is in frame with respect to the interrupted gene. Such fusion proteins typically demonstrate β-galactosidase activity (1). MM54 formed white colonies on MacConkey agar and produced no detectable β-galactosidase activity. When the fusion plasmids pMS47, pMS48, and pMS49 were introduced into MM54, the resulting antibiotic-resistant strains were red on MacConkey agar. To confirm that this red phenotype was due to increased production of β-galactosidase, enzyme activity was assayed as described by Miller (12). MM54 and MM54 harboring pMS48 produced 0 and 270 U of β-galactosidase activity, respectively. Immunoprecipitation of fusion proteins containing β-galactosidase was performed as described by Maihle et al. (10). Antiserum directed against β-galactosidase was a gift from Nita Maihle.

Gene expression directed by the T7 promoter. Plasmid proteins were labeled with the T7 RNA polymerase-promoter system by a modification of the procedure of Tabor and Richardson (25). Strains were constructed that contained two plasmids: pGP1-2, carrying the gene coding for the T7 RNA polymerase under the control of the inducible lambda p_L promoter and the gene coding for the heat-sensitive lambda repressor *c*I857; and a derivative of pT7-3 that carries the T7 promoter either without (pT7-3) or with (pMS2573, pMS2574, and pJF20) the *mgtA* locus cloned downstream. In plasmid pMS2574, the orientation of the *mgtA* locus is opposite that in pMS2573 and pJF20. Strains containing both the T7 polymerase plasmid and a T7 promoter plasmid were grown at 30°C in kanamycin and ampi-

cillin (to maintain selection for both plasmids). Overnight cultures were diluted 1:100 into LB with kanamycin and ampicillin and grown to an optical density at 600 nm of approximately 0.6. A 0.7-ml sample was removed from each culture, washed five times in M9 medium (25), suspended in 3.5 ml of M9 containing 0.01% of 18 amino acids (minus cysteine and methionine), and incubated at 30°C for 1 h. Samples of 2.4 ml were removed from each tube and incubated at 42°C for 15 min. The remaining 1.1 ml constituted the uninduced control. The heat-induced samples were split into 1.2-ml fractions, and rifampin added to one fraction at a final concentration of 400 μg/ml. Rifampin inhibits the *S. typhimurium* but not the T7 RNA polymerase. Incubation of both fractions was continued at 42°C for 20 min, followed by a 20-min incubation at 37°C. Each fraction, including the uninduced control, then received 15 μCi of [^{35}S]methionine. After 5 min at 30°C, the three fractions from each culture were placed on ice. Samples of 1 ml were pelleted and washed once with M9 medium before resuspension in 100 μl of SDS sample buffer and electrophoresis on a 10% polyacrylamide gel. Labeling of each plasmid resulted in three samples: uninduced; induced; and induced, rifampin treated.

Cell fractionation. Cells were fractionated into periplasmic, cytosolic, and membrane components by a modification of the method of Harayama et al. (3). Maxicell samples of 100 μl labeled with [^{35}S]methionine were pelleted for 2 min in an Eppendorf microcentrifuge. The cell pellet was washed twice with 1 ml of 10 mM Tris–30 mM NaCl (pH 7.3) and suspended in 200 μl of 30 mM Tris (pH 7.3)–20% sucrose–0.1 mM EDTA. The cells were incubated in this buffer for 10 min, pelleted as described above, and suspended in 600 μl of cold 0.5 mM $MgCl_2$. After the cells were again pelleted, the supernatant was removed and stored at −20°C. This represented the periplasmic fraction. The cell pellet was suspended in 350 μl of 30 mM Tris (pH 8.0)–5 mM EDTA–1 mg of lysozyme (Sigma Chemical Co.) per ml, incubated at 35°C for 60 min, frozen at −20°C, and thawed slowly to room temperature. Samples were chilled in an ice-salt bath and sonicated for 2 min (four times for 30 s each at 60-s intervals). Unbroken cells were removed by centrifugation for 2 min in the microcentrifuge. The supernatant was removed and centrifuged again. The resulting supernatant was then centrifuged in a Beckman Ti50 rotor for 2.5 h at 200,000 × *g* to pellet the cell membrane fragments. The supernatant (cytosolic fraction) was removed and stored at −20°C. The pellet (membrane fraction) was washed twice in 20 mM Tris (pH 7.5)–0.85% NaCl and suspended in 200 μl of SDS sample buffer. The membrane fraction was then boiled for 5 min and stored at −20°C. Samples of the periplasmic (400 μl) and the cytosolic (200 μl) fractions were concentrated to 50 μl in Centricon-10 microconcentrators (Amicon Corp.). A 50-μl amount of 2× SDS sample buffer as added to each of these fractions, and both were boiled for 5 min. Samples were visualized by SDS-polyacrylamide gel electrophoresis, followed by autoradiography. To distinguish integral and peripheral membrane proteins, the membrane fraction was treated with the chaotropic agent urea. The membrane fraction was split into two portions. Each was pelleted and suspended in buffer (10 mM Tris [pH 7.2], 30 mM NaCl) with or without urea at a final concentration of 6 M and incubated at 37°C for 20 min; membranes were repelleted and subjected to SDS-polyacrylamide gel electrophoresis.

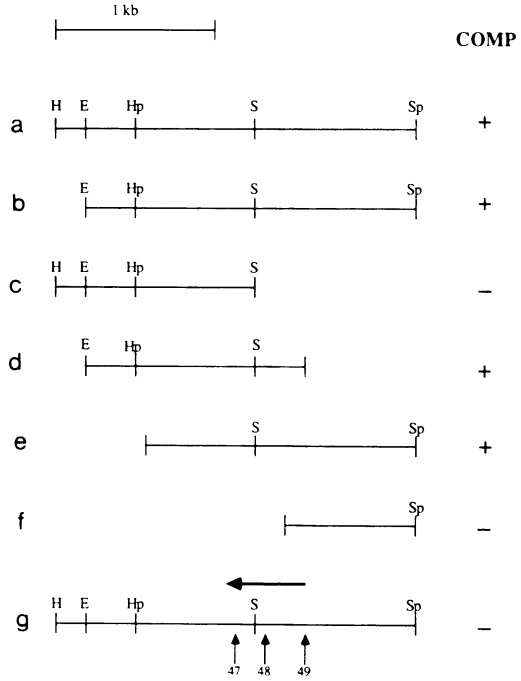

FIG. 1. Restriction maps of pSPH25 (a) and derivatives pSPH35 (b), pSPH36 (c), pD2-5 (d), pD1-2 (e), and pD1-7 (f). Only the insert DNA is shown. Deletions in pSPH35 and pSPH36 were generated by religation of pSPH25 after digestion with restriction endonucleases. Deletions in pD2-5, pD1-2, and pD1-7 were generated by using exonuclease III (see Materials and Methods). Mu dK insertions into pSPH25 were constructed as described in Materials and Methods. (g) Locations and allele numbers for three *corA*::Mu dK (↑); insertions; *corA47*::Mu dK, *corA48*::Mu dK, and *corA49*::Mu dK correspond to insertions in pMS47, pMS48, and pMS49, respectively. ←, Direction of transcription determined from the orientations of the insertions. The size of the arrow does not indicate the size of the coding region. The ability of each plasmid to complement the *corA27* mutation is indicated on the right. Restriction sites: H, *Hin*dIII; E, *Eco*RI; Hp, *Hpa*I; S, *Sal*I; Sp, *Sph*I.

RESULTS

Identification of the *corA* gene product. pSPH25 is a pBR328-derived plasmid that contains the *corA* locus on a 2.2-kilobase-pair (kbp) chromosomal DNA insert into the *Bam*HI site (Fig. 1). When introduced into a *corA* strain, pSPH25 restores Co^{2+} sensitivity, $^{60}Co^{2+}$ uptake, and $^{28}Mg^{2+}$ uptake. The locus contained in pSPH25 maps to the chromosomal *corA* locus identified previously (5). The presence of pSPH25 in the Mg^{2+}-dependent triply mutant strain MM206 (*corA mgtA mgtB*) allowed growth on LB agar without Mg^{2+} supplementation. Deletion of a *Hin*dIII-*Eco*RI fragment from the chromosomal insert in pSPH25 resulted in a plasmid, pSPH35 (Fig. 1), that still complemented by restoring growth on low-Mg^{2+} medium to MM206. Removal of a *Sal*I-*Sph*I fragment from pSPH25 resulted in pSPH36. When pSPH36 was introduced into MM206, the strain remained Mg^{2+} dependent.

Insertion mutations were also generated by using pSPH25. Three plasmids were constructed in which Mu dK was inserted into the cloned DNA in pSPH25 as described in Materials and Methods. The positions of the three insertions in pMS47, pMS48, and pMS49 were determined by restriction endonuclease mapping (Fig. 1). All three of these plasmids produced β-galactosidase and had lost the ability to

complement the Mg^{2+} dependence of MM206. The positions of the insertions suggest that the *corA* gene is located toward the center of the 2-kbp insert in pSPH35, consistent with the loss of complementation resulting from the deletion in pSPH36. In addition, because the *lacZ* coding sequence must be transcribed from the *corA* promoter, the orientation of the *Mu* d(*lac*) elements in pSPH25 indicates the direction of transcription of the *corA* gene (Fig. 1).

Further localization of the *corA* gene was obtained by using deletions generated by exonuclease III (Fig. 1). The *Eco*RI-*Sph*I fragment of the chromosomal insert from pSPH35 was subcloned into pBSM13SK (Stratagene) to form pMS41. Deletions into the insert DNA were generated as described in Materials and Methods. Removal of 400 bp starting at the *Eco*RI site or 600 bp starting at the *Sph*I site in pMS41 resulted in plasmids pD1-2 and pD2-5, respectively, which still complemented the Mg^{2+} dependence of MM206 (Fig. 1). Deletion of another 700 bp from the *Eco*RI site yielded pD1-7 and destroyed the ability to restore growth on low-Mg^{2+} medium to MM206. These deletions localize the coding region of the *corA* gene to a region of approximately 1.2 kbp in the insert DNA of pSPH25 defined by the deletion endpoints in pD1-2 and pD2-5.

To identify the gene product(s) coded for by the plasmids that complemented the Mg^{2+} dependence of MM206, plasmid DNA was transformed into the *E. coli* maxicell strain CSR603 (18), and the plasmid-encoded gene products were labeled as described in Materials and Methods. The putative *E. coli corA* gene product was examined as a positive control. Since the *E. coli corA* gene complements the *corA* mutation in *S. typhimurium* (5), we also expected that the two *corA* genes would yield similar gene products.

pBR328 without insert (Fig. 2) expressed the gene products responsible for antibiotic resistance: β-lactamase, 29 kilodaltons (kDa) (18); chloramphenicol acetyltransferase, 20 kDa (21); and the tetracycline resistance (*tet*) gene product, which runs at 31 kDa on denaturing gels (18). The *E. coli* (pMS4) and *S. typhimurium* (pSPH25) *corA*$^+$ plasmids expressed β-lactamase and chloramphenicol acetyltransferase, but instead of the *tet* gene product, they expressed single, additional polypeptides of 39 kDa (*E. coli*) and 42 kDa (*S. typhimurium*; Fig. 2). A protein of molecular weight 42,000 would require a coding region of around 1 kbp. Thus, the size of the putative *corA* gene product is consistent with the size of the insert fragment shown to be necessary for complementation.

pSPH35, which retained the ability to complement the Mg^{2+} dependence, produced the 42-kDa protein (Fig. 1a and Fig. 2); this plasmid did not confer chloramphenicol resistance (Cam^r) and did not produce chloramphenicol acetyltransferase. However, pSPH36 (Fig. 1c) did not complement the Mg^{2+}-dependence of MM206 and failed to produce the 42-kDa protein (Fig. 2), suggesting that the 42-kDa protein is the product of the *corA* gene. Examination of the proteins produced by pMS48 containing the *corA48*::Mu dK protein fusion supported the identification of the 42-kDa protein as the *corA* gene product. pMS48 failed to complement the *corA* mutation. pMS48 did not produce the 42-kDa protein made by pSPH25 but did produce a 130-kDa protein (Fig. 3). This protein was larger than β-galactosidase, indicating the production of a hybrid protein. An antiserum directed against β-galactosidase precipitated the 130-kDa protein (Fig. 3), confirming that it is a fusion product.

Identification of the *mgtA* gene product. Plasmid pSPH37 contains the *mgtA* locus on a 7.1-kbp chromosomal insert. To identify the region responsible for complementation of the Mg^{2+} dependence of MM206, various fragments of the chromosomal insert were subcloned (Fig. 4a to f). Plasmid pJF21 contains the *Cla*I-*Sal*I fragment from pSPH37 cloned into pBR328 and fails to restore growth on LB agar to MM206. pJF22 and pJF23 contain the *Pvu*II-*Sal*I and *Sph*I-*Sal*I fragments from pSPH37 cloned into pBR328; both of these plasmids complement the Mg^{2+} dependence of MM206. In addition, pMS2573 and pJF20 contain the *Sal*I-*Pst*I and *Sph*I-*Pst*I fragments of pSPH37, respectively, cloned into the expression vector pT7-3 (25). Compared with pSPH37, which gave normal-size colonies after overnight incubation, strains carrying either pMS2573 or pJF20 gave only weak complementation, as shown by the appearance of tiny colonies after at least 24 h of incubation. Furthermore, strains carrying these two plasmids showed Mg^{2+} dependence intermediate between that of the Mg^{2+}-dependent strain MM206 and either the wild-type strain or MM206 carrying pSPH37. These data indicate that the fragment necessary for complementation of the *mgtA* mutation resides in a fragment that includes the *Cla*I-*Pst*I region of pSPH37. The weak complementation by pMS2573 and pJF20 is discussed further below.

Insertion mutations were generated in pSPH37 by using Mu dJ (Fig. 4g). Five distinct insertions that eliminated the ability of pSPH37 to complement the Mg^{2+} dependence of MM206 were identified; the locations of the insertions were determined by restriction endonuclease mapping (Fig. 4g). The location of the *mgtA* locus suggested by these insertions is consistent with the localization suggested by the deletions in pJF21, pJF22, and pJF23. In addition, expression of

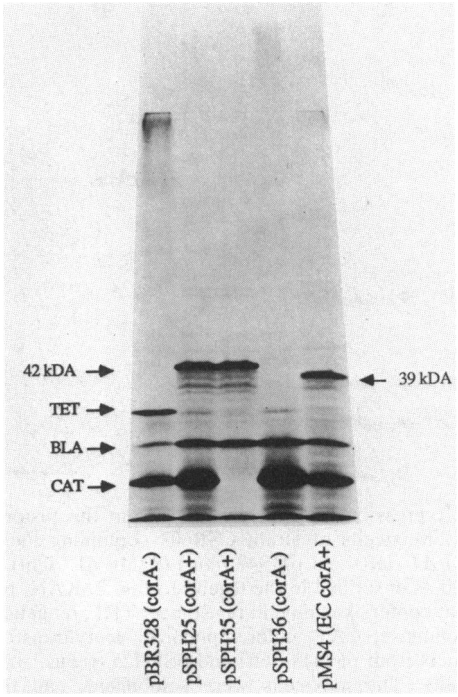

FIG. 2. Gene products expressed by *corA* plasmids. Gene products were expressed in *E. coli* maxicells labeled with [^{35}S]methionine. Proteins were separated on an SDS–10% polyacrylamide gel and visualized by autoradiography. Maxicells of strain CSR603 contained the plasmids indicated. Gene products coded for by genes conferring antibiotic resistance: TET, *tet* gene product; BLA, β-lactamase; CAT, chloramphenicol acetyltransferase. Also shown are the molecular sizes of insert-encoded gene products.

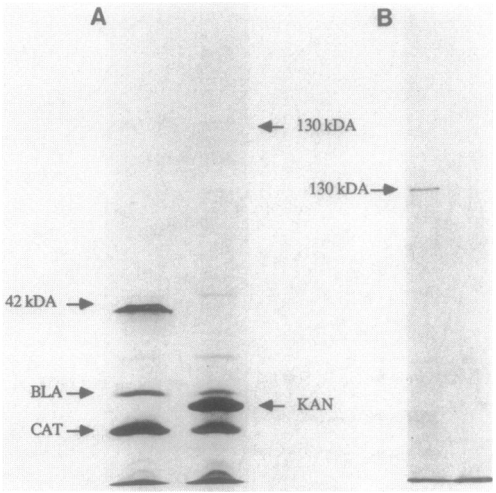

FIG. 3. Expression of gene products from the fusion plasmid pMS48. (A) Maxicells of strain CSR603 containing the plasmids pSPH25 (*corA*$^+$, left) and pMS48 (*corA48*::Mu dJ, right). Samples were treated as described in the legend to Fig. 2. KAN, product of the gene that confers kanamycin resistance; TET, *tet* gene product; BLA, β-lactamase; CAT, chloramphenicol acetyltransferase. (B) Gene products from pMS48 (left) and pSPH25 (right) labeled in *E. coli* maxicells. The maxicells were solubilized, and the fusion protein was immunoprecipitated with anti-β-galactosidase antibodies (10).

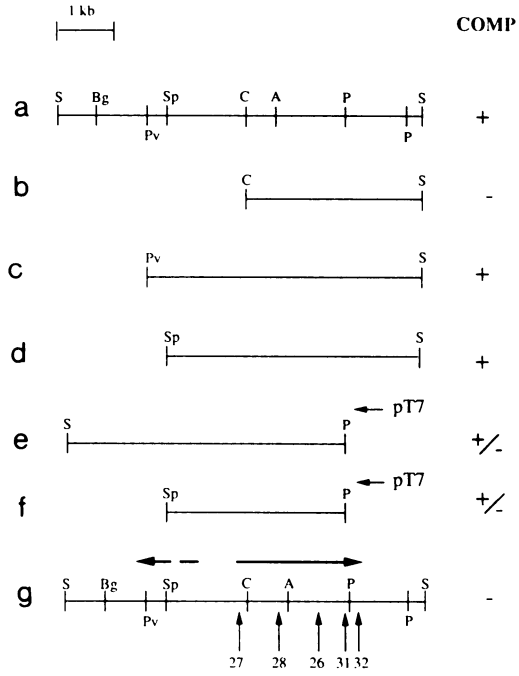

FIG. 4. Restriction maps of insert DNA from *mgtA* plasmids pSPH37 (a), pJF21 (b), pJF22 (c), pJF23 (d), pMS2573 (e), and pJF20 (f) and the insertion mutation *mgtA*::Mu dJ (g). In maps e and f, the *Sal*I-*Pst*I and *Sph*I-*Pst*I fragments from pSPH37 were cloned into the expression vector pT7-3 (described in Materials and Methods). Direction of transcription from the T7 promoter is indicated by the small horizontal arrows. The large horizontal solid arrow indicates the direction of transcription of the locus coding for the 91-kDa protein (see text); the dashed arrow indicates the direction of transcription of the locus coding for the 37-kDa gene product (see text). The sizes of the arrows do not indicate the sizes of the coding regions. Vertical arrows in map g mark the positions of *mgtA*::Mu dJ insertions. The ability of each plasmid to complement the Mg^{2+} dependence of MM206 is indicated on the right. Restriction sites: S, *Sal*I; Bg, *Bgl*II; Pv, *Pvu*II; Sp, *Sph*I; C, *Cla*I; A, *Acc*I; P, *Pst*I.

β-galactosidase from the insertions (unpublished observations) indicates that transcription proceeds as indicated in Fig. 4g.

Labeling of the gene products encoded by pSPH37 revealed that the insert DNA coded for two gene products with apparent molecular sizes of 37 and 91 kDa (Fig. 5). The noncomplementing plasmid pJF21 made neither gene product, whereas the complementing plasmid pJF22 expressed proteins of 35 and 91 kDa (Fig. 5B). The 35-kDa gene product is presumably a truncated form of the 37-kDa protein expressed by pSPH37. Thus, the coding region for the 37-kDa protein appears to be interrupted by the *Pvu*II site on the left side of the insert in pSPH37 (Fig. 4). pJF23 expressed only the 91-kDa protein and complemented the Mg^{2+} dependence of MM206, suggesting that the 91-kDa gene product is sufficient for complementation of the *mgtA7* allele in MM206.

The insertion mutations in pJF27, pJF28, pJF31, and pJF32 (Fig. 4g) eliminated complementation and expression of the 91-kDa protein. All of these plasmids still expressed the 37-kDa gene product. Furthermore, pJF26 failed to complement and produced, in addition to the 37-kDa protein, a 73-kDa protein (presumably a truncated 91-kDa protein). This result supports a direction of transcription for the 91-kDa gene product opposite that of the 37-kDa protein (Fig. 4g). Thus, only the plasmids that produce the intact 91-kDa protein fully complement the Mg^{2+} dependence of MM206.

Plasmid pMS2573 (Fig. 4e) expressed only the 37-kDa protein in *E. coli* maxicells or when transcription is initiated at the T7 promoter present on pT7-3 (see Materials and Methods). Neither the 37- nor the 91-kDa protein was expressed in pMS2574, in which the orientation of the insert relative to the T7 promoter is reversed. These results support the conclusion presented above indicating that the 37- and 91-kDa proteins are transcribed in opposite directions (Fig. 4). Since both pMS2573 and pMS2574 weakly complement the Mg^{2+} dependence of MM206, this result would seem to conflict with those presented above. However, pJF20 contains a shorter insert than pMS2573, expresses neither the 37- nor the 91-kDa protein in *E. coli* maxicells (data not shown), and complements weakly. Thus, production of the 37-kDa protein does not seem to be required for complementation. Weak complementation in the absence of the 91-kDa protein might be explained by production of an unstable truncated form, since the plasmids that weakly complement both terminate at the *Pst*I site that seems to be very near the 3′ terminus of the 91-kDa protein (see Discussion). It should be noted that in complementation studies and experiments using maxicells, genes contained on the insert DNA are transcribed from native promoters. Transcription from the T7 promoter occurs only under specific conditions (see Materials and Methods).

Identification of the *mgtB* gene product. pSPH39 carries a 7.4-kbp chromosomal DNA insert containing the *mgtB* locus. After integration into the chromosome, the insert maps at the *mgtB* locus (4), and introduction of pSPH39 into MM206 restores ability to grow on low-Mg^{2+} medium (4). When a 3.5-kbp *Hin*dIII-*Sau*3A fragment was removed from pSPH39 to form pSPH42 (Fig. 6a and b), the ability to relieve

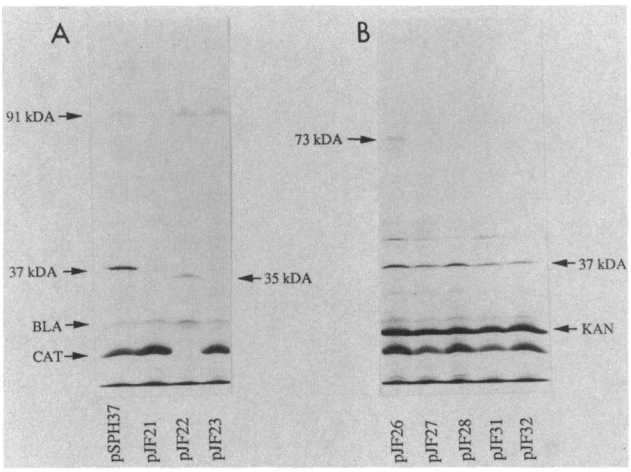

FIG. 5. Gene products expressed by *mgtA* plasmids. Gene products were expressed in *E. coli* maxicells labeled with [^{35}S]methionine. Proteins were separated on an SDS–10% polyacrylamide gel and visualized by autoradiography. Maxicells of strain CSR603 contained the plasmids indicated. Gene products coded for by genes conferring antibiotic resistance: BLA, β-lactamase; CAT, chloramphenicol acetyltransferase; KAN, kanamycin resistance. Also shown are the molecular sizes of insert-encoded gene products.

the Mg^{2+} dependence of MM206 was lost. In addition, several insertions of Mu dJ into pSPH39 were isolated (4). Plasmids pMM10, pMM11, and pMM12 contain Mu dJ integrated into the insert DNA of pSPH39 at distinct locations (Fig. 6c). All three of these plasmids fail to complement the Mg^{2+} dependence of MM206 (4). The positions of the three insertions localize the *mgtB* gene to a 2.7-kbp region near the center of the 7.4-kbp insert in pSPH39 (Fig. 6c). The fact that they span the *Hin*dIII site is consistent with the loss of complementing activity in pSPH42. Insertions *mgtB11*::

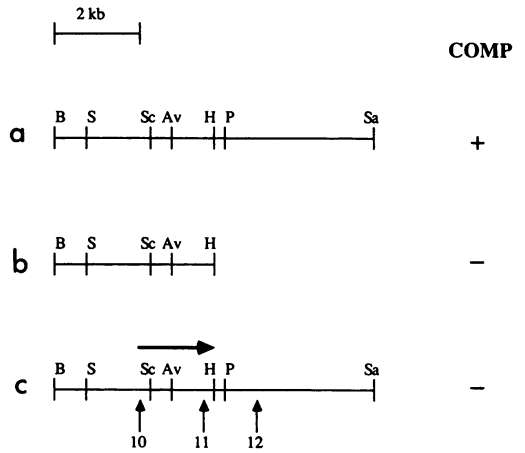

FIG. 6. Constructions of *mgtB* plasmids pSPH39 (a) and pSPH42 (b) and the insertion mutation *mgtB*::Mu dJ (c). Only the insert DNA is shown for pSPH39 and its derivatives. In the *mgtB*::Mu dJ construction, *mgtB* alleles 10, 11, and 12 (3) correspond to the insertions in plasmids pMM10, pMM11, and pMM12, respectively. →, Direction of transcription determined from the orientations of the insertions. The size of the arrow does not indicate the size of the coding region. The ability of each plasmid to complement the Mg^{2+} dependence of MM206 is indicated on the right. Restriction sites: B, *Bam*HI; S, *Sal*I; Sc, *Sac*I; Av, *Ava*I; H, *Hin*dIII; P, *Pst*I; Sa, *Sau*3A.

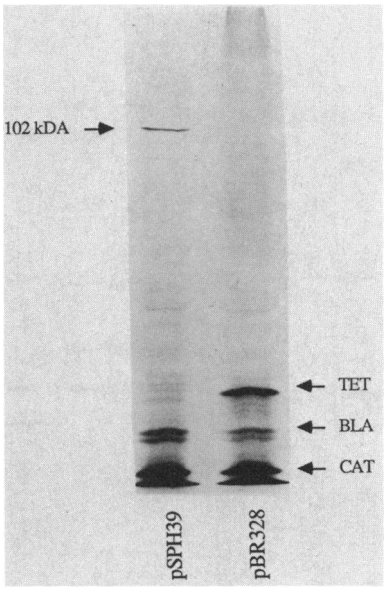

FIG. 7. Gene products produced by an *mgtB* plasmid. *E. coli* maxicells contained the indicated plasmids. Gene products (TET, *tet* gene product; BLA, β-lactamase; CAT, chloramphenicol acetyltransferase) were expressed and visualized as described in Materials and Methods. Proteins were separated on an SDS–7.5% polyacrylamide gel.

Mu dJ and *mgtB12*::Mu dJ express β-galactosidase from the *mgtB* promoter, whereas *mgtB10*::Mu dJ contains the Mu d(*lac*) element in the opposite orientation and does not express β-galactosidase activity (unpublished observations). These results indicate the direction of transcription of the *mgtB* gene (Fig. 6).

Expression of plasmid-encoded products in maxicells revealed a single polypeptide with an apparent molecular size of 102 kDa, coded for by the 7.4-kbp insert in pSPH39 (Fig. 7). This protein was not expressed by the noncomplementing plasmid pSPH42 (Fig. 7) or pMM10 (data not shown). These data support the identity of this protein as the *mgtB* gene product.

Cellular localization of *corA*, *mgtA*, and *mgtB*. The cellular location of the gene products identified was investigated, since at least some components of each Mg^{2+} transport system would be associated with the cell membrane. pSPH25, pSPH37, and pSPH39 were transformed into CSR603, the maxicells were labeled with [^{35}S]methionine, and the resulting samples were split into two parts. One part for each plasmid was solubilized by heating in SDS sample buffer and represented total cellular protein; the other was fractionated into periplasmic, membrane, and cytosolic components by the procedure of Harayama et al. (3). Although a portion of the 42-kDa *corA*, the 37- and 91-kDa *mgtA*, and the 102-kDa *mgtB* gene products were found in the cytosol, all were clearly associated with the membrane fraction (Fig. 8). The lack of chloramphenicol acetyltransferase, a cytoplasmic protein, and β-lactamase, a periplasmic protein, in the membrane fraction (Fig. 8A) illustrates that contamination of the membrane fractions by cytoplasmic and periplasmic proteins was negligible. Contamination of the cytosolic fraction by periplasmic and membrane-associated proteins has been observed by others (6) and could be due to a variety of reasons. For example, the formation of small vesicles after sonication may prevent quantitative sedimentation, a

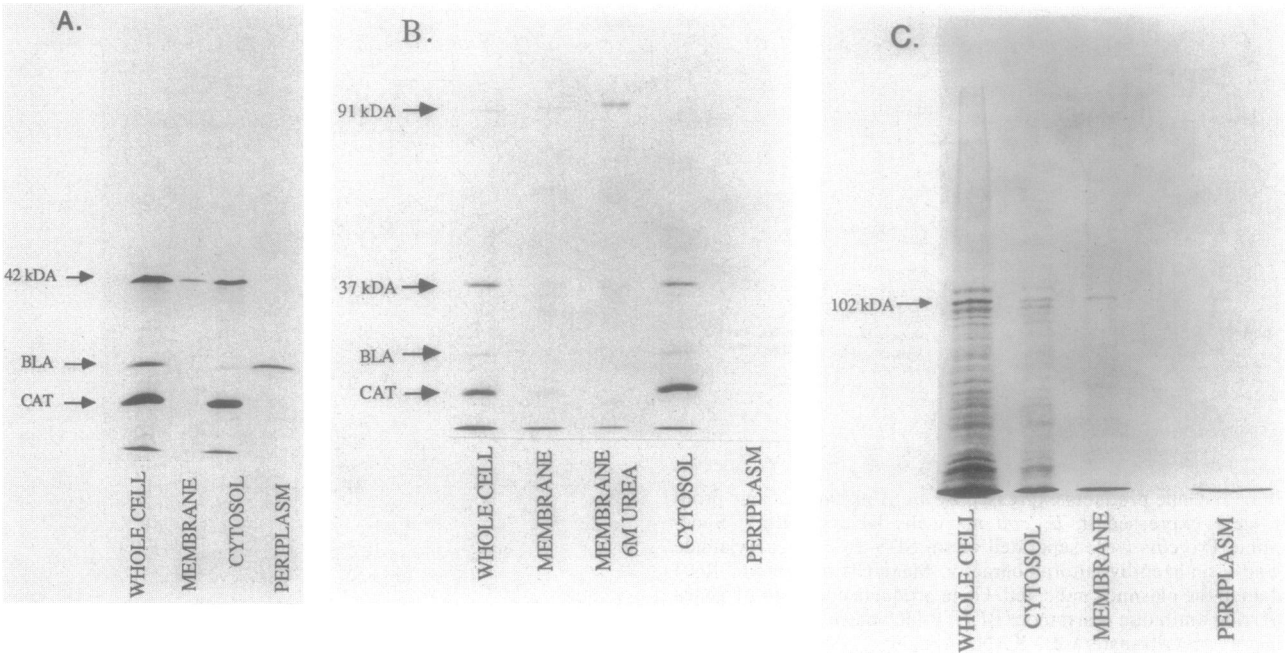

FIG. 8. Localization of proteins involved in Mg^{2+} transport. *E. coli* maxicells containing various plasmids were fractionated into membrane, periplasmic, and cytosolic components (see Materials and Methods). The plasmid-encoded, [^{35}S]methionine-labeled gene products were separated on SDS-polyacrylamide gels (A and B, 10% gels; C, 7.5% gel) and visualized by autoradiography. Maxicells contained pSPH25 (*corA*$^+$) (A), pSPH37 (*mgtA*$^+$) (B), and pSPH39 (*mgtB*$^+$) (C). In panel B, the membrane fraction was incubated in the presence of 6 M urea for 20 min at 37°C as described in Materials and Methods.

cytosolic precursor for exported proteins may exist, or the membrane may not be able to incorporate the entire amount of a membrane-associated protein produced from a high-copy-number plasmid. That the *corA*, *mgtA*, and *mgtB* gene products (Fig. 8) were all found in the membrane fraction is consistent with their identification as transport proteins.

The membrane fraction was further treated with the chaotropic agent urea (6 M) to distinguish peripheral from integral membrane proteins. Urea treatment had no effect on the 91-kDa *mgtA* gene product but greatly decreased the amount of the membrane-associated 37-kDa *mgtA* gene product (Fig. 8B). Urea also had no effect on the 42-kDa *corA* or the 102-kDa *mgtB* gene product (data not shown). These data suggest that the *corA*, the 91-kDa *mgtA*, and the *mgtB* gene products are all tightly associated with the membrane, whereas the 37-kDa gene product from the *mgtA* plasmid is likely a peripheral membrane protein. Association of the various gene products with the inner versus the outer membrane was not tested; however, association with the outer membrane seems highly unlikely, since Mg^{2+} passes freely through the porins of the outer membrane (14).

DISCUSSION

The *corA* locus. In this study, we have tentatively identified gene products for each of three Mg^{2+} transport systems in *S. typhimurium*. The evidence indicates that the 42-kDa *corA* gene product is an ion-transporting protein. First, in the presence of the mutant allele of *corA*, Mg^{2+} uptake is diminished and Co^{2+} uptake is absent (5). Reintroduction of a single copy of the wild-type allele into the chromosome restores Mg^{2+} and Co^{2+} uptake. Second, addition of a plasmid containing the *corA* gene to a *corA* mutant strain restores both Mg^{2+} and Co^{2+} uptake in a manner dependent on the copy number of the vector into which it is cloned. With increasing copy number, the V_{max} of uptake increases but the K_m remains unchanged (5; unpublished observations), implying that an increased number of transport proteins is being synthesized. Third, the 42-kDa protein is the only protein produced from the 2.2-kbp insert in pSPH25, and its production by various plasmids corresponds exactly with the ability of those plasmids to complement the Mg^{2+} dependence of MM206. Finally, the 42-kDa *corA* gene product is tightly associated with the membrane, as would be expected for a cation transporter.

It is conceivable that interruption of the coding sequence of this protein by deletions or insertions has polar effects on the expression of downstream genes that do not produce visible products in the labeling experiments. However, we consider this possibility unlikely. From the orientation of the inserted Mu d(*lac*) elements in pMS47, pMS48, and pMS49, transcription proceeds from right to left (Fig. 1). A 42-kDa protein requires approximately 1 kbp of coding sequence. Since the 600-bp deletion in pD2-5 and the 400-bp deletion in pD1-2 failed to eliminate complementation, the approximately 1.2 kbp between the endpoints of these two deletions must come very close to defining the two ends of the *corA* coding sequence.

The *mgtA* locus. Results obtained with deletions and Mu dJ insertions into the chromosomal insert in pSPH37 strongly suggest that the 91-kDa protein is responsible for complementation of the *mgtA7* allele in MM206. The 91-kDa protein is tightly associated with the membrane, and plasmids that express this gene product complement regardless of whether the 37-kDa protein is expressed. The positions of the Mu dJ insertions and their orientations localize the 91-kDa protein in the insert DNA. The coding region for the *mgtA* gene product must span the *Cla*I-*Pst*I fragment, extending past the restriction sites on both sides. The production of a

73-kDa truncated protein by pJF26 supports this interpretation. On the basis of these data, the predicted size of the coding region would be approximately 2 kbp, close to the amount of DNA necessary to code for a 91-kDa polypeptide.

The 37-kDa protein is a peripheral membrane protein and is obviously not required for complementation of the *mgtA7* allele. However, it may still be involved in transport by the MgtA system if that system has multiple components. The fact that the 37- and 91-kDa proteins are divergently transcribed shows that they are not members of an operon. The 37-kDa protein might still be a component of the *mgtA* system, however. For example, since transcription of *mgtA* is altered in response to changes in the extracellular Mg^{2+} concentration, the 37-kDa protein could be a divergently transcribed regulatory protein analogous to MerR of the mercury resistance operon (15). The truncated gene product expressed by pJF22 indicates that nearly all of the coding region for the 37-kDa protein is to the right of the *Pvu*II site in Fig. 4.

The fact that pMS2573 and pJF20 fail to express the 91-kDa protein and yet complement weakly requires explanation. MM206 carrying either of these two plasmids grows slowly and has an intermediate Mg^{2+} dependence. The most likely possibility is that these plasmids produce an unstable, slightly truncated form of the 91-kDa protein that, although not visible in the expression studies because of its low concentration, is sufficient to allow growth of MM206 on LB agar. In support of this interpretation, the inserts in both of these plasmids terminate at the *Pst*I site, which is likely very close to the 3′ end of the coding region for the 91-kDa gene product. Enough of a truncated *mgtA* gene product might be formed from the plasmid to allow growth of MM206 on medium containing less than 10 mM Mg^{2+}. Such truncated proteins might be produced as shown by the 73-kDa polypeptide from pJF26. In addition, pJF31 and pJF32 contain Mu dJ insertions near the *Pst*I site at the 3′ end but fail to complement possibly because of the formation of products even more unstable than those produced by pMS2573 and pJF20.

The *mgtB* locus. Insertions and deletions in pSPH37 that eliminate the ability to complement the Mg^{2+} dependence of MM206 also eliminate the production of the 102-kDa protein identified in *E. coli* maxicells (Fig. 7). The tight membrane association of the 102-kDa protein is consistent with involvement in cation transport, as is the abolishment of MgtB-mediated transport by the Mu dJ insertions. An approximate location is identified by the spacing of the insertions in pMM10 and pMM12; the insertions are approximately 2.5 kbp apart. Since both insertions eliminate complementation, they may define the two ends of the putative *mgtB* gene. Thus, the coding region for the 102-kDa protein appears to reside in the center of the insert. Nonetheless, a polar effect of one of the insertions on expression of the 102-kDa protein cannot be excluded, leaving the exact location of the 102-kDa polypeptide on the insert uncertain. Since a 102-kDa protein requires approximately 2.5 kbp of coding sequence, there is more than 4 kbp of insert DNA that could code for other proteins. It is somewhat surprising that only one gene product, albeit a large one, is expressed in maxicells from more than 7 kbp of DNA.

Evidence in this and the accompanying report (4) indicates that the *corA*, *mgtA*, and *mgtB* genes each express a distinct product that (i) restores growth on low-Mg^{2+} medium to a Mg^{2+}-dependent strain, (ii) restores $^{28}Mg^{2+}$ uptake in a strain lacking detectable Mg^{2+} transport, (iii) has a distinct sensitivity to other divalent cations, and (iv) is membrane bound. The data suggest that the *corA*, *mgtA*, and *mgtB* genes represent three distinct transport systems, each capable of supporting Mg^{2+} movement through the membrane. This interpretation is confirmed by the transport data in the accompanying report (23).

ACKNOWLEDGMENTS

We thank Nita Maihle for numerous technical discussions and Paul Hmiel for the original isolation of some plasmids.

This work was supported by Public Health Service grant GM26340 (to M.E.M.) from the National Institutes of Health. M.D.S. was supported by a fellowship from the Northeast Ohio Chapter of the American Heart Association.

LITERATURE CITED

1. **Castilho, B. A., P. Olfsen, and M. J. Casadaban.** 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposons. J. Bacteriol. **158**:488–495.
2. **Grubbs, R. D., and M. E. Maguire.** 1987. Magnesium as a regulatory cation: criteria and evaluation. Magnesium **6**:113–127.
3. **Harayama, S., J. Bollinger, T. Iino, and G. L. Hazelbauer.** 1983. Characterization of the *mgl* operon of *Escherichia coli* by transposon mutagenesis and molecular cloning. J. Bacteriol. **153**:408–415.
4. **Hmiel, S. P., M. D. Snavely, J. Florer, M. E. Maguire, and C. G. Miller.** 1989. Magnesium transport in *Salmonella typhimurium*: genetic characterization and cloning of three distinct magnesium transport loci. J. Bacteriol. **171**:4742–4751.
5. **Hmiel, S. P., M. D. Snavely, C. G. Miller, and M. E. Maguire.** 1986. Magnesium transport in *Salmonella typhimurium*: characterization of magnesium influx and cloning of a transport gene. J. Bacteriol. **168**:1444–1450.
6. **Horazdovsky, B. F., and R. W. Hogg.** 1987. High-affinity L-arabinose transport operon. Gene product expression and mRNAs. J. Mol. Biol. **197**:27–35.
7. **Jasper, P., and S. Silver.** 1978. Magnesium transport in microorganisms, p. 7–47. *In* E. D. Weinberg (ed.), Microorganisms and minerals. Marcel Dekker, Inc., New York.
8. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
9. **Lusk, J. E., and E. P. Kennedy.** 1969. Magnesium transport in *Escherichia coli*. J. Biol. Chem. **244**:1653–1655.
10. **Maihle, N. J., M. A. Raines, T. W. Flickinger, and H.-J. Kung.** 1988. Proviral insertional activation of c-*erbB*: differential processing of the protein products arising from two alternate transcripts. Mol. Cell. Biol. **8**:4868–4876.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Nelson, D. L., and E. P. Kennedy.** 1972. Transport of magnesium by a repressible and a nonrepressible system in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **69**:1091–1093.
14. **Nikaido, H., and M. Vaara.** 1987. Outer membrane, p. 7–22. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
15. **O'Halloran, T. V., B. Frantz, M. K. Shin, D. M. Ralston, and J. G. Wright.** 1989. The MerR heavy metal receptor mediates positive activation in a topologically novel transcription complex. Cell **56**:119–129.
16. **Palva, E. T., P. Liljestrom, and S. Harayama.** 1981. Cosmid cloning and transposon mutagenesis in *Salmonella typhimurium* using phage (*lambda*) vehicles. Mol. Gen. Genet. **181**:153–157.
17. **Park, M. H., B. B. Wong, and J. E. Lusk.** 1976. Mutants in three genes affecting transport of magnesium in *Escherichia coli*: genetics and physiology. J. Bacteriol. **126**:1096–1103.
18. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method

for identification of plasmid-coded proteins. J. Bacteriol. **137:** 692–693.

19. **Sancar, A., and C. S. Rupert.** 1978. Determination of plasmid molecular weights from ultraviolet sensitivities. Nature (London) **272:**471–472.
20. **Sanderson, K. E., and J. R. Roth.** 1988. Linkage map of *Salmonella typhimurium*, edition VII. Microbiol. Rev. **52:**485–532.
21. **Shaw, W. V., L. C. Packman, B. D. Burleigh, A. Dell, H. R. Morris, and B. S. Hartley.** 1979. Primary structure of a chloramphenicol acetyltransferase specified by R plasmid. Nature (London) **282:**870–872.
22. **Silver, S.** 1969. Active transport of magnesium in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **62:**764–771.
23. **Snavely, M. D., J. B. Florer, C. G. Miller, and M. E. Maguire.** 1989. Magnesium transport in *Salmonella typhimurium*: $^{28}Mg^{2+}$ uptake by the CorA, MgtA, and MgtB systems. J. Bacteriol. **171:**4761–4766.
24. **Sorge, J., and S. H. Hughes.** 1982. Polypurine track adjacent to the U3 region of the Rous sarcoma virus provides a *cis*-acting function. J. Virol. **43:**482–488.
25. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
26. **Walker, G. M.** 1986. Magnesium and cell cycle control: an update. Magnesium **5:**9–23.
27. **Walker, G. M., and John H. Duffus.** 1983. Magnesium as the fundamental regulator of the cell cycle. Magnesium **2:**1–16.
28. **Webb, M.** 1970. Interrelationships between the utilization of magnesium and the uptake of other bivalent cations by bacteria. Biochim. Biophys. Acta **222:**428–439.