Selective regulation of *ptsG* expression by Fis: formation of either activating or repressing nucleoprotein complex in response to glucose

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SUMMARY

Transcription of \( ptsG \) encoding glucose-specific permease, enzyme \( IICB_{\text{Glc}} \), in \( Escherichia coli \) is initiated from two promoters, P1 and P2. \( ptsG \) transcription is repressed by Mlc, a glucose-inducible regulator of carbohydrate metabolism. The regulation of \( ptsG \) P1 transcription is also under positive control by cyclic AMP receptor protein and cyclic AMP complex (CRP-cAMP) as observed in other Mlc regulon. We report here that Fis, one of the nucleoid-associated proteins, plays a key role in glucose induction of Mlc regulon. \( ptsG \) transcription was induced when wild-type cells were grown in the presence of glucose. However, in a \( fis \) mutant, the basal level of \( ptsG \) transcription was higher, but decreased when cells were grown in the presence of glucose, which implies the possibility of regulatory interactions among Fis, Mlc, and CRP-cAMP. Footprinting experiments with various probes and transcription assays revealed that Fis assists both Mlc repression and CRP-cAMP activation of \( ptsG \) P1 through the formation of Fis-CRP-Mlc or Fis-CRP nucleoprotein complex at \( ptsG \) P1 promoter depending on the availability of glucose in the growth medium. \( ptsG \) P2 transcription was inhibited by Fis and Mlc. Tighter Mlc repression and enhanced CRP-cAMP activation of \( ptsG \) P1 by Fis enable cells to regulate Mlc regulon efficiently by selectively controlling the concentration of enzyme \( IICB_{\text{Glc}} \) that modulates Mlc activity.
INTRODUCTION

PEP:carbohydrate phosphotransferase system (PTS) of bacteria catalyzes concomitant uptake and phosphorylation of sugars (1). In *E. coli*, glucose is translocated and phosphorylated via the membrane-bound glucose permease, enzyme IIICB\textsuperscript{Gle} (EIICB\textsuperscript{Gle}), which is a component of glucose-specific PTS (1). EIICB\textsuperscript{Gle} is encoded by *ptsG* located at 25 min of the chromosomal locus. Kimata *et al.* (2) showed that functional CRP-cAMP complex is essential for the expression of *ptsG*, which is induced by glucose (3-5), and that EIICB\textsuperscript{Gle} is a key mediator of diauxic growth in glucose-lactose medium and of inducer-exclusion.

*ptsG* transcription is regulated through two global systems, positively by CRP-cAMP and negatively by Mlc (6, 7), as observed in all Mlc-regulated genes (8-12). Our and two other groups have demonstrated that unphosphorylated EIICB\textsuperscript{Gle} formed upon glucose uptake can relieve the repression of Mlc regulon by sequestering Mlc through direct protein-protein interaction (13-15). Our group found that glucose-induced expression of *ptsG* is enhanced significantly under heat shock, and, thus, proposed that this enhancement may counteract the highly increased *mlc* expression, whereby normal glucose metabolism is maintained even at high growth temperature (16). In addition to the regulations at the transcriptional level, expression of *ptsG* is regulated posttranscriptionally via modulation of *ptsG* mRNA stability in response to glycolytic flux in the cells (17). These observations suggest that the expression of *ptsG* is regulated in a highly complex and dynamic manner in response to various growth environments as well as to the availability of glucose.

Several types of small DNA-binding proteins, known as nucleoid-associated or histone-like proteins, exist in *E. coli* (18), among which Fis is the most abundant in
exponentially growing cells (19). Fis is known to be involved in site-specific DNA recombination and participates in the regulation of growth-related genes, including those for rRNA, tRNA, and DNA replications (20). It also regulates genes related to the catabolism of sugars and nucleic acids (21). The expression of fis is dependent on the growth phase of cells; the expressed fis mRNA reaches maximum level at the early-exponential phase then decreases rapidly (22). Fis exists at maximum over 50,000 molecules per cell, bringing about the assumption that Fis binds to every 200 to 300 bp of E. coli chromosomal DNA (19, 22).

We found several Fis-like sites, which are homologous to the known degenerate-consensus binding sequence of Fis upstream of ptsG gene. ptsG P1 transcription is induced by glucose in the wild-type strain (wt) (6). However, the opposite effect of glucose on the ptsG P1 activity in fis mutant compared with wt raises the possibility that either Fis is involved in Mlc-mediated repression or CRP-cAMP-dependent activation of the promoter. We report here that E. coli can regulate the expression of ptsG prior to the modulation of other Mlc regulon due to the selective enhancement of Mlc-dependent repression as well as CRP-dependent activation of ptsG in the presence of Fis.
EXPERIMENTAL PROCEDURES

Materials
Cyclic AMP was obtained from Sigma (St. Louis, MI). RNA polymerase saturated with σ^{30}, nucleotide triphosphates, [γ-32P]ATP, and [α-32P]UTP were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The cycle sequencing kit was from Epicentre Technologies (Madison, WI).

Bacterial strains and growth condition
All E. coli strains used in this study are MC4100 (araD139 ΔargF-lacU169 rpsL150 thiArelA1flb5301deoc1ptsF25rbsR) derivatives. To construct a fis mutant strain, SR507, fis::Km region of SA37 strain (23) (a gift from Altuvia, S.) was transferred into MC4100 by P1 transduction. SR504 (MC4100 ΔptsG::Cm) was made by P1 transduction of the CmR region of ZSC112LΔG strain (24) (a gift from Erni, B.). fis::Km was transferred into SR505 (MC4100 mlc::Tc) (16) and SR504, through which SR575 (MC4100 fis⁻ mlc⁻) and SR574 (MC4100 fis⁻ ptsG⁻), respectively, were constructed. All strains were grown in Luria-Bertani (LB) medium (25) aerobically at 37°C.

Plasmid construction
Basic cloning protocols described by Sambrook and Russell (26) were used. PCR cloning of the ptsG promoter was carried out using primers that have unique restriction sites in their sequences. The clone was verified by DNA sequencing. The supercoiled plasmid pGX12 containing entire ptsG promoter region, and pGX10 carrying only
ptsG P1 promoter were made by inserting DNA fragments from base pairs -305 ~ +132 and -75 ~ +132 between EcoRI and PstI sites in front of the rpoC terminator in plasmid pSA600, respectively (27).

**Primer extension analysis**

Cells were grown aerobically at 37°C overnight in LB. Overnight cultures were diluted 1:100 into fresh LB with or without 1% glucose. Total RNA was isolated from E. coli cells grown to mid-exponential phase (OD$_{600}$ = 0.5 ~ 0.6) using Trizol reagent (Life Technologies, Inc). To study ptsG transcription, 50,000 cpm of $^{32}$P-labeled primer PG1 (5’-AATTGAGAGTGCTCCTGAGTATGGGTGC-3’, complementary to +74 ~ +102) was coprecipitated with 30 µg of total cell RNA. Primer extension reactions were performed as described by Ryu and Garges (27).

**In vitro transcription assay**

Single-round *in vitro* transcription reactions were performed in a 20-µl total volume containing 20 mM Tris acetate (pH 8.0), 3 mM magnesium acetate, 200 mM potassium glutamate, 1 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 0.2 mM CTP, 0.02 mM UTP, 10 µCi of [$\alpha$-$^{32}$P] UTP (800 Ci/mmol), 2 nM supercoiled DNA template, 5-20 nM RNA polymerase, 100 µg/ml bovine serum albumin, and 5% glycerol. Regulator proteins such as CRP, Mlc or Fis were added to the reaction as described in the “Results”. All components except nucleotides were incubated at 37°C for 10 min. Transcription was started by the addition of nucleotides containing 200 µg/ml of heparin and terminated after 10 min by adding 20 µl of formamide loading buffer. RNA was resolved by electrophoresis on 6% polyacrylamide gel containing 8 M urea.
The amounts of transcripts were measured using a phoshpoimage analyzer, BAS2500 (Fuji Photo Film Co.).

**Gel shift assay**

A DNA fragment covering *ptsG* P1 promoter region was amplified by PCR using a pair of primers, PG1 and PGR3 (complementary to-54 ~ -78). PCR products were purified from 6% polyacrylamide gel as described by Sambrook and Russel (26) and labeled with [$\gamma$-32P]ATP. Purified protein(s), DNA probe, and 50 $\mu$g/ml of poly (dI-dC)$\bullet$poly (dI-dC) were mixed in the same buffer used for *in vitro* transcription at a 15-$\mu$l total volume. The binding mixture was incubated for 20 min at 37°C. In the reaction containing RNA polymerase, heparin (200 $\mu$g/ml) was added and further incubated for 5 min. The reaction was analyzed by electrophoresis on 5% polyacrylamide gel. In the reaction containing CRP-cAMP complex, 200 $\mu$M of cAMP was also added into the gel and the running buffer in the upper reservoir.

**DNase I footprinting analysis**

DNA fragment carrying *ptsG* promoter region was amplified by PCR using either 5’-end-labeled PG1 or PGR2 (5’-ATAACTTCGCCCGTCTGTTTCACATCG-3’, -248 ~ -222). The PCR product was purified using polyacrylamide gel. Purified protein(s) and DNA fragment were incubated in 40 $\mu$l of *in vitro* transcription assay buffer. Five microliters of DNase I solution (10 ng DNase I per reaction) was added to the binding mixture, which was then placed at room temperature for 1 min. DNase I reaction was terminated by the addition of 200 $\mu$l stop solution containing 0.4 M sodium acetate, 10 mM EDTA, and 100 $\mu$g/ml yeast tRNA. After phenol extraction and ethanol
precipitation, the pellet was dissolved in a sequencing dye and resolved on 8% polyacrylamide gel containing 8 M urea.

**Potassium permanganate reactivity assay**

Supercoiled plasmid DNA (2 nM), 20 nM of RNA polymerase, and regulator proteins were incubated for 20 min at 37°C in 20 μl transcription buffer containing 100 mM each of two initiating nucleotides, ATP and UTP. After the addition of 2 μl of 100 mM KMnO₄, the reaction mixture was incubated for 30 s at room temperature. To terminate the reaction, 2 μl of 14 M β-mercaptoethanol was added. The reaction was precipitated, and the pellet was washed with 70% ethanol and resuspended in water, which was used as a template for 5 cycles of PCR with end-labeled PG1 primer (28). The PCR products were resolved on 6% sequencing gel.

**Dimethyl sulfate (DMS) footprinting**

Two nM of supercoiled plasmid DNA and proteins were incubated in 40 μl of the transcription buffer. DMS methylation was started by adding 2.5 μl of DMS (150 mM). After incubation at 37°C for 5 min, the reaction was stopped by adding β-mercaptoethanol. The reaction was precipitate with ethanol in the presence of sodium acetate and salmon sperm DNA. The pellet was resuspended in water, which was used as a template for 5 cycles of PCR as described in potassium permanganate assay. The PCR products were analyzed on 6% sequencing gel.
RESULTS

Fis is required for both full repression and activation of ptsG

Role of Fis on ptsG expression was examined by analyzing the effects of fis mutation on ptsG transcription with various genetic backgrounds using primer extension assay. When wt was grown in the absence of glucose, only the basal level of ptsG P1 expression was observed, whereas P1 promoter activity was highly induced in cells grown in the presence of glucose (Fig. 1A). However, the effect of glucose on ptsG P1 expression was reversed in the fis- strain. ptsG P1 expression in fis- strain grown in the absence of glucose was markedly increased compared with wt (Fig. 1A, lanes 1 and 2) and was reduced in the presence of glucose. These results suggest that Fis is required for both the repression of ptsG P1 by Mlc and the activation of ptsG P1 under limited concentration of CRP-cAMP (6, 7). However, the interaction between Mlc and EIICB^{Glc} was not affected by Fis, because the P1 expression also increased in the absence of Fis, even in ptsG mutant that cannot produce EIICB^{Glc} (Fig. 1B, lane 2). These observations imply that Fis is involved directly in the Mlc binding and/or repression of ptsG P1 promoter.

It is possible that Fis also assists CRP-dependent activation of ptsG P1, because P1 promoter activity decreased markedly in the fis- strain when cells were grown in the presence of glucose (Fig. 1A, lanes 3 and 4). The effect was not dependent on Mlc because the ptsG P1 promoter activity high in mlc mutant also decreased in mlc and fis double mutant regardless of the presence of glucose (Fig. 1B, lanes 3-8). We also determined the effect of exogenous cAMP on ptsG P1 expression in the fis- mlc- strain based on the report of González-Gil et al. (29) that crp expression is increased in fis-
strains. However, cAMP addition to the medium did not restore the P1 promoter activity (Fig. 1B, lanes 5 and 8), and the level of mlc expression was not changed in the fis' strain compared with wt (data not shown). These results imply that reduction of the CRP-dependent P1 expression in the fis mutant cannot be explained by changes in the level of either Mlc or CRP-cAMP. Furthermore, the reduction of ptsG P1 transcription in the fis' mlc' strain grown without glucose was lower than that in the fis' strain grown in the presence of glucose (Fig. 1). Thus, Fis effect is more prominent in the CRP-dependent ptsG P1 transcription activation under limited intracellular concentration of CRP-cAMP.

In the case of ptsG P2 expression, the P2 promoter activity was also induced by glucose (Fig. 1A) (7) and increased in the fis mutant (Fig. 1A, lanes 2 and 4). These results suggest that ptsG P2 transcription is inhibited by Fis as well as Mlc in vivo.

Fis, CRP, and Mlc can bind simultaneously to ptsG P1, forming nucleoprotein complexes

In order to examine whether Fis influences ptsG expression directly or not, we performed DNase I footprinting assay using ptsG promoter DNA region from -248 to +103 (all numberings in this study are based on the transcription start site of the ptsG P1 promoter as shown in Fig. 2). At least five putative Fis-binding sites were found on the ptsG promoter DNA (Figs. 2 and 3), each of which showed high homology with the Fis-binding sequence (20, 30). Fis sites I and II, centered at positions +3 and -19, respectively, were detected between +18 and -32 of the ptsG promoter region. Fis site II was positioned between the CRP and Mlc sites of the P1 promoter, and Fis site I partially overlapped the Mlc site as well as the P1 transcription start site (Fig. 2B). Fis
occupied from positions -65 to -90 (site III), which overlapped CRP site II centered at position -95. Fis also protected DNA from positions -105 to -130 (site IV), and Fis site V centered at -145 overlapped the P2 promoter (Figs. 2 and 3).

Two CRP sites have been identified at the ptsG promoter. We observed two hypersensitive bands upon CRP-cAMP binding at CRP site I centered at -40.5, which is a typical pattern of CRP binding (Fig. 3, lane 4) (31). It is likely that both CRP-cAMP and Fis could bind to the ptsG P1, because the two bands, which appeared upon CRP-cAMP binding, remained and DNA protection by Fis at sites I and II was maintained in the presence of both CRP-cAMP and Fis (Fig. 3). CRP-cAMP bound at CRP site II protected DNA from -85 to -105. In the presence of both CRP-cAMP and Fis, the hypersensitive cleavage site around -90 generated by Fis binding at site III disappeared, suggesting the possibility that these two proteins compete for the binding sites (Fig. 3, lane 3). However, the relevance of CRP binding at site II in ptsG expression has not yet been fully elucidated (6).

Fis sites I and II partially overlapped Mlc site I located at -8. Presence of Mlc protected DNA between +5 and -17 from DNase I attack (Fig. 3, lane 6). Fis and Mlc could bind simultaneously to the P1 promoter, even though their binding sites overlapped. The weak bands remaining around the Mlc binding site in the presence of Fis were further weakened by Mlc addition (Fig. 3B, compare lanes 2 and 5). At the same time, Fis binding at sites I and II was observed clearly in the presence of Mlc as shown by the protection of two bands around positions -32 and +18 (Fig. 3). When all three regulators, Fis, CRP, and Mlc were present, similar DNase I cleavage pattern observed between Fis and CRP and between Fis and Mlc was reproduced (Fig. 3, lane 8). These findings suggest that all these proteins could bind to ptsG P1 promoter
simultaneously, forming a highly ordered protein-DNA complex.

**RNA polymerase binding at ptsG P1 promoter**

We also examined the effect of Fis and CRP-cAMP on RNA polymerase binding to ptsG P1 using DMS footprinting experiment. When Fis was incubated with supercoiled pGX12 carrying both P1 and P2 promoters of ptsG, Fis binding to site I protected guanine residues at -5 and +11, but increased DMS reactivity of -3 G. Fis binding at site II also generated characteristic changes in DMS reactivity at ptsG P1 promoter, increasing reactivity at -25 G and near -20 G (Fig. 4). Binding of CRP-cAMP to site I completely protected G residues at -47, -45, and -38 (Fig. 4, lane 7). Consistent with our DNase I footprinting experiment, DMS reactivity patterns at the P1 promoter shown in the presence of either Fis or CRP-cAMP were retained in the presence of both Fis and CRP-cAMP (Fig. 4, lane 10). Addition of RNA polymerase alone did not change DMS reactivity at the ptsG P1 promoter, and the presence of Fis showed no effect on RNA polymerase binding to the P1 promoter (lanes 2, 3, 5, and 6 of Fig. 4). However, strong protection of G residues at -5 and -3 by RNA polymerase binding was detected in the presence CRP-cAMP (Fig. 4, lanes 8 and 9).

When Fis, CRP-cAMP, and RNA polymerase were added at various combinations, complete protection of -5 G and significant reduction of DMS reactivity at -3 G was observed, whereas DMS reactivity at +11 G was restored (Fig. 4). However, the altered DMS reactivity by Fis binding to site II and CRP binding to site I was retained under the same condition. These results strongly suggest that ptsG P1 promoter can be occupied simultaneously by CRP-cAMP (at site I), Fis (at site II), and RNA polymerase, but Fis bound to site I is excluded by RNA polymerase in the presence of
CRP·cAMP.

**ptsG P1 transcription is better repressed by Mlc in the presence of Fis**

Influence of Fis on the Mlc repression of *ptsG* P1 was studied through *in vitro* transcription assay. Addition of Mlc reduced the CRP-activated P1 promoter activity to 50% of its full activity (Fig. 5A). However, the P1 activity was repressed further to 32% when both Mlc and Fis were added to the reaction. The effect was specific to *ptsG* P1, because the repression of *ptsH* P0 promoter activity by Mlc was not affected by Fis (data not shown). These findings, together with the DNase I footprinting results, strongly suggest that more effective repression of the *ptsG* P1 activity could be accomplished through the formation of nucleoprotein complex containing Fis at *ptsG* P1.

**Fis increases CRP-dependent *ptsG* P1 activity**

We also investigated the effect of Fis on CRP-dependent transcription activation of *ptsG* using *in vitro* transcription assay. *ptsG* P1 is known as a typical class II CRP-dependent promoter (6, 7). RNA polymerase alone could not initiate transcription from the P1 promoter, and the addition of Fis showed no effect on the P1 transcription (Fig. 5B). However, P1 transcription was activated in the presence of CRP·cAMP, and Fis further increased CRP-activated transcription of P1 (Fig. 5B). However, Fis did not affect the activation of *ptsH* P0 by CRP·cAMP (data not shown).

In the case of P2 promoter, 308-nt of transcripts were made from the P2 promoter regardless of the CRP·cAMP presence in the reactions (Fig. 5B). As expected from DNase I footprinting experiment, addition of Fis repressed transcription initiation at P2
promoter, which suggests that Fis binding at site V represses the P2 promoter activity.

**Fis enhances CRP-dependent open complex formation at *ptsG* P1 promoter**

To analyze the effect of Fis on the *ptsG* transcription, open complex formation at the promoter was probed with potassium permanganate. When RNA polymerase was incubated with supercoiled pGX12 carrying the entire *ptsG* promoter region, open complex was not formed at *ptsG* P1 regardless of the presence of Fis in the reaction. Addition of CRP-cAMP increased the KMnO₄ reactivity at positions, +2, -2, and -6, and the KMnO₄ reactivity of these bases were further increased in the presence of both CRP-cAMP and Fis (Fig. 6A). These results show that Fis can enhance CRP-dependent open complex formation at *ptsG* P1 promoter, resulting in the increased transcription initiation at the promoter.

Fis sites I and II were sufficient to show the Fis effect on the open complex formation at P1 promoter. Fis could enhance CRP-dependent open complex formation at *ptsG* P1 promoter, even when DNA containing only Fis site I and site II (pGX10) was used as a template (Fig. 6A, compare lanes 7, 8, and 9). Similar results were obtained through gel shift assay using DNA fragment ranging from -78 to +102 of *ptsG* promoter.

Incubation of either Fis or CRP-cAMP with the DNA probe generated a single retarded band (Fig. 6B). When both Fis and CRP-cAMP were present in the reaction, Fis-DNA complex disappeared, whereas Fis-CRP-DNA complex was observed, an indication that Fis and CRP could bind simultaneously to the promoter region (Fig. 6B, lane 4). Incubation of the DNA probe with RNA polymerase only and subsequent heparin challenge did not generate any RNA polymerase-DNA complex as expected from KMnO₄ reactivity assay (Fig. 6B, lane 5). When the same reaction was done in the
Shin et al.

presence of CRP-cAMP, a heparin-resistant complex was detected, which was further increased by the addition of Fis (Fig. 6B, compare lanes 7 and 8). However, addition of Fis only showed no effect on the stable RNA polymerase-DNA complex formation. These results suggest that CRP binding to site I is sufficient for the effective open complex formation at \( \text{ptsG} \) P1 promoter, and Fis binding to sites I and II can co-activate \( \text{ptsG} \) P1 transcription with CRP-cAMP.
DISCUSSION

In this report, we demonstrate that two different types of nucleoprotein complexes containing Fis are formed at \( ptsG \) P1 promoter in response to glucose in the growth medium for optimal regulation of \( ptsG \) transcription. Several lines of evidences suggest that Fis assists both repression by Mlc and activation by CRP-cAMP of the \( ptsG \) P1.

Mlc site I is located between Fis sites I and II and partially overlaps both sites. Fis and Mlc could bind simultaneously to their binding sites of \( ptsG \) promoter (Fig. 3), and Mlc showed better repression of \( ptsG \) P1 in the presence of Fis (Fig. 5). It is possible that Mlc and Fis repress \( ptsG \) P1 independently; however, their effects are additive, because we could not detect cooperative binding between Fis and Mlc to their respective binding sites (data not shown). Corepression of \( ptsG \) P1 by Fis and Mlc is distinct from the mechanism described by Nasser et al. (32) that one of the two divergent promoters are repressed by either Fis or CRP.

CRP-cAMP binding to site I centered at -40.5 is essential for the activation of \( ptsG \) P1 promoter, a typical class II CRP-dependent promoter (33, 34). Fis further increased CRP-dependent transcription of \( ptsG \) P1 promoter (Fig. 5). However, Fis alone showed no effect on the transcription initiation at P1 promoter. Our KMnO\(_4\) reactivity assay demonstrated that the increased \( ptsG \) P1 transcription initiation by Fis results from enhanced open complex formation at the promoter in the presence of both CRP-cAMP and Fis (Fig. 6). Moreover, nucleoprotein complex formations on CRP site I and Fis site II are responsible for the increased open complex formation at \( ptsG \) P1 promoter, because Fis could increase the open complex formation, even when the shortened \( ptsG \) promoter without the Fis sites III, IV, and V was used as a DNA template (Fig. 6) and Fis binding to site I was excluded upon RNA polymerase binding to the P1 promoter in
the presence of CRP-cAMP (Fig. 4). Thus, to our knowledge, this is the first case known in class II CRP-dependent promoter that Fis assists CRP-dependent transcription. This is different from the coactivation of proP P2 promoter by Fis and CRP-cAMP, where the CRP binding occurs at –121 (35). We are presently attempting to elucidate the mechanisms of how Fis assists CRP-dependent activation, because no evidence of cooperative binding could be found between Fis and CRP.

What is the biological significance of these findings in ptsG expression? As E. coli cells are grown in the presence of glucose, ptsG expression is highly induced, because Mlc action is relieved via direct interaction with unphosphorylated EIICB\textsuperscript{Glc} during the glucose transport (13-15). However, transport of glucose into the cells results in the reduction of both CRP and cAMP (36). Thus, the level of ptsG expression is a sum of the two effects, i.e., derepression of Mlc and catabolite repression upon glucose uptake. Plumbridge reported that ptsG expression in wt grown with glucose was 42% compared to that in mlc\textsuperscript{-} strain grown with glycerol, which indicates the degree of catabolite repression in this gene (7). The level of ptsG expression during E. coli growth on glucose appears to be sufficient to derepress Mlc action completely, considering that the level of ptsG expression was almost equal in both mlc\textsuperscript{+} and mlc\textsuperscript{-} cells grown in the presence of glucose (7). Regulation of manXYZ, one of the Mlc regulon, is mediated also by CRP-cAMP and Mlc (10). CRP binding to -40.5 of manX promoter, which has been categorized as a class II CRP-dependent promoter, is essential for its expression (10). Other than ptsG, manX regulation is subject to more severe catabolite repression upon glucose uptake; the promoter activity in mlc\textsuperscript{+} cells grown with glucose was no less than 20% of mlc\textsuperscript{-} cells grown with glycerol (10). Thus, we propose that Fis can alleviate catabolite repression on ptsG P1 through the
formation of coactivating complex with CRP in cells grown with glucose. On the other hand, upon glucose depletion in the medium, phosphorylated form of EII\textsubscript{CB}\textsuperscript{Glc} begins to appear, after which Mlc is free from sequestration by EII\textsubscript{CB}\textsuperscript{Glc} (14). Under this circumstance, Mlc may bind more preferentially to \textit{ptsG} P1 promoter than to other competing promoters through the interaction with Fis-CRP nucleoprotein complex already formed at the promoter region. Consequently, this selective shut-off of \textit{ptsG} expression will lead to the efficient repression in other Mlc-regulated genes. Speculating that \textit{ptsG} expression triggers cascades of gene expressions for glucose uptake, Fis facilitates rapid adaptation of \textit{E. coli} to different nutritional environment through the formation of nucleoprotein complex with either CRP-cAMP in the presence of glucose or Mlc in the absence of glucose.

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Shin et al.

2885


Legends to figures

Fig. 1. Fis effects on *ptsG* expression in *E. coli* analyzed by primer extension analysis.

*In vivo* effects of Fis on *ptsG* expression were studied using primer extension assay. Thirty micrograms of total *E. coli* RNA, extracted at mid-exponential growth phase, was coprecipitated and annealed with end-labeled PG1 primer. Reactions were performed as described in “Experimental Procedures”. The products were resolved on 6% sequencing gel. (A) Expression of *ptsG* in *E. coli* MC4100 strain (lanes 1 and 3) and isogenic *fis* mutant strain (lanes 2 and 4) was examined. The strains in lanes 1 and 2, and lanes 3 and 4 were grown in LB without and with 1% glucose, respectively. (B) *ptsG* P1 expression was monitored at various mutation backgrounds. *ptsG* P1 mRNA from *ptsG* and *fis* *ptsG* strains grown with glucose were analyzed as shown in lanes 1 and 2, respectively. *mlc* mutant (lanes 3 and 6) and *fis mlc* double mutant strains (lanes 4, 5, 7, and 8) were grown in the presence (lanes 3-5) or absence (lanes 6-8) of 1% glucose. cAMP (2 mM) was added into the culture media of lanes 5 and 8.

Fig. 2. Regulatory region of *ptsG* gene in *E. coli*.

(A) Schematic diagram of *ptsG* promoter region. The two transcription initiation sites of *ptsG* (7) are shown with arrows. All numberings of this figure are based on the transcription start point of the P1 promoter. The binding sites of two major regulators of *ptsG* expression, CRP and Mlc, are indicated in boxes.

(B) Nucleotide sequences of *ptsG* promoter region. The binding sites of CRP and Mlc are indicated with gray boxes. The five protection regions against DNase I digestion by
Fis binding are shown with dashed lines under the sequence. The best-matched sequence with the proposed 15-bp of highly degenerate Fis binding site, Gnn(c/t)(A/g)(a/t)(a/t)(T/A)(t/a)(T/c)(g/a)nnC (20), is indicated with a dot over the sequence.

Fig. 3. DNase I footprinting analysis
Effect of Fis on the binding of Mlc and CRP at the ptsG promoter. The ptsG promoter DNA fragment, end-labeled on the lower strand, was incubated with individual regulator proteins or in combinations as shown at the bottom of the figure. The products were analyzed on 8% acrylamide gel containing 8 M urea. Final concentrations of the proteins were; 20, 60, and 10 nM of CRP, Fis, and Mlc, respectively. The protection regions by the binding of proteins are indicated with vertical lines on the right side of the figure. The positions of the two hypersensitive cleavages by CRP binding are shown with arrowheads.

Fig. 4. Effects of Fis and CRP-cAMP on RNA polymerase binding to ptsG P1 promoter.
DMS footprinting was performed to investigate RNA polymerase interactions at ptsG P1 promoter. Two nM of supercoiled pGX12 was incubated with proteins as shown at the bottom of the figure and treated with DMS. Changes in DMS reactivity at guanine residues on the top strand of ptsG promoter are indicated with arrows. Concentrations of RNA polymerase, CRP, and Fis were 20 or 40, 40, and 80 nM, respectively.
**Fig. 5.** Effects of Fis on transcription of *ptsG* P1 analyzed by *in vitro* transcription assay.

(A) To examine the effect of Fis on Mlc repression of *ptsG* P1 promoter activity, single-round *in vitro* transcription assay was carried out. The supercoiled template was incubated with CRP (40 nM), Fis (80 nM), and/or Mlc (10 nM). After incubation with 20 nM of RNAP, the reaction was started and stopped by adding NTP solution containing heparin and loading dye, respectively, and analyzed on 6% sequencing gel. The 106/107-nt *rep* transcripts were used as internal controls for normalization of *in vitro* transcription results. (B) Effects of Fis on transcription activation of *ptsG* by CRP-cAMP *in vitro*. Two nM of supercoiled template was incubated with 40 nM of CRP and/or 50 nM of Fis. Subsequently, 5 nM of RNA polymerase was added, and the template was further incubated to form an open complex. After the addition of heparin (200 μg/ml), the reactions were started and stopped by adding NTP solution and loading dye, respectively, and were resolved on 6% sequencing gel.

**Fig. 6.** Fis increases CRP-dependent open complex formation at *ptsG* P1.

(A) To study Fis effect on promoter opening at *ptsG* P1, KMnO₄ footprinting assay was performed. KMnO₄ reactivity patterns at top strand of pGX12 (lanes 1-6) and pGX10 (lanes 7-9) are indicated with arrows. Concentrations of RNA polymerase, CRP, and Fis were 20, 40, and 50 (lanes 3, 5, and 8) and 100 nM (lanes 6 and 9), respectively. The reaction in lane 1 was treated with KMnO₄ in the absence of protein. (B) Effect of Fis on the open complex formation at *ptsG* P1 was studied further using heparin challenge experiment. Two nM of end-labeled DNA fragment carrying only P1 promoter region (+102 ~ -78) was incubated with proteins as shown at the bottom of
the figure. Heparin was added to lanes 5-8 before gel loading. Each protein-DNA complex is indicated with arrow. Concentrations of proteins were 20, 40, and 60 nM of RNA polymerase, CRP, and Fis, respectively.
Fig. 1
Shin et al.
Fig. 2
Shin et al.

A

Mlc II (-175) → P2 (-140) CRP II (-95) CRP I (-40.5) → P1 (+1) ptsG

Fis V (-145) Fis IV (-119) Fis III (-80) Fis II (-19) Fis I (+3)

B

-200 ATCCGTTGAATGAGTTTTTTAAAGCTCGTAATTAATGGCTAAAACGAGTAAAGTTCACC -141

P2

-140 GCCGAAAATTGGCGGTGAATAACCACGTTTGAAATATTGTGACATATGTTTTGTCAAAA -81

Mlc II

Fis V

-80 TGTGCAACCTCTCTCAATGATCTGAAGTTGAAACGTGATAGCCGTCAAACAAATTGGCACT -21

CRP I

Fis III

-20 GAATTATTTTACTGTGAATAAAATAAAAAGGCGCTTAGATGCCCTGTACACGGCGAGGCT +40

Mlc I

Fis I
Fig. 3

Shin et al.
Fig. 4

Shin et al.
Fig. 5
Shin et al.
Fig. 6

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