Purification of Mlc and Analysis of Its Effects on the pts Expression in Escherichia coli*

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Products of the pts operon of Escherichia coli have multiple physiological roles such as sugar transport, and the operon is controlled by two promoters, P0 and P1. Expression of the pts P0 promoter that is increased during growth in the presence of glucose is also activated by cAMP receptor protein-cAMP. Based on the existence of a sequence that has a high similarity with the known Mlc binding site in the promoter, the effects of the Mlc protein on the pts P0 promoter expression were studied. In vivo transcription assays using wild type and mlc-negative E. coli strains grown in the presence and absence of glucose indicate that Mlc negatively regulates expression of the P0 promoter, and Mlc-dependent repression is relieved by glucose in the growth medium. In vitro transcription assay using purified recombinant Mlc showed that Mlc repressed transcription from the P0 but did not affect the activity of the P1. DNase I footprinting experiments revealed that a Mlc binding site was located around +1 to +25 of the promoter and that Mlc inhibited the binding of RNA polymerase to the P0 promoter. Cells overexpressing Mlc showed a very slow fermentation rate compared with the wild type when grown in the presence of various phosphoenolpyruvate-carbohydrate phosphotransferase system sugars but few differences in the presence of non-phosphoenolpyruvate-carbohydrate phosphotransferase system sugars except maltose. These results suggest that the pts operon is one of major targets for the negative regulation by Mlc, and thus Mlc regulates the utilization of various sugars as well as glucose in E. coli. The possibility that the inducer of Mlc may not be sugar or its derivative but an unknown factor is proposed to explain the Mlc induction mechanism by various sugars.

The phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) catalyzes the phosphorylation and transportation of a large number of its sugar substrates (1). The phosphoryl group from phosphoenolpyruvate is sequentially transferred to enzyme I, to HPr, to the sugar-specific enzyme II complex, and finally to the substrate as it is transported across the membrane. The ptsH, ptsI, and crr genes encode HPr, enzyme I, and enzyme IIIC, respectively, constitute the pts operon. The pts operon is regulated in a complex fashion by P0 and P1, each of which has two different transcription start sites depending on the availability of CRP-cAMP and on the topology of DNA (2). Transcription of the pts promoters increases in the presence of CRP-cAMP as well as glucose in vivo (3, 4). The glucose- and cAMP-mediated activations occur independently of each other (2, 4, 5), even though glucose lowers the level of CRP and cAMP in the cell (6). It has been known that the target promoter that is activated when cells are grown in the presence of glucose is the P0 promoter, but the mechanism by which glucose activates the P0 transcription is not known. Transcription from the P0 is fully activated only when cells grow in the presence of both glucose and exogenous cAMP (2, 4, 7). The possibility of the presence of a repressor that is sensitive to glucose has been suggested based on these observations (7).

Mlc (making large colonies) is a newly identified global regulator of carbohydrate metabolism (8–11). The mlc gene was originally found to cause the reduction of acetate accumulation when the overexpressing cells grow in the presence of glucose (12). The mlc gene was also shown to be identical with the previously characterized dgsA gene (10, 13, 14). All genes that are so far known to be under the control of Mlc are also regulated by CRP-cAMP. We noticed a sequence that has a high homology with the known Mlc binding site between +1 and +25 of the pts P0 promoter region (Fig. 1). In this study, a procedure is described for purification of the active form of Mlc to homogeneity, and the effects of Mlc on the expression of the pts P0 promoter were studied to determine whether the in vivo glucose effect on pts operon expression is mediated by Mlc.

EXPERIMENTAL PROCEDURES

Materials—Cyclic AMP was obtained from Sigma. RNA polymerase and nucleotide triphosphates were purchased from Amersham Pharmacia Biotech. The cycle sequencing kit was from Epicentre Technologies (Madison, WI). [γ-32P]ATP and [α-32P]UTP were from Amersham Pharmacia Biotech. Klenow polymerase was from New England Biolabs, Inc. (Beverly, MA).

Bacterial Strains—SR702 (MC4100, suhX1) is a derivative of MC4100 (araD139 dargF-locI168purL150 thiA1 relA1 fliB5301 deoC1 ptsF25 rbsR) that expresses a high level of GroEL because of an IS1 element inserted upstream of the groE gene. It was constructed from KY1603 (15) by P1 transduction. The chaperonin GroEL protects RNA from nucleases (16) so that the unstable mRNA from the P0 promoter (17) is stabilized in suhX1 strain background because of a high level of GroEL. SR703 (SR702, mlc) was constructed from CP1036 by P1 transduction. Glutamine at residue 369 of Mlc is replaced by a stop codon (UAG) in CP1036 (18).

Primer Extension—Cells were grown in tryptone broth (1% Bacto tryptone, 0.8% NaCl) with or without 0.2% glucose. Total Escherichia
coli RNA was purified using Trizol reagent (Life Technologies, Inc.). RNA was resuspended in sterile distilled water. Purified γ-32P-end labeled primer P1 (5'-GCCAGTTTTTAAACAGGCAGCGACCGAAG-3') (7) was coprecipitated with 30 μg of total cell RNA, and the pellet was resuspended in 20 μl of 250 mM KCl, 2 mM Tris-HCl, pH 7.9, and 0.2 mM EDTA. Primer extension reactions were done as described by Ryu and Garges (2).

Construction of Expression Vector pNS100 and Overexpression of Recombinant Mlc—The DNA sequence from base 194 to 1490 of the E. coli mlc gene (GenBank accession code D32222) was amplified by polymerase chain reaction. The forward polymerase chain reaction primer (5'-GGGAAAATTATAGGGAGTATCATGGTGC-3') contained an engineered NdeI site (underlined) containing the ATG start codon (boldface, the original GTG was changed into ATG for better expression). The reverse polymerase chain reaction primer (5'-CTTTCTCTGCCCCAAATTTGGAATCCCGGAAA-3') contained an engineered BamHI site (underlined) located 33 base pairs downstream from the TAA stop codon. The polymerase chain reaction product was cloned into the NdeI and BamHI cloning sites of the vector pRE1 (19), and the sequence of the recombinant plasmid, pNS100, was verified by a cycle sequencing kit. The pNS100 was electroporated with an E. coli pulser (Bio-Rad) into E. coli strain GI698. In plasmid pNS100, the mlc gene is under the control of the strong AP, promoter-cI ribosome binding site combination. The lacI repressor gene is under the control of the trp promoter in GI698 (20). E. coli GI698 transformed with plasmid pNS100 was cultured at 30 °C in 200 ml of synthetic medium (20) supplemented with 0.1 mg/ml ampicillin. When the culture reached an A600 = 0.5, tryptophan (0.1 mg/ml) was added to induce the expression of Mlc. The cells harvested 20 h after induction were washed once with 20 mM Tris-HCl, pH 7.5, containing 50 mM NaCl and stored at −20 °C.

RESULTS

Effect of Mlc on pts Expression in E. coli

Effect of glucose on transcription from the P0 promoter in the wild type (SR702) and mlc-negative strain (SR703). Total RNA was isolated from cells grown in tryptone broth with or without glucose. Primer extension analysis of 30 μg of total RNA/reaction was done as described under “Experimental Procedures.” WT, wild type.

mlc Is a Repressor of the pts P0 Promoter—We have suggested the presence of a repressor that is inducible by glucose in the pts P0 promoter (2). The presence of a sequence that has a high similarity with the known Mlc binding site (9, 10) at 30 with respect to the transcription start site of the P0 promoter is shown. The −10 and −35 region of the P0 promoter and the CRP binding site are underlined. The Mlc binding sites are boxed. The sequence is identical to the consensus Mlc binding sequence proposed by Kimata et al. (9) with the exception of one nucleotide. The DNA sequence from base 194 to 1490 of the E. coli mlc gene (GenBank accession code D32222) was amplified by polymerase chain reaction. The forward polymerase chain reaction primer (5'-GGGAAAATTATAGGGAGTATCATGGTGC-3') contained an engineered NdeI site (underlined) containing the ATG start codon (boldface, the original GTG was changed into ATG for better expression). The reverse polymerase chain reaction primer (5'-CTTTCTCTGCCCCAAATTTGGAATCCCGGAAA-3') contained an engineered BamHI site (underlined) located 33 base pairs downstream from the TAA stop codon. The polymerase chain reaction product was cloned into the NdeI and BamHI cloning sites of the vector pRE1 (19), and the sequence of the recombinant plasmid, pNS100, was verified by a cycle sequencing kit. The pNS100 was electroporated with an E. coli pulser (Bio-Rad) into E. coli strain GI698. In plasmid pNS100, the mlc gene is under the control of the strong AP, promoter-cI ribosome binding site combination. The lacI repressor gene is under the control of the trp promoter in GI698 (20). E. coli GI698 transformed with plasmid pNS100 was cultured at 30 °C in 200 ml of synthetic medium (20) supplemented with 0.1 mg/ml ampicillin. When the culture reached an A600 = 0.5, tryptophan (0.1 mg/ml) was added to induce the expression of Mlc. The cells harvested 20 h after induction were washed once with 20 mM Tris-HCl, pH 7.5, containing 50 mM NaCl and stored at −20 °C.

Mlc protein was purified to characterize its effects on the pts expression in E. coli. Mlc was monitored by SDS-polyacrylamide gel electrophoresis, and the system (Bio-Rad).

The amounts of transcripts were measured using a Molecular Imager and terminated after 10 min by the addition of 25 μl of formamide loading buffer (80% formamide, 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol). RNA was resolved on an 8% polyacrylamide gel.

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the system, the protein was found in the insoluble fraction of the lysate (lanes 3 and 4 of Fig. 3). Various methods as suggested by Rudolph and Lilie (21) were tried to increase the solubility of Mlc in the cell extract, and we found that Mlc was soluble in glycine-NaOH buffer, pH 9.5. Mlc was purified using a combination of anion-exchange and gel filtration chromatography. The overall purification was 3.8-fold from the crude extract to the final preparation (Table I). From 100 ml of culture (about 350 mg of wet weight cell), a yield of 5.5 mg of purified Mlc, which is 44 kDa, was obtainable with about 6

Effect of Mlc on the pts P0 Transcription in Vitro—The effect of Mlc on the P0 transcription was studied further by an in vitro transcription assay using purified proteins. The supercoiled pHX DNA containing both P0 and P1 promoters was used. A 185-base transcript from the P0 and 85- and 78-base transcripts from the P1a and P1b promoters are shown as P0, P1a, and P1b, respectively. The transcripts from plasmid origin of replication (106/107 bases) are shown as

FIG. 3. Expression, solubilization and purification of recombinant Mlc from E. coli. The recombinant Mlc was overexpressed in E. coli GI698 containing pNS100. Aliquots (20 μl) of bacterial cell lysates or purified Mlc were subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. Lane M, prestained SDS-polyacrylamide gel electrophoresis standards, low range (Bio-Rad); lane 1, crude cell extract before induction; lane 2, crude cell extract 20 h after induction of Mlc; lanes 3–5, supernatants after centrifugation at 100,000 × g of sonicated culture following suspension of cells in buffers 10 mM Na-acetate buffer with 50 mM NaCl (pH 4.5), 10 mM potassium phosphate buffer with 50 mM NaCl (pH 7.0), and 10 mM glycine-NaOH buffer with 50 mM NaCl (pH 9.5) containing 1 mM DTT, respectively; lane 6, 1 μg of purified Mlc, which is 44 kDa.

FIG. 4. Effects of Mlc on the activity of the pts promoter in vitro. The supercoiled plasmid pHX, which has both P0 and P1 promoters, was used. A, Mlc specifically inhibited the P0 promoter activity. A 185-base transcript from the P0 and 85- and 78-base transcripts from the P1a and P1b promoters are shown as P0, P1a, and P1b, respectively. The transcripts from plasmid origin of replication (106/107 bases) are shown as rep. B, effect of Mlc on the P0 promoter activity in the presence (closed circle) and absence (open circle) of 40 nm of CRP-cAMP. 0, 6.3, 12.5, 25, 50, 100, and 200 ng of Mlc were tested in each experiment.

Effect of Mlc on pts Expression in E. coli

Phenotype of E. coli Overexpressing Mlc under Various Conditions

was not significant, and Mlc is a stronger regulator.

Binding of Mlc to the pts P0 Promoter Region—We tested the binding of Mlc on the P0 promoter region to determine the mechanism of Mlc action. Binding of Mlc, CRP-cAMP, and RNA polymerase on the P0 promoter region was studied using a DNase I footprinting experiment. Fig. 5 showed that Mlc bound to the P0 promoter region, and the binding of both Mlc and CRP-cAMP to the P0 promoter region was independent of each other (compare lanes 2, 3, and 7). DNase I footprinting results also showed that the binding of RNA polymerase to the P0 promoter region was inhibited in the presence of Mlc (compare lanes 2, 4, and 5 in Fig. 5).

Phenotype of E. coli Overexpressing Mlc under Various Conditions

Unaltered phenotype

- Growth in rich media
- Normal motility
- Normal flagella
- Normal colony morphology
- Normal aerobility

Altered phenotype

- Reduced growth in rich media
- Reduced motility
- Reduced flagella
- Shrunken colony morphology
- Reduced aerobility

Table I

| Purification step   | Total protein | Mlc | Purity | Yield | Purification fold |
|--------------------|---------------|-----|--------|-------|-------------------|
| Crude extract      | 55            | 13.7| 25     | 100   | 1.00              |
| 100,000 × g supernatant | 41           | 11.5| 28     | 84    | 1.12              |
| Mono-Q chromatography | 12           | 7.2 | 60     | 53    | 2.4               |
| Superdex 75 chromatography | 5.8      | 5.5 | 95     | 40    | 3.8               |
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Growth Conditions—The phenotype of E. coli overexpressing Mlc was examined on MacConkey agar plates containing various PTS and non-PTS sugar substrates. The overall colony size of GL698 harboring pNS100 (containing the mlc gene) was larger than the strain harboring a control vector, pRE1, regardless of the sugar substrates, and colonies on plates containing readily fermentable sugars showed a reddish purple color 6 h after spotting at 37 °C. When non-PTS sugars such as lactose, maltose, melibiose, and sucrose were used as substrate, there were no remarkable differences between GL698 harboring pNS100 and the strain containing pRE1 except for maltose (Table II). When PTS sugars were included in MacConkey plates, however, Mlc overexpression seriously retarded the fermentation rate of those PTS sugars regardless of the sugar substrates (Table II). Repressing activity of Mlc and derepression of Mlc activity by readily fermentable sugar substrates have been shown for several sugar-specific transporter genes and the pts operon (8–10). According to these observations, the wild type cells grown in the presence of these sugar substrates should show the similar fermentation rate compared with the mlc-negative cells. When we compared fermentation patterns of the mlc mutant and the wild type cells to the GI698 strain harboring pRE1 or pNS100, the mlc-negative mutant showed no remarkable difference in fermentation patterns with the wild type cells or the GI698 strain harboring pRE1, as expected. These results imply that Mlc, which mainly affects utilization of PTS sugars, is a global regulator of carbohydrate metabolism.

DISCUSSION

Because of the multiple roles exerted by the gene products of the pts operon (22, 23), the intricate regulation of expression of the operon is crucial for the survival of the organism. The expression level of pts gene products in E. coli was reported to change 2- to 3-fold in response to the environmental changes such as the availability of the sugar substrates and oxygen (1). It has been known that the pts P0 promoter is activated when cells are grown in the presence of glucose. Examination of the pts P0 promoter region revealed a site that has a high similarity to the sequence of the known Mlc binding sites. The mlc encodes a 44-kDa protein, Mlc, that has been shown to regulate the expression of ptsG encoding IICB(3-5), manXYZ encoding enzyme II of the mannose PTS, malT encoding the activator of maltose regulon, and mlc itself (8–10). Mlc is proposed to be a regulatory gene product of carbohydrate metabolism (8, 9).

Comparison of transcription from the pts P0 promoter in wild type and mlc-negative mutant suggests that Mlc is a repressor of the P0 promoter. The P0 promoter was activated in the wild type, whereas it was repressed in a mlc-negative mutant when cells were grown in the presence of glucose (Fig. 2). The probable reason for the reduction of the activity of the P0 promoter when the mlc-negative strain grows in the presence of glucose is that the concentration of an activator of the P0 promoter, CRP-cAMP, was lowered in that condition (1, 6). These results also indicated that the action of Mlc in the regulation of the pts operon is dominant over that of CRP-cAMP as proposed for the regulation of ptsG expression by Mlc recently (9).

It has been suggested that the concentration of Mlc in E. coli is limiting (9). Expression of the gene is autoregulated (8), and the translation efficiency of the mlc is low because the translation initiation codon of the mlc is GTG (12). We cloned the mlc under the control of Ap and changed the GTG to ATG in our expression vector pNS100 for better expression of the gene. We could get more than 95% pure Mlc using a combination of ion-exchange and gel filtration chromatography. This is the first report describing the purification of Mlc. In vitro transcription assays with purified Mlc clearly showed that Mlc specifically inhibited transcription from the P0 promoter but had no effect on the P1 promoter (Fig. 4A). CRP-cAMP reduced the action of Mlc a little but could not prevent Mlc from inhibiting the P0 promoter. It is, however, possible that the modest influence of CRP-cAMP on Mlc action revealed by in vitro transcription assay can be significant in vivo because Mlc concentration is limiting in E. coli (9). In vitro transcription assay results together with the in vivo Mlc effects on the pts P0 promoter activity strongly demonstrated that Mlc was a repressor of the pts P0 promoter that can be induced by glucose. We identified one Mlc binding site centered at +13 of the promoter. The sequence has high homology with the tentative consensus sequence of the Mlc binding site as proposed by Kimata et al.
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The binding sites of CRP-cAMP and Mlc do not overlap and their binding to the P0 promoter region was independent of each other as expected from the previous finding that the glucose- and cAMP-mediated activations of the pts promoter were independent of each other (2). Mlc inhibited the P0 promoter activity by interfering with the binding of RNA polymerase to the promoter (Fig. 5).

The common feature of five operons (manXYZ, malT, mlc, ptsG, and pts) identified as the Mlc regulon so far is that all of them have at least one CRP binding site as well as a Mlc binding site, and thus they are under the dual regulation. This seems to be necessary for the fine control of expression of these genes to respond to various environmental conditions. It is interesting that E. coli overexpressing Mlc showed the same phenotype (the cells having a very slow fermentation rate) on N-acetylglucosamine and mannitol indicator plates as on glucose and mannose plates (Table II). The nagE gene of N-acetylglucosamine PTS and the mtlA gene of mannitol PTS correspond to the ptsG gene of glucose PTS and the manXYZ gene of mannose PTS in that all of them encode the major sugar-specific transporters, enzyme II. It is not known if Mlc is involved in the regulation of the nagE and mtlA genes, even though positive regulation of nagE by the CRP-cAMP complex was reported recently (24). In any case, we can expect that Mlc regulates utilization of many PTS sugars because it modulates the expression of the pts that encodes general proteins for all PTSs, enzyme I, and HPr. To serve as a common regulator of metabolism of many sugars, Mlc should be induced by different kinds of sugar. The inducer of Mlc has not been identified despite repeated attempts to identify the inducer that affects the Mlc binding to its binding site by many researchers (8, 9).

We examined the possibility that the inducer relieves repression by Mlc without affecting the binding of Mlc to its binding site using in vitro transcription assay. However, none of the sugars or their derivatives tested (glucose, glucose-6-P, maltose, mannitol, mannose, xylose, melibiose, pyruvate, N-acetylglucosamine, methyl β-D-thiogalactoside) showed any effect on the repression by Mlc. These results suggest the possibility that the inducer of Mlc may not be the sugar or its derivative. It has been known that glucose-mediated activation of the pts operon is dependent on the enzyme IICB<sup>Glc</sup>, which may act as a sensor protein (25). In this view, Mlc can act as a response regulator whose activity is modulated by enzyme IICB<sup>Glc</sup>. However, the possibility that Mlc is phosphorylated by enzyme IICB<sup>Glc</sup> is low because we could not get any evidence of phosphorylation of Mlc, even though we tried several different approaches employing [<sup>32</sup>P]phosphoenolpyruvate or [γ-<sup>32</sup>P]ATP mixed with purified Mlc in the presence and absence of E. coli cell-free extracts to phosphorylate Mlc (data not shown). These may imply that the activity of Mlc is modulated by interaction with an unknown factor probably through protein-protein interaction as in the case of anti-σ factors (26, 27). Glucose may affect the interaction by changing the phosphorylation state of enzyme IICB<sup>Glc</sup>, and other sugars may also affect the interaction through their own enzyme II. The unknown factor could be any one of PTS proteins or an unknown novel factor yet to be identified.

We are testing this possibility, and further work is needed to discover the inducer of Mlc would be.

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