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A novel regulatory role of glucose transporter of *Escherichia coli*: membrane sequestration of a global repressor Mlc

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External glucose stimulates transcription of several genes including *ptsG* encoding IICB^{Glc}, a membrane component of the phosphotransferase system (PTS), by relieving the negative regulation of a global repressor Mlc in *Escherichia coli*. We investigate here how glucose modulates Mlc action. The Mlc-mediated repression is eliminated by a *ptsI* mutation, while Mlc is constitutively active in a *ptsG* mutant. We show that IICB^{Glc}-FLAG interacts physically with Mlc in crude extracts prepared from cells in which IICB^{Glc} is supposed to exist as the non-phosphorylated form. The IICB^{Glc}–Mlc interaction is no longer observed when IICB^{Glc} is phosphorylated. Exogenously added purified Mlc binds to purified IICB^{Glc}-FLAG. We also demonstrate that Mlc is associated with membrane when IICB^{Glc} is dephosphorylated while it is in the cytoplasm when IICB^{Glc} is phosphorylated or absent. We conclude that IICB^{Glc} regulates the cellular localization of Mlc, depending on its phosphorylation state, which is determined by the availability of external glucose. Thus, glucose induces the transcription of Mlc-regulated promoters by sequestering Mlc to the membrane through dephosphorylation of IICB^{Glc}.

Keywords: glucose transporter/membrane sequestration/Mlc/PTS/spatial regulation

Introduction

How *Escherichia coli* cells respond to external glucose is a classical paradigm in bacterial signal transduction and global gene regulation. Early studies on the glucose effect led to important concepts on gene regulation. Subsequent studies have revealed that the phosphoenolpyruvate (PEP)-dependent carbohydrate phosphotransferase system (PTS) is primarily responsible for various effects of glucose on cellular activities (reviewed by Meadow *et al*., 1990; Postma *et al*., 1996; Saier *et al*., 1996). The PTS consists of two common cytoplasmic proteins, enzyme I and HPr, and an array of sugar-specific enzyme II complexes (EIIs). The phosphoryl group from PEP is sequentially transferred to enzyme I, to HPr, to EIIs and finally to the substrate as it is translocated across the membrane. The glucose-specific EII (glucose transporter) consists of cytoplasmic protein IIA^{Glc} and membrane receptor IICB^{Glc}.

Recent genetic and biochemical studies have revealed several new aspects of glucose signaling in *E.coli*. For example, we found that the inhibitory effect of glucose on the utilization of lactose is due primarily to the inhibition of lactose uptake (inducer exclusion) by non-phosphorylated IIA^{Glc} and therefore the well-known cAMP signaling model is no longer true (Inada *et al*., 1996). In addition, one of the real roles of cAMP in the preferential utilization of glucose over lactose was shown to support inducer exclusion by activating the expression of the *ptsG* gene (Kimata *et al*., 1997).

Another new aspect of cellular response to glucose is the induction of gene expression by glucose. It is now established that glucose stimulates transcription from certain promoters. For example, the expression of the *pts* operon, composed of *ptsH*, *ptsI* and *crr* encoding HPr, enzyme I and IIA^{Glc}, respectively, is known to be stimulated by external glucose, and one of the two major *ptsH* promoters, *ptsH* P0, is subject to this glucose induction (De Reuse and Danchin, 1988, 1991; De Reuse *et al*., 1992; Fox *et al*., 1992; Ryu and Garges, 1994). A more dramatic inducible effect of glucose was observed with the *ptsG* in which a major promoter is the target of glucose induction (Kimata *et al*., 1998; Plumbridge, 1998b). An important finding concerning the mechanism of glucose induction was that the *ptsG* and *ptsH* P0 promoters are under the negative control of a global repressor, Mlc, and that mutations in the *mlc* gene lead to constitutive expression of the *ptsG* and *ptsH* promoters (Kimata *et al*., 1998; Plumbridge, 1998b, 1999; Kim *et al*., 1999; Tanaka *et al*., 1999). Mlc also regulates the expression of *manXYZ*, encoding three proteins of the mannose PTS, *malT*, encoding the activator of maltose regulon, and *mlc* itself (Decker *et al*., 1998; Plumbridge, 1998a).

While glucose induces the expression of target genes apparently by relieving Mlc-mediated repression, how glucose modulates Mlc action was not known. Several lines of genetic evidence suggested that PTS proteins are involved in the signal transduction pathway from glucose to Mlc. First, the *ΔptsHI crr*, *ΔptsI crr* or *ptsI* mutation causes constitutive expression of both *ptsG* and *ptsH* (De Reuse and Danchin, 1991; Plumbridge, 1999). Secondly, the constitutive expression of *ptsG* and *ptsH* no longer occurs when the *ptsG* gene has been further disrupted (Plumbridge, 1999). Thirdly, mutation in *mlc* leads to constitutive expression of *ptsG* and *ptsH* in all strains tested (Plumbridge, 1999). Fourthly, in addition to glucose several other PTS sugars could also induce the expression of *ptsG* and *ptsH* to various extents (De Reuse and Danchin, 1991; Plumbridge, 1999). However, the actual roles of PTS proteins in glucose induction and/or in modulation of Mlc action remain to be studied.

An attractive model for the mechanism of Mlc regulation by glucose has been proposed in which IICB^{Glc} interacts physically with Mlc depending on its

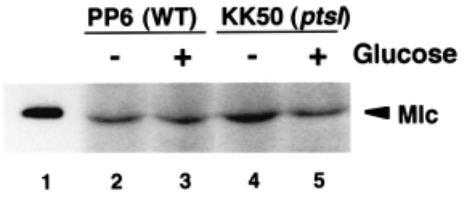

Fig. 1. Effect of glucose on the expression of Mlc protein. Crude extracts equivalent to an OD_{600} of 0.1, prepared from *E.coli* PP6 (lanes 2 and 3) or KK50 (lanes 4 and 5) grown in LB medium with and without 1% glucose, were analyzed by western blotting using anti-Mlc antibody as described in Materials and methods. Lane 1 corresponds to 5 ng of purified Mlc.

phosphorylation state to sequester Mlc away from the DNA (Plumbridge, 1999). Here, we present evidence that $IICB^{Glc}$ interacts physically with Mlc when $IICB^{Glc}$ exists as non-phosphorylated form. More importantly, we demonstrate that the cellular localization of Mlc varies depending on the phosphorylation state of $IICB^{Glc}$. Our data clearly indicate that the Mlc protein shuttles between the cytoplasm, where it binds operators to repress the transcription of target promoters, and the membrane, where it presumably fails to access DNA. Thus, glucose induces the transcription of Mlc-regulated promoters by sequestering Mlc to the membrane through dephosphorylation of $IICB^{Glc}$. This is the first instance in which a membrane component of PTS is involved in spatial regulation of a transcription factor via protein–protein interaction depending on its phosphorylation state.

Results

Effect of external glucose on Mlc expression

There are several possible mechanisms by which glucose induces transcription from Mlc-repressible promoters. First, glucose could stimulate the target promoters by reducing the Mlc level. In fact, the *mlc* gene appears to be under the positive control of CRP–cAMP (Decker *et al*., 1998). This raises a possibility that glucose downregulates Mlc expression through the catabolite repression mechanism although the situation is complicated because *mlc* is also negatively regulated by Mlc itself (Decker *et al*., 1998). Thus, glucose could affect Mlc expression in principle simultaneously through two control circuits, positive regulation mediated by CRP–cAMP and negative regulation mediated by Mlc as in other Mlc-repressible operons. The net effect of glucose on Mlc expression must be tested experimentally. Thus, we analyzed the effect of external glucose on Mlc expression in wild-type cells (PP6) by western blotting using anti-Mlc antibody. The Mlc level in cells grown in the presence of glucose was essentially equivalent to that in cells grown in the absence of glucose (Figure 1, lanes 2 and 3). Thus, external glucose does not significantly affect the Mlc level no matter how two control circuits are involved in *mlc* regulation. We conclude that fluctuation in Mlc level can not be responsible for the inducible effect of glucose on target promoters.

Effects of pts mutations on Mlc action

Secondly, glucose and/or its metabolites could act as an allosteric effector to downregulate Mlc activity. However,

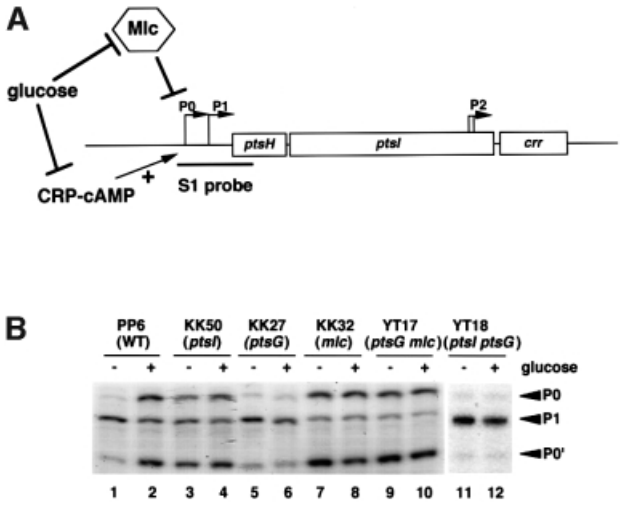

Fig. 2. (**A**) Schematic representation of the *pts* operon and its regulation. The open bars represent the coding region for *ptsH*, *ptsI* and *crr*. P0 and P1 are two major promoters. Transcription from P0 is regulated both negatively and positively, by Mlc and CRP–cAMP, respectively. Glucose affects P0 transcription through two regulatory pathways. The DNA fragment used for S1 nuclease assay is shown below the map. (**B**) Effect of *pts* mutations on *pts* mRNA expression. The indicated strains were grown in LB medium with and without 1% glucose. Total RNAs (30 μg) were subjected to S1 analysis. P0 and P1 transcripts along with the presumed processed RNA (P0′) are shown by arrowheads.

this model also appears to be less likely because glucose and its related compounds had no effect on Mlc action *in vitro*, and attempts to identify a direct inducer for Mlc was unsuccessful (Decker *et al*., 1998; Kimata *et al*., 1998; Plumbridge, 1998b, 1999). Instead, previous studies have suggested that PTS proteins are involved in the modulation of Mlc action (De Reuse and Danchin, 1991; Plumbridge, 1999).

We examined the effects of *pts* mutations on *ptsH* mRNA expression by S1 nuclease analysis. We found that the *ptsH* P0 promoter, which is both negatively and positively regulated, by Mlc and CRP–cAMP, respectively (Kim *et al*., 1999; Plumbridge, 1999; Tanaka *et al*., 1999), was constitutively expressed in *ptsI* cells (KK50) regardless of the availability of glucose (Figure 2B, lanes 3 and 4) as in the case of *mlc* cells (KK32) (Figure 2B, lanes 7 and 8). The P0 mRNA level was slightly lower in the *ptsI* cells than in the *mlc* cells. This is presumably due to the reduced cAMP level caused by the *ptsI* mutation (Takahashi *et al*., 1998). In contrast, the *ptsH* P0 promoter was little expressed in *ptsG* cells (KK27) (Figure 2B, lanes 5 and 6). These results are consistent with earlier gene fusion studies (De Reuse and Danchin, 1991; Plumbridge, 1999) and strongly suggest that PTS proteins affect the Mlc-dependent negative regulatory pathway.

How does the *ptsI* mutation cause the constitutive expression of *ptsH* P0? This could happen again if the Mlc level were reduced by the *ptsI* mutation. We found, however, that this is not the case because the *ptsI* mutation caused a slight (in the absence of glucose, Figure 1, lane 4) or no (in the presence of glucose, Figure 1, lane 5) increase in the Mlc level. This implies that the derepression of P0 promoter in the *ptsI* mutant is not due to a reduction in Mlc level. Thus, the *ptsI* mutation may inhibit the repressor activity of Mlc by some unknown mechanism(s).

Implication of IICB^{Glc} in glucose–Mlc signaling

The membrane component of glucose transporter IICB^{Glc} is obviously important for glucose signaling because it acts as a sensor for external glucose. The IICB^{Glc} catalyzes PEP-dependent phosphorylation of glucose in the presence of EI, HPr and IIA^{Glc}. It is known that IICB^{Glc} is phosphorylated as a catalytically important intermediate at Cys421 during phosphotransfer reactions (Meins *et al*., 1993). IICB^{Glc} would exist as the non-phosphorylated form in *ptsI* cells because the phosphorylation chain from PEP via EI, HPr, and IIA^{Glc} to IICB^{Glc} is not functional. In wild-type cells, it is expected that IICB^{Glc} is predominantly phosphorylated in the absence of external glucose and dephosphorylated in the presence of glucose. In addition, disruption of the *ptsG* gene led to repression of the *ptsH* P0 promoter in the *ptsI* strain (Figure 2B, lanes 11 and 12) but the P0 promoter was constitutively expressed in a *mlc ptsG* double mutant (Figure 2B, lanes 9 and 10). Taken together, Mlc is apparently active to repress transcription of target promoters when IICB^{Glc} is phosphorylated or when IICB^{Glc} is absent, while it is inactive when IICB^{Glc} is dephosphorylated.

How does IICB^{Glc} modulate Mlc action? It had been proposed that IICB^{Glc} may act as a sensor protein of a two-component system in the glucose-mediated regulation of the *ptsHI* operon because the C-terminus of IICB^{Glc} exhibits a sequence similarity to the transmitter consensus motif of sensor proteins (De Reuse and Danchin, 1991). This raises a possibility that the activity of Mlc could be modulated by IICB^{Glc}-dependent phosphorylation. However, Mlc shows no homology to any known regulators of the two-component system (Plumbridge, 1999). In fact, there is no evidence so far that Mlc is phosphorylated (Kim *et al*., 1999).

Expression and properties of IICB^{Glc}-FLAG

Another possibility regarding the role of IICB^{Glc} in Mlc regulation is that IICB^{Glc} interacts physically with Mlc depending on its phosphorylation state, thus sequestering Mlc away from the DNA (Plumbridge, 1999). To test this hypothesis, we constructed a low-copy plasmid pYT100 in which *ptsG* is under the control of the *bla* promoter. Then, the *ptsG* gene was manipulated to construct a variant *ptsG^{FL}* gene encoding IICB^{Glc}-FLAG fusion protein, in which the FLAG peptide was attached to the C-terminus of IICB^{Glc} (Figure 3A). The resulting plasmid containing the *ptsG^{FL}* gene was designated pYT101. As expected, IICB^{Glc} and IICB^{Glc}-FLAG were expressed constitutively in cells harboring pYT100 or pYT101 (Figure 3B, lanes 5–8), while the expression of IICB^{Glc} from the chromosomal *ptsG* gene was stimulated by external glucose (Figure 3B, lanes 1 and 2). The expression level of IICB^{Glc}-FLAG was significantly lower than that of IICB^{Glc} (Figure 3B, lanes 5–8). This is presumably due to a reduced stability of mRNA caused by the lack of the Rho-independent terminator of *ptsG* in pYT101 (see Figure 3A).

Before using IICB^{Glc}-FLAG as a tool to study the possible IICB^{Glc}–Mlc interaction, we examined whether the IICB^{Glc}-FLAG fusion protein functions in cells. Glucose fails to inhibit *lac* expression in the presence of lactose in cells lacking the chromosomal *ptsG* gene (Figure 3C, lane 2) because glucose-specific PTS is not functional and IIA^{Glc} remains phosphorylated. The

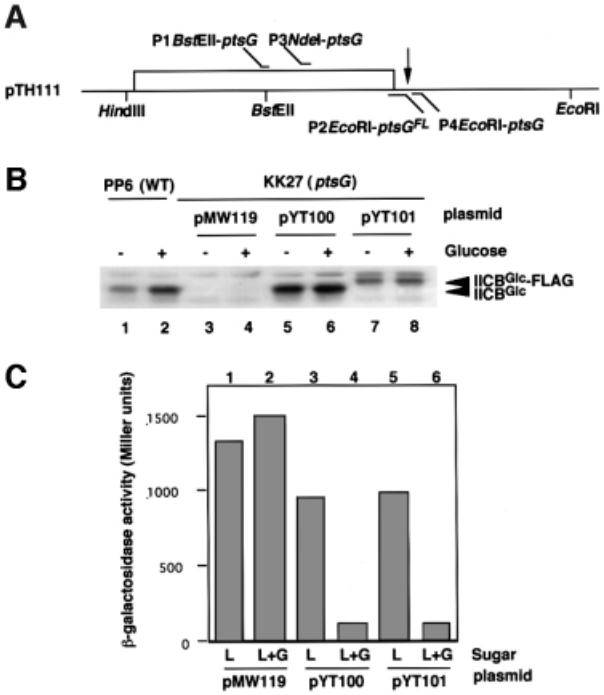

Fig. 3. Expression and properties of IICB^{Glc}-FLAG. (**A**) The *ptsG* gene on pTH111. The open bar represents the coding region for *ptsG*. The vertical arrow indicates the position of the Rho-independent terminator of *ptsG*. The locations of primers used for PCR amplification are shown. (**B**) Expression of IICB^{Glc} and IICB^{Glc}-FLAG. Crude extracts equivalent to an OD_{600} of 0.01, prepared from PP6 (lanes 1 and 2), KK27 harboring pMW119 (lanes 3 and 4), KK27 harboring pYT100 (lanes 5 and 6), KK27 harboring pYT101 (lanes 7 and 8) grown in LB medium with and without 1% glucose, were analyzed by western blotting using anti-IIB^{Glc} antibody. Both IICB^{Glc} and IICB^{Glc}-FLAG are shown by arrowheads. (**C**) Effect of IICB^{Glc}-FLAG on the expression of β-galactosidase. KK27 harboring pMW119 (lanes 1 and 2), pYT100 (lanes 3 and 4) and pYT101 (lanes 5 and 6) were grown in LB medium with 1% lactose (L) or with 1% lactose + 1% glucose (G). Culture samples were taken at OD_{600} of 0.6 and β-galactosidase activities were determined.

introduction of pYT100 or pYT101 into *ptsG* cells restored the inducer exclusion, resulting in a large decrease in the level of β-galactosidase activity (Figure 3C, lanes 4 and 6). Thus, IICB^{Glc}-FLAG fusion protein is functional with respect to the transport and phosphorylation of glucose, resulting in dephosphorylation of IIA^{Glc}. In fact, the direct assay for the phosphorylation state of IIA^{Glc} revealed that the IICB^{Glc}-FLAG fusion protein could cause dephosphorylation of IIA^{Glc} in the presence of glucose (data not shown).

Evidence for a physical interaction between Mlc and IICB^{Glc}

To examine a possible interaction between IICB^{Glc} and Mlc, crude extracts were prepared from the *ptsI* cells harboring pYT100, pYT101 or pYT102 expressing IICB^{Glc}, IICB^{Glc}-FLAG or CRP-FLAG, respectively. IICB^{Glc} or IICB^{Glc}-FLAG is expected to exist as a non-phosphorylated form in *ptsI* cells because the PEP-dependent phosphorylation pathway is blocked. The crude extracts, which also contain Mlc expressed from the chromosomal *mlc* gene, were incubated with anti-FLAG M2–agarose affinity gel, washed extensively with a buffer, and then proteins bound to the beads were eluted

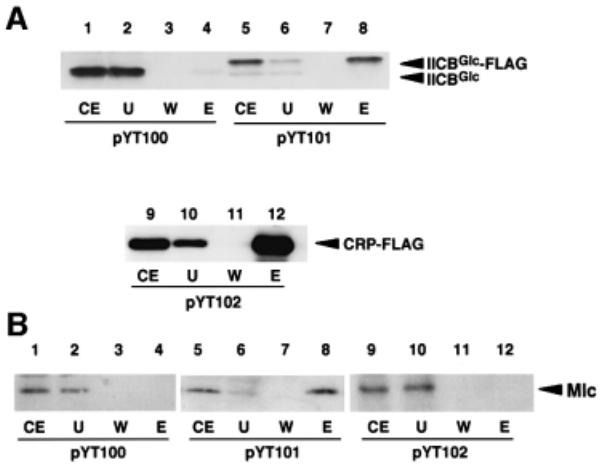

Fig. 4. Physical interaction between endogenous Mlc and IICB^{Glc}. Assay for interaction between endogenous Mlc and IICB^{Glc} was performed as described in Materials and methods. Cell extracts of YT18 harboring pYT100 (lanes 1–4), pYT101 (lanes 5–8) or pYT102 (lanes 9–12) were incubated with anti-FLAG M2–agarose, washed extensively with a buffer, and proteins bound to the beads were eluted with SDS sample buffer. The fractions were subjected to immunoblot analysis using anti-IIB^{Glc} (**A**, lanes 1–8), anti-CRP (A, lanes 9–12) or anti-Mlc (**B**) antibodies. The protein samples used are: crude extracts (CE), 2 µl; unbound fraction (U), 10 µl, wash fraction 4 (W) 10 µl; elution fraction (E), 20 µl.

with SDS sample buffer. The protein samples were first analyzed by western blotting using anti-IIB^{Glc} antibody. As expected, a significant amount of IICB^{Glc}-FLAG bound to anti-FLAG M2–agarose was eluted from the beads when extracts contained IICB^{Glc}-FLAG (Figure 4A, lane 8) while no IICB^{Glc} was detected in the eluate when extracts containing normal IICB^{Glc} were used (Figure 4A, lane 4). A control CRP-FLAG fusion protein also effectively bound to the anti-FLAG M2–agarose and it was detected by anti-CRP antibody (Figure 4A, lane 12). We next analyzed the protein samples by western blotting using anti-Mlc antibody. Mlc was clearly detected in the eluate from the anti-FLAG M2–agarose when IICB^{Glc}-FLAG was co-expressed (Figure 4B, lane 8). No detectable Mlc was found in the eluate when IICB^{Glc} was co-expressed with Mlc (Figure 4B, lane 4). In addition, Mlc was not associated with a control protein CRP-FLAG (Figure 4B, lane 12), indicating that FLAG peptide itself has no ability to interact with Mlc. These results suggest strongly that IICB^{Glc}-FLAG could interact with endogenous Mlc either directly or indirectly at least in the *ptsI* cells.

Purified Mlc binds to affinity-purified IICB^{Glc}-FLAG

In order to examine whether exogenously added purified Mlc could bind to purified IICB^{Glc}, we attempted to purify IICB^{Glc}-FLAG with anti-FLAG M2–agarose from crude extracts prepared from the *ptsI mlc* cells (YT19) harboring pYT103 in which IICB^{Glc}-FLAG is expressed ~10-fold more compared with cells harboring pYT101 (Figure 5A, lane 3). Crude extracts were also prepared from the *ptsI mlc* cells harboring pTH111 in which IICB^{Glc} is overexpressed (Figure 5A, lane 1). The extracts were incubated with anti-FLAG M2–agarose and the beads were washed extensively with several buffers as described in Materials and methods. The proteins bound to the beads were eluted with

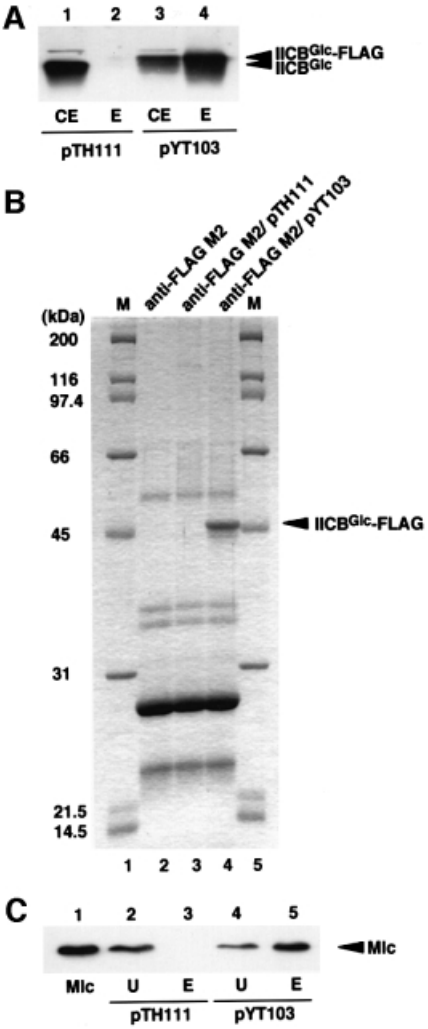

Fig. 5. Purification of IICB^{Glc}-FLAG and binding of exogenously added Mlc to purified IICB^{Glc}-FLAG. IICB^{Glc}-FLAG was overproduced by introducing pYT103 into *ptsI mlc* cells and purified with anti-FLAG M2 affinity gel as described in Materials and methods. (**A**) Immunoblot analysis of IICB^{Glc}-FLAG. Lane 1, 1 µl of crude extracts of cells harboring pTH111; lane 2, 4 µl of eluate of anti-FLAG M2–agarose treated with crude extracts of cells harboring pTH111; lane 3, 1 µl of crude extracts of cells harboring pYT103; lane 4, 4 µl of eluate of anti-FLAG M2–agarose containing purified IICB^{Glc}-FLAG. (**B**) SDS–PAGE analysis of purified IICB^{Glc}-FLAG. Lanes 1 and 5, marker proteins; lane 2, 20 µl of eluate of anti-FLAG M2–agarose alone; lane 3, 20 µl of eluate of anti-FLAG M2–agarose treated with crude extracts of cells harboring pTH111; lane 4, 20 µl of eluate of anti-FLAG M2–agarose containing purified IICB^{Glc}-FLAG. (**C**) Analysis of the interaction between purified Mlc and IICB^{Glc}-FLAG by western blotting. Purified Mlc (20 ng) was incubated with anti-FLAG M2–agarose suspensions (10 µl) with (lanes 4 and 5) or without (lanes 2 and 3) purified IICB^{Glc}-FLAG in 50 µl of TBS buffer, washed extensively with a buffer. Proteins bound to the beads were eluted with 50 µl of SDS sample buffer. Unbound (lanes 2 and 4) and bound (lanes 3 and 5) fractions of 20 µl each were subjected to immunoblot analysis using anti-Mlc antibody. Lane 1 is 10 ng of purified Mlc.

SDS sample buffer and analyzed by SDS–PAGE. Coomassie Blue staining of the gel revealed that a ~50 kDa protein corresponding to IICB^{Glc}-FLAG was eluted as a major band along with several bands when crude extracts containing IICB^{Glc}-FLAG were used (Figure 5B, lane 4). Western blotting showed that the ~50 kDa protein is indeed IICB^{Glc}-FLAG (Figure 5A, lane 4). Other bands are apparently derived from anti-

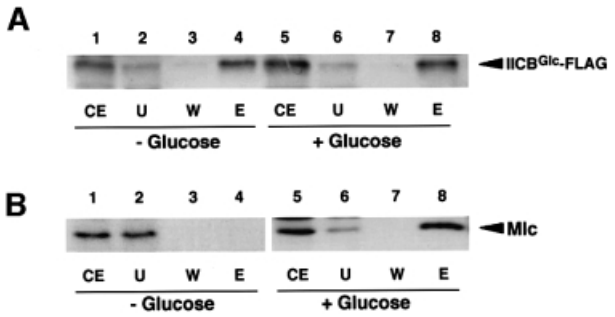

Fig. 6. Effect of glucose on the formation of the Mlc–IICB^{Glc} complex. *Escherichia coli* KK27 harboring pYT101 was grown in LB medium with (lanes 1–4) or without (lanes 5–8) 1% glucose. Cell extracts were prepared and binding experiments were performed as described in Materials and methods. The fractions were subjected to immunoblot analysis using anti-IIB^{Glc} (**A**) or anti-Mlc (**B**) antibodies. The protein samples used are: crude extracts (CE), 2 µl; unbound fraction (U), 10 µl; wash fraction 4 (W) 10 µl; elution fraction (E), 20 µl.

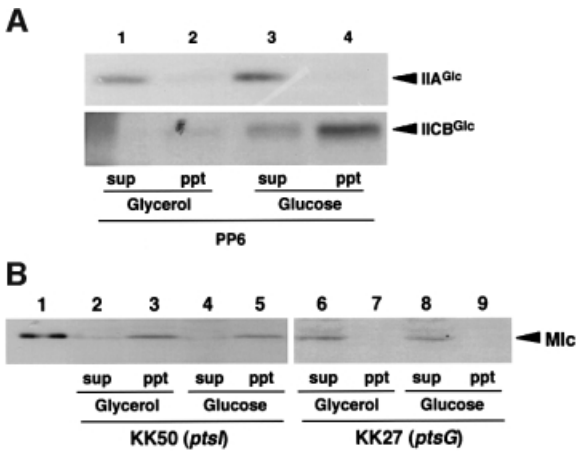

Fig. 7. Analysis of cellular localization of proteins. (**A**) Localization of IIA^{Glc} and IICB^{Glc}. *Escherichia coli* PP6 was grown in MOPS medium with 0.4% glycerol (lanes 1 and 2) or 0.2% glucose (lanes 3 and 4). The cytoplasm and membrane fractions were prepared as described in Materials and methods and 10 µl of each sample were subjected to immunoblot analysis using anti-IIA^{Glc} or anti-IIB^{Glc} antibody. (**B**) Effect of *pts* mutations on the localization of Mlc. KK50 (lanes 2–5) or KK27 (lanes 6–9) was grown in MOPS medium with 0.4% glycerol or 0.2% glucose. The cytoplasm and membrane fractions were prepared and 10 µl of each sample were subjected to immunoblot analysis using anti-Mlc antibody. Lane 1 corresponds to 2 ng of purified Mlc.

FLAG M2–agarose because they were also eluted from the beads alone. When crude extracts containing IICB^{Glc} were used, neither IICB^{Glc} nor other cellular proteins were detected (Figure 5A, lane 2 and B, lane 3). These results clearly indicate that IICB^{Glc}-FLAG has been highly purified.

We then tested whether the affinity-purified immobilized IICB^{Glc}-FLAG could bind to exogenously added Mlc. The immobilized IICB^{Glc}-FLAG was incubated with purified Mlc. The beads were separated by centrifugation and washed with a buffer. The proteins bound to the beads were eluted with SDS sample buffer and analyzed by western blotting using anti-Mlc antibody. The added Mlc was found predominantly in the eluate (Figure 5C, lanes 4 and 5). The Mlc was found in the unbound fraction but not in the eluate when control beads were used (Figure 5C, lanes 2 and 3). These results imply that the purified IICB^{Glc}-FLAG could bind directly to purified Mlc.

Glucose promotes the Mlc–IICB^{Glc} interaction

We next addressed questions of whether IICB^{Glc} could associate with Mlc in cells where the glucose PTS is functional and if so how the phosphorylation state of IICB^{Glc} affects the IICB^{Glc}–Mlc interaction. For this, we introduced pYT101 expressing IICB^{Glc}-FLAG into *ptsG* cells. The crude extracts of cells harboring pYT101 grown both in the presence and absence of glucose were incubated with anti-FLAG M2–agarose beads, washed extensively with a buffer, and then bound proteins were eluted with SDS sample buffer. The protein samples were analyzed by western blotting using both anti-IIB^{Glc} and anti-Mlc antibodies. IICB^{Glc}-FLAG was recovered in the eluate from the anti-FLAG M2–agarose beads regardless of the presence of glucose in the culture medium (Figure 6A, lanes 4 and 8). However, Mlc was detected in the eluate only when crude extracts were prepared from cells grown in the presence of glucose (Figure 6B, lanes 4 and 8). It is expected that IICB^{Glc} is phosphorylated in the absence of external glucose while it is dephosphorylated in the presence of glucose. Thus, it is highly possible that Mlc interacts strongly with IICB^{Glc} when IICB^{Glc} is dephosphorylated.

Localization of Mlc in ptsI or ptsG cells

Does the Mlc–IICB^{Glc} interaction mentioned above influence the cellular localization of Mlc? In order to answer this question, we performed cellular localization assay of proteins. First, the localization of two control proteins, cytoplasmic protein IIA^{Glc} and transmembrane protein IICB^{Glc} was examined with wild-type cells grown on either glucose or glycerol by western blotting using anti-IIA^{Glc} and anti-IIB^{Glc} antibodies. As expected, IIA^{Glc} appeared exclusively in the soluble cytoplasm fraction regardless of the carbon source in the medium (Figure 7A, upper panel). On the other hand, IICB^{Glc} appeared predominantly in the membrane fraction although a little IICB^{Glc} was detected in the glycerol medium (Figure 7A, lower panel). If the interaction between IICB^{Glc} and Mlc sequesters Mlc to the membrane, one may expect that Mlc protein is located in the membrane fraction in *ptsI* cells because IICB^{Glc} is dephosphorylated. In contrast, Mlc is expected to be present in the cytoplasm in the *ptsG* cells lacking IICB^{Glc}. We found that this is exactly the case. Mlc appeared predominantly in the membrane fraction in *ptsI* cells (Figure 7B, lanes 2–5) while it was found in the cytoplasmic soluble fraction in *ptsG* cells (Figure 7B, lanes 6–9). These results suggest strongly that Mlc is sequestered at the membrane via the physical interaction with the non-phosphorylated form of IICB^{Glc}.

Glucose promotes the membrane association of Mlc

We already showed that external glucose promotes the physical interaction between IICB^{Glc} and Mlc presumably by facilitating dephosphorylation of IICB^{Glc} (Figure 6B). To examine if the enhanced interaction between IICB^{Glc} and Mlc by glucose leads to membrane sequestration of Mlc, we determined the distribution of Mlc between two

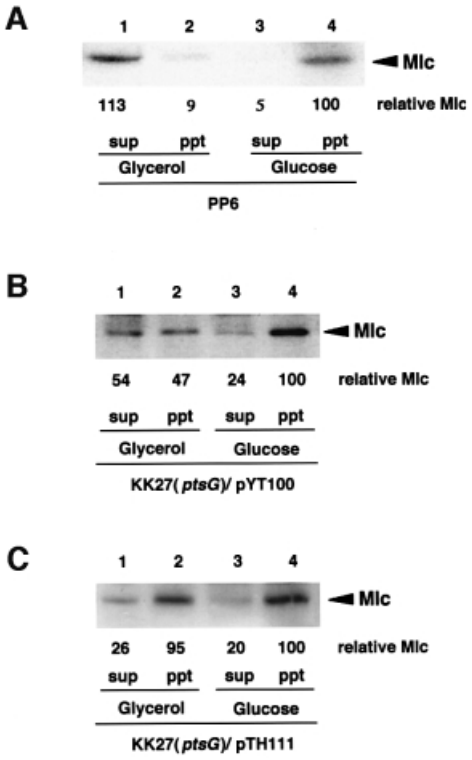

Fig. 8. Effects of glucose and IICB^{Glc} on localization of Mlc. *Escherichia coli* PP6 (**A**), KK27 harboring pYT100 (**B**) and KK27 harboring pTH111 (**C**) were grown in MOPS medium with 0.4% glycerol (lanes 1 and 2) or 0.2% glucose (lanes 3 and 4). The cytoplasm and membrane fractions were prepared and 10 µl of each sample were subjected to immunoblot analysis using anti-Mlc antibody. Mlc levels were quantified by densitometric scanning and expressed relative to that of the membrane fraction of glucose-grown cells (=100).

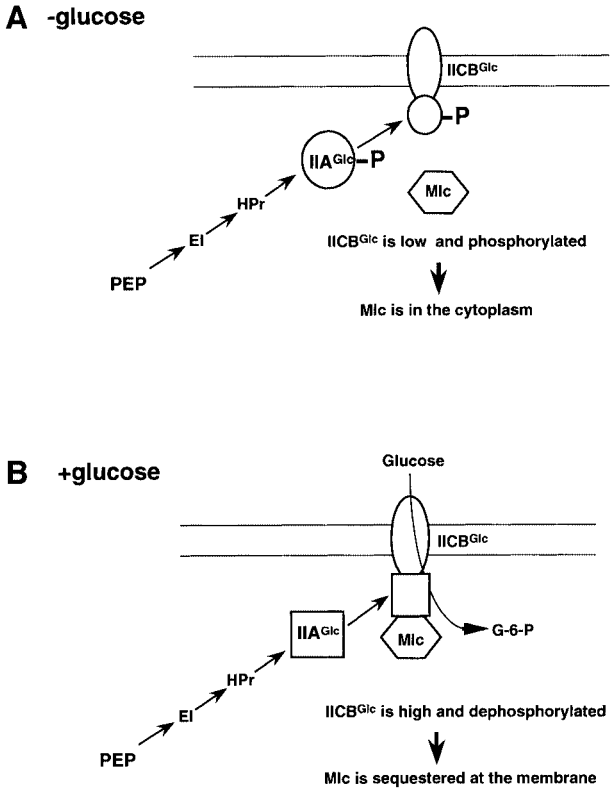

Fig. 9. Model for the regulation of Mlc by IICB^{Glc} and glucose. (**A**) In the absence of glucose, IICB^{Glc} is expressed at a low level and it is phosphorylated. Mlc binds the phosphorylated IICB^{Glc} only weakly and most Mlc is in the cytoplasm to inhibit the target promoters. (**B**) In the presence of glucose, transport and phosphorylation of glucose leads to dephosphorylation of IICB^{Glc}. Mlc binds strongly to the non-phosphorylated form of IICB^{Glc} and it is trapped at the membrane resulting in derepression of target promoters including *ptsG*. Increased IICB^{Glc} further contributes to the efficient sequestration of Mlc.

fractions in wild-type cells grown on either glucose or glycerol. Mlc was found mostly in the cytoplasmic fraction when cells were grown on glycerol, while it appeared in the membrane fraction when cells were grown on glucose (Figure 8A). This implies that glucose promotes the membrane association of Mlc in wild-type cells by dephosphorylating IICB^{Glc} and therefore by enhancing the Mlc–IICB^{Glc} interaction. It should be noted, however, that the level of IICB^{Glc} is low in the absence of glucose in wild-type cells. This raises a possibility that Mlc is located in the cytoplasm simply due to the limited amount of IICB^{Glc} in the absence of glucose. Then, we determined the distribution of Mlc in *ptsG* cells harboring pYT100 expressing IICB^{Glc} constitutively at a moderate level. Mlc appeared again in the membrane fraction when cells were grown on glucose, while it appeared both in the cytoplasm and membrane fractions when cells were grown on glycerol (Figure 8B). Furthermore, when IICB^{Glc} was overproduced by introducing a multicopy plasmid containing the *ptsG* gene, Mlc appeared predominantly in the membrane even in the glycerol medium (Figure 8C). These results suggest that not only the dephosphorylation of IICB^{Glc} but also the increased amount of IICB^{Glc} caused by external glucose plays a role in the effective membrane sequestration of Mlc in wild-type cells.

Discussion

The present study demonstrates that sequestration of Mlc to the membrane via the physical interaction with IICB^{Glc} is a key event in the glucose induction of Mlc-repressible genes. A plausible scenario of glucose induction of the Mlc-repressible promoters is depicted in Figure 9. In the absence of external glucose, the IICB^{Glc} level is low and it exists predominantly as the phosphorylated form. The affinity of phospho-IICB^{Glc} for Mlc is presumably not strong enough to sequester Mlc at the membrane. Thus, Mlc is mostly in the cytoplasm to bind its operators. The transport and phosphorylation of glucose through glucose-specific PTS leads to dephosphorylation of IICB^{Glc}. The non-phosphorylated IICB^{Glc} then associates with Mlc, which in turn prevents Mlc binding to operators resulting in derepression of Mlc-repressible operons. In addition, the increased level of IICB^{Glc} by glucose is certainly responsible for an effective membrane sequestration of Mlc. Thus, the induction process by glucose apparently consists of a positive feedback system. These conclusions are based on the following observations: (i) endogenous Mlc is associated with IICB^{Glc}-FLAG in *ptsI* cells in which IICB^{Glc}-FLAG is expected to exist as the non-phosphorylated form (Figure 4); (ii) exogenously added Mlc binds to highly purified immobilized IICB^{Glc}-FLAG (Figure 5); (iii) Mlc is associated with IICB^{Glc}-FLAG in wild-type

cells grown in the presence of glucose but not in the absence of glucose (Figure 6); and (iv) Mlc is found in the membrane fraction when $IICB^{Glc}$ is dephosphorylated either by the mutation in *ptsI* or by the transport of glucose in wild-type cells. Mlc is found in the cytoplasm when $IICB^{Glc}$ is phosphorylated due to the absence of external glucose or when it is lacking due to the mutation in the *ptsG* gene (Figure 7).

If $IICB^{Glc}$ and Mlc interact with each other, one may expect to obtain mutations in both *mlc* and *ptsG* genes that impair the physical interaction between the two proteins. It has been shown recently that some mutations in *ptsG*, originally called the *umgC* mutation (Jones-Mortimer and Kornberg, 1980), cause an increase in *ptsG* expression even in the absence of PTS sugars (Notley-McRobb and Ferenci, 2000; Zeppenfeld *et al*., 2000; J.Plumbridge, personal communication). It would be interesting to examine whether these mutations affect the $IICB^{Glc}$–Mlc interaction and the cellular localization of Mlc.

How does the membrane sequestration of Mlc via the interaction with $IICB^{Glc}$ lead to the dissociation of Mlc from its operators? The binding of Mlc to the membrane may simply compete with its binding to DNA. Thus, the concentration of free Mlc would be expected to drop below that required for repression of the target promoters when Mlc becomes associated with the membrane. However, it remains to be studied whether the membrane association of Mlc could also cause an allosteric change in the Mlc conformation that decreases its affinity for DNA. In addition, although we have shown that a highly purified $IICB^{Glc}$-FLAG could interact with purified Mlc, this does not completely exclude the possibility that additional proteins may also be involved in the $IICB^{Glc}$–Mlc interaction and therefore the sequestration of Mlc to the membrane in intact cells.

While the primary functions of PTS are sugar reception, transport and phosphorylation, it is well recognized that the PTS is involved in the regulation of a variety of physiological processes acting as a major signal transduction system (Meadow *et al*., 1990; Postma *et al*., 1996; Saier *et al*., 1996). For example, the involvement of PTS in bacterial chemotaxis had been recognized, although how PTS works for chemoreception activity is not yet elucidated (Lux *et al*., 1995, 1999). The best-known regulatory function of PTS is the regulation of metabolism and uptake of various carbohydrates by IIA^{Glc}. This cytoplasmic component of glucose PTS is known to act as both a positive effector of adenylate cyclase activity and a negative effector of the transport or use of non-PTS sugars, depending on its phosphorylation state, which is determined by the availability of external glucose (Postma *et al*., 1996; Takahashi *et al*., 1998). The BglF is an excellent example in which a membrane component of PTS plays a unique role in gene regulation. It modulates the phosphorylation state, therefore the activity of transcriptional antiterminator BglG by monitoring the availability of β-glucosides (Amster-Choder *et al*., 1989; Schnetz and Rak, 1990). Our study has shed light on a novel regulatory role of the membrane component of the glucose transporter $IICB^{Glc}$. This is the first case in which the regulatory role of $IICB^{Glc}$ has been revealed.

Dynamic changes in cellular localization have been recognized as a common feature of regulatory and structural proteins even in bacteria (Shapiro and Losick, 2000). There are several known systems where the membrane association of a transcription factor plays a key role in the regulation of bacterial gene expression. For example, Pro-σ^E is sequestered from RNA polymerase at the cytoplasmic membrane as a membrane-associated protein in the predivisional state in *Bacillus subtilis* (Hofmeister, 1998). Following cell division, pro-σ^E is processed and σ^E is released into the cytoplasm of mother cells where it associates with RNA polymerase. Regulation of the proline utilization *put* operon in enteric bacteria is a well known example in which membrane sequestration of a transcriptional repressor was shown to be the molecular basis of the induction of the operon (Ostrovsky de Spicer and Maloy, 1993; Muro-Pastor *et al*., 1997). When the intracellular concentration of proline is low, PutA remains in the cytoplasm to act as a repressor, but when the intracellular concentration of proline is high, it binds to the membrane and acts as an oxidation enzyme of proline. Thus, the PutA protein shuttles between the membrane and the cytoplasm, depending on the proline concentration. Another less-characterized example is the regulation of the transcriptional activator MalT by MalK, a component of the ABC-type transporter for maltose in *E.coli* (Panagiotidis *et al*., 1998). It was shown that MalK interacts physically with MalT to downregulate its transcriptional activity although the full biological significance of this regulation was not known.

The regulation of Mlc localization by $IICB^{Glc}$ presented in this study has provided a clear case for dynamic and spatial regulation of transcription by a membrane protein in *E.coli*. A striking feature of the $IICB^{Glc}$–Mlc system is that a membrane component of PTS acts as both a sensor and a modulator of a transcription factor. In other words, it determines the cellular location of a global repressor protein for genes involved in carbon metabolism depending on the availability of the sugar substrate therefore depending on the phosphorylation state of a transporter protein. It remains to be studied whether any other systems exist where the membrane sequestration of a transcription factor is used for gene regulation in response to an environmental change in *E.coli.*

Materials and methods

Media and growth conditions

Cells were grown aerobically at 37°C in Luria–Bertani (LB) medium or in minimal MOPS medium (Neidhardt *et al*., 1974). Antibiotics were used at the following concentrations: ampicillin (50 μg/ml), kanamycin (50 μg/ml) and tetracycline (15 μg/ml). Bacterial growth was monitored by determining the optical density at 600 nm.

Bacterial strains and plasmids

The *E.coli* K12 strains and plasmids used in this study are listed in Table I. PP6 was used as a parent wild-type strain. KK32 and KK50 were described previously. The *ptsG*::Tn*5* region of IT1168 was transferred to PP6 and to KK50 by P1 transduction to construct KK27 and YT18, respectively. Similarly, the *mlc1157*::Tn*10* region of KK32 was transferred to KK27 and KK50 to construct YT17 and YT19.

The 2.5 kb *Hin*dIII–*Eco*RI fragment containing the *ptsG* gene derived from pTH111 was cloned into pMW119 (Nippon Gene) to construct pYT100. A DNA fragment corresponding to the C-terminal half of $IICB^{Glc}$-FLAG was amplified by PCR from pTH111 using two primers: P1*Bst*EII–*ptsG* (5′-CGTTATATGGCGGGTGACCCGACTGC-3′) and P2*Eco*RI–*ptsG*^{FL} (5′-CGCGAATTCTTACTTGTCATCGTCATCCTTGTAGTCGTGGTTACGGATGTACTCATCC-3′). The amplified fragment

Table I. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant genotype and property	Source
Strain		
PP6	wild type	Kimata *et al*. (1998)
IT1168	W3110 *ptsG*::Tn*5*	Kimata *et al*. (1997)
KK27	PP6 *ptsG*::Tn*5*	this study
KK32	PP6 *mlc1157*::Tn*10*	Kimata *et al*. (1998)
KK50	PP6 *ptsI*::Tn*5*	Tanaka *et al*. (1999)
YT17	KK27 *mlc1157*::Tn*10*	this study
YT18	KK50 *ptsG*::Tn*5*	this study
YT19	KK50 *mlc1157*::Tn*10*	this study
Plasmid		
pTH111	derivative of pBR322 containing *ptsG* expressed from the *bla* promoter	Takahashi *et al*. (1998)
pYT100	derivative of pMW119 containing *ptsG* expressed from the *bla* promoter	this study
pYT101	derivative of pYT100 containing $ptsG^{FL}$ expressed from the *bla* promoter	this study
pYT103	derivative of pBR322 containing $ptsG^{FL}$ expressed from the *bla* promoter	this study
pETIIB387	derivative of pET3a containing the 3′ portion of *ptsG* under T7 promoter	this study
pHA7M	derivative of pHA7 in which a *Mlu*I site is introduced near last codon of *crp*	Abo *et al*. (2000)
pYT102	derivative of pBR322 containing crp^{FL} expressed from the *bla* promoter	this study
pTH219	derivative of pBR322 containing *crr* expressed from the *bla* promoter	Takahashi *et al*. (1998)

of ~0.8 kb was digested with *Bst*EII and *Eco*RI, and substituted for the corresponding 1.5 kb region of pYT100 to construct pYT101 carrying the $ptsG^{FL}$ gene encoding $IICB^{Glc}$-FLAG. Similarly, the *Bst*EII–*Eco*RI fragment was cloned into the corresponding site of pTH111 to construct pYT103 carrying the $ptsG^{FL}$ gene encoding $IICB^{Glc}$-FLAG. To construct pETIIB387 expressing a cytoplasmic domain (IIB^{Glc}) of $IICB^{Glc}$, a DNA fragment encoding the C-terminal portion of $IICB^{Glc}$ consisting of 91 amino acid residues was amplified by PCR from pTH111 using two primers: P3*Nde*I–*ptsG* (5′-GCACTCTCACATATGGAAGACGCGA-CTG-3′) and P4*Eco*RI–*ptsG* (5′-GCGCGAATTCTCAGCATTACC-GGCACG-3′). The amplified fragment was digested with *Nde*I and *Eco*RI, and the resulting fragment was cloned into the corresponding sites of pET3a (Takara Shuzo, Kyoto). To construct pYT102 containing the crp^{FL} gene encoding FLAG-tagged CRP, a DNA fragment was amplified by PCR from pHA7M using two primers: pBR–*Bam*HI (5′-GAGCCC-GATCTTCCCCATCG-3′) and *Mlu*I–*crp* (5′-GCGCACGCGTTACTTG-TCATCGTCATCCTTGTAGTCGCCGTAAACGACGATGGTTTTA-CCG-3′). The amplified fragment was digested with *Hin*dIII and *Mlu*I, and the resulting fragment was cloned into the corresponding sites of pHA7M.

Immunoblot analysis

Mlc purification was described previously (Tanaka *et al*., 1999). To purify the IIB^{Glc} domain $IICB^{Glc}$, BL21 cells (Takara Shuzo) harboring pETIIB387 were grown at 37°C in LB medium, and the expression of IIB^{Glc} was induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside. The IIB^{Glc} protein was purified from extracts of the induced cells using DEAE-Sephacel and MONO-Q columns, and finally by SDS–PAGE followed by protein extraction from the gel. IIA^{Glc} was purified from the extracts of MC4100 cells harboring pTH219 containing the *crr* gene, using DEAE Toyo-pearl 650 and Sephacryl HR and DEAE Toyo-pearl 650 columns. Anti-Mlc, anti-IIB^{Glc} and anti-IIA^{Glc} polyclonal antibodies were obtained by immunizing rabbits with purified Mlc, IIB^{Glc} and IIA^{Glc} proteins, respectively. Anti-CRP polyclonal antibody was described previously (Ishizuka *et al*., 1993). For immunoblot analysis, protein samples were loaded onto 0.1% SDS–12% polyacrylamide gels and electrophoresed. Western blotting was performed as described previously (Ishizuka *et al*., 1993) using polyclonal anti-IIA^{Glc}, anti-IIB^{Glc}, anti-Mlc and anti-CRP antibodies and a horseradish peroxidase-conjugated secondary antibody.

S1 nuclease analysis

The S1 nuclease assay was performed as described previously (Aiba *et al*., 1981). Total cellular RNAs were isolated from cells grown in LB medium to an OD_{600} of 0.6. The 741 bp *Hin*dIII–*Xho*I fragment ^{32}P-labeled at its 5′ ends was used as DNA probe for *pts* mRNA. The DNA probe and total RNA were hybridized and treated with 40 U of S1 nuclease at 37°C for 10 min. The reaction products were analyzed on an 8% polyacrylamide–8 M urea gel.

Analysis of interaction between Mlc and $IICB^{Glc}$-FLAG

Cells were grown in 20 ml of LB medium to an OD_{600} of 0.8, harvested and washed with 1 ml of 50 mM Tris–HCl pH 8.0, 5 mM EDTA. The cell pellets were suspended in 0.5 ml of ice-cold 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, CompleteTM Protease inhibitor Cocktail Tablets (Boehringer Mannheim). The cells were sonicated for 5 s four times at 0°C, with 10 s rest periods in between, using a special micro tip of a Heat Systems Ultrasonics Sonicator. The disrupted cells were centrifuged at 12 000 *g* for 5 min at 4°C. The supernatants were used as crude extracts. Crude extracts (50 µl) were incubated with 0.2 ml of TBS buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl) and 50 µl of anti-FLAG M2–agarose suspension (Sigma) for 15 min at 4°C. The mixtures were centrifuged and the supernatants were used as unbound fractions. The pellet beads were washed four times with 0.25 ml of TBS buffer. The supernatants were used as wash fractions. The proteins bound to the beads were eluted with 50 µl of SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 62.5 mM Tris–HCl pH 6.8, 10% glycerol, 0.1% bromophenol blue) at 100°C for 5 min and used as bound fractions. The protein samples at each step were subjected to western blot analysis.

Binding of purified Mlc to purified $IICB^{Glc}$-FLAG

Cells harboring pYT103 carrying the $ptsG^{FL}$ gene encoding $IICB^{Glc}$-FLAG were grown in 20 ml of LB medium to an OD_{600} of 0.8. Crude extracts (0.5 ml) were prepared as mentioned above. To purify $IICB^{Glc}$-FLAG, 100 µl of anti-FLAG M2–agarose suspension (50 µl bed volume) were added to the crude extracts and the mixtures were incubated for 1 h at 4°C. The mixtures were centrifuged and the beads were washed twice with 1 ml of 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, twice with 1 ml of 50 mM Tris–HCl pH 7.5, 500 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and twice with 1 ml of TBS buffer by centrifugation. The pellet beads were suspended with 50 µl of TBS buffer. The presence and purity of $IICB^{Glc}$-FLAG in the agarose beads were examined by western blotting and SDS–PAGE followed by Coomassie Blue staining. Crude extracts were also prepared from cells harboring pTH111 carrying the wild-type *ptsG* gene encoding $IICB^{Glc}$ and treated with anti-FLAG M2–agarose. For the Mlc binding assay, 10 µl of agarose suspension with or without the purified $IICB^{Glc}$-FLAG was incubated with 20 ng of purified Mlc in 50 µl of TBS buffer for 15 min at 4°C. The mixtures were centrifuged and the supernatants were used as unbound fractions. The pellet beads were washed four times with 0.25 ml of TBS buffer by centrifugation. The proteins bound to the beads were eluted with 50 µl of SDS sample buffer at 100°C for 5 min and used as bound fractions. The protein samples were subjected to western blot analysis.

Protein localization assay

Cells were grown in minimal MOPS medium containing either glycerol or glucose to an OD_{600} of 0.6, and 0.5 ml of cell cultures were directly

sonicated for 5 s twice at 0°C, with a 10 s interval in between. The sonicated samples were centrifuged at 15 000 *g* (4°C) for 3 min. The pellets were dissolved in 30 µl of SDS sample buffer and used as the membrane fractions. The supernatant fraction was precipitated with cold trichloroacetic acid (5% final concentration). The precipitates were collected by centrifugation, washed with 70% acetone, dissolved in 30 µl of SDS sample buffer and used as the soluble cytoplasmic fractions. The supernatant and membrane fractions were subjected to western blot analysis.

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