Heat Shock RNA Polymerase (Eσ32) Is Involved in the Transcription of mlc and Crucial for Induction of the Mlc Regulon by Glucose in Escherichia coli*

Received for publication, February 26, 2001, and in revised form, April 17, 2001
Published, JBC Papers in Press, May 4, 2001, DOI 10.1074/jbc.M101757200

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Mlc is a global regulator of carbohydrate metabolism. Recent studies have revealed that Mlc is depressed by protein-protein interaction with enzyme IICB(32), a glucose-specific permease, which is encoded by ptsG. The mlc gene has been previously known to be transcribed by two promoters, P1(+1) and P2(+13), and have a binding site of its own gene product at +16. However, the mechanism of transcriptional regulation of the gene has not yet been established. In vitro transcription assays of the mlc gene showed that P2 promoter could be recognized by RNA polymerase containing the heat shock sigma factor σ32 (Eσ32) as well as Eσ70, while P1 promoter is only recognized by Eσ70. The cyclic AMP receptor protein and cyclic AMP complex (CRP-cAMP) increased expression from P2 but showed negative effect on transcription from P1 by Eσ70, although it had little effect on transcription from P2 by Eσ32 in vitro. Purified Mlc repressed transcription from both promoters, but with different degrees of inhibition. In vivo transcription assays using wild type and mlc strains indicated that the level of mlc expression was modulated less than 2-fold by glucose in the medium with concerted action of CRP-cAMP and Mlc. A dramatic increase in mlc expression was observed upon heat shock or in cells overexpressing σ32, confirming that Eσ32 is involved in the expression of mlc. Induction of ptsG P1 and pts P0 transcription by glucose was also dependent on Eσ32. These results indicate that Eσ32 plays an important role in balancing the relative concentration of Mlc and EIICB(Glc) in response to availability of glucose in order to maintain inducibility of the Mlc regulon at high growth temperature.

When Mlc is overproduced on a multicopy plasmid in Escherichia coli grown in the presence of glucose, it causes reduction of acetate accumulation and E. coli makes large colonies (1). Mlc has been proposed to be a new global regulator of carbohydrate metabolism (2–5). It has been reported that Mlc regulates manXYZ encoding enzyme II of the mannose PTS (4, 12, 13). Decker et al. (14, 15) have not yet been reported. Mlc is a global regulator of carbohydrate metabolism. Several genes that are necessary to respond to various environmental or nutritional changes require specific recognition by RNA polymerase containing the heat shock sigma factor, σ70 (14, 15). Several genes that are necessary to respond to various environmental or nutritional changes require specific recognition by RNA polymerase associated with the alternative sigma factors, σ32 (16), σE (17), or σ8 (19). The heat shock response in E. coli is mediated by Eσ32 (20) and it is known that expression of at least 26 genes is induced by heat shock in E. coli (21). Many essential genes in E. coli have multiple promoters including one recognized by Eσ32 in order to respond to various environmental conditions (22–24). It has been shown that the pts P0 promoter is recognized by Eσ32 as well as Eσ70 (25) as is expected for a system as central to carbohydrate metabolism as the PTS. In this work, we studied the transcriptional regulation of the mlc gene in vitro as well as in vivo and the role of Eσ32 in maintaining glucose-dependent induction of the Mlc regulon at high growth temperature.

EXPERIMENTAL PROCEDURES

Materials—Cyclic AMP was obtained from Sigma, RNA polymerase saturated with σ70, nucleotide triphosphates, [γ-32P]ATP, and [α-32P]UTP were purchased from Amersham Pharmacia Biotech. The cycle sequencing kit was from Epicentre Technologies (Madison, WI). Bacterial Strains—MC4100 (araD139 ΔargF-lacU169::rpsL105 thiA relA1 flb5301 deoC1 ptsF25 rbsR) was used as a wild type strain in this study. The mlc strain SR505 is the same as KD413 (MC4100, mlc::Tn10Tet). To construct the rpoH-deleted mutant, SR701 (SR702, ΔrpoH), ΔrpoH allele of KY1603 (26) was transferred to SR702

* This work was supported in part by Grant 2000-2-20200-006-3 from the Basic Research Program of the Korea Science & Engineering Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Supported by the Brain Korea21 Project.
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Mlc and Garges (29) in MC4100 cells harboring the pKV10 plasmid were grown on 12872 Eco
cmlc
equilibrated with TGEDN. After the column
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reported in order to repress the P1 transcription (Fig. 3B). For half-
repression of the E.
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expression, a plasmid pKV10 in which the
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ation cloning of the

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promoter region, was made by inserting the DNA segment from

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RESULTS

The mlc Promoter Is Recognized by Both E.
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such as 40 nM CRP, 100 μM CAMP, or 0–1.5 μM Mlc were added to the
reaction as needed. All components except nucleotides were incubated
at 37 °C for 10 min. Transcriptions were started by the addition of
nucleotides 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).
DNA was resolved by electrophoresis on an 8 M urea, 6% polyacrylamide
gel. The amounts of transcribed were measured using a phosphoimage
analyzer, BAS2500 (Fuji Photo Film Co.).

RESULTS

The mlc Promoter Is Recognized by Both E.
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Reactions were done as described by

Plasmid Construction and Preparation—Basic cloning protocols used
were described in Sambrook and Russell (27). Polymerase chain reac-
tion cloning of the

cmlc
terminated with RNase-free condition for

Basic cloning protocols used

were carried out using primers that have unique restriction sites in their sequences. The clones were verified
by DNA sequencing. The supercoiled plasmid pMX, which contains the
mlc
promoter region, was made by inserting the DNA segment from base pair −272 to +105 (all of these numbering are based on the transcription start site of the P1 promoter of mlc in Fig. 1) between the EcoRI and the PstI sites in front of the rpoC promoter in plasmid pSA600 (28). Supercoiled DNA was prepared by Concert kit (Life Tech-
nologies, Inc.) in RNase-free condition for

in vitro

transcription assay.

Primer Extension—Cells were grown aerobically at 30 °C in tryptone broth (1% Bacto-tryptone, 0.8% NaCl) either in the presence or absence of
0.2% glucose. At A600 = 0.5, growth temperature was shifted to 42 °C
and incubation was continued for the designated time and total E.
ccoli RNA was purified using Trizol reagent (Life Technologies, Inc.) to study the heat shock effects on mlc and pshG transcription. Purified RNA was
resuspended in sterile distilled water. To study mlc transcription, 32P-
labeled primer MLR (5′-ATGATTATGAGTCGGCAGCGTGTGTTGACTG-GCT-3′), which is complementary to +162 to +192, was co-precipitated with 40 μg of total cell RNA. The primer PMG (5′-AATGGATAATGCTGCTTGGATT-GGTGCT-3′, complementary to +74 to +102) and the primer PM6 (6) were co-precipitated with 30 μg of total RNA to study pshG and pshT transcription, respectively. 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 (29).

Purification of RNA Polymerase Holoenzyme Containing σ70—E.
ccoli MC4100 cells harboring the pBK10 plasmid were grown on 2 × LB (1%
Bacto-tryptone, 0.5% yeast extract, 2% NaCl) in a 5-liter fermenter at
30 °C. At A600 = 1.0, 1 mM isopropyl-β-D-thigalactopyranoside (IPTG)
and 0.2 M NaCl were added to the cultures. The cells were harvested 10 min after a temperature shift from 30 to
42 °C at A600 = 2.5 in late exponential growth phase. RNA polymerase
was purified by fast protein liquid chromatography according to Harger
et al. (30) with some modifications as described by Sukhodolts et al.
(31). Crude RNA polymerase was obtained from 10 g of wet cell paste by
Polymin P precipitation and the drained pellet was solubilized with
TGED (10 mM Tris, pH 7.9, 5% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM
dithiothreitol) containing 0.2 mM NaCl (TGED3). This enzyme solution
was loaded on a single-stranded DNA-agarose column (Amersham
Pharmacia Biotech) pre-equilibrated with TGED6. After the column
was washed with 3 column volumes of TGED5, RNA polymerase was
eluted with a linear salt gradient (from TGED3 to TGED containing 1.0
mM NaCl) at a flow rate of 2 ml/min. The elution of RNA polymerase
was monitored by the presence of β2′ on a sodium dodecyl sulfate-polyacry-
lamide gel. The fractions containing RNA polymerase (from 0.3 to 0.5
mM NaCl gradient) were loaded onto a 5/5 Mono-Q column (Amersham
Pharmacia Biotech), and RNA polymerase containing σ70 was eluted
with a linear gradient of NaCl (0.3 to 0.5 M) in TGED at 1 ml/min. The
yield for purified RNA polymerase was 1.5 mg.

In Vitro Transcription Assay—Reactions were done as described by
Ryu and Garges (29) in a 25-μl volume containing the following: 20 mM
Tris acetate, pH 8.0, 3 mM magnesium acetate, 200 mM potassium
phosphate, 1 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 0.2 mM CTP, 0.02 mM UTP, 10 μCi of [α-32P]UTP (800 Ci/mmol), 2 nmol supercoiled
dNA template, 20 mM RNA polymerase saturated with σ70 or σ70, 100
μg/ml bovine serum albumin, and 5% glycerol. Additional regulators
of mlc expression, a plasmid pKV10 in which the

cmlc
sequence is based on the transcription start point of the P1 promoter.

Regulation of mlc in E.
ccoli

FIG. 1. Nucleotide sequences of the promoter region of mlc. The transcription
start points of P1 (+1) and P2 (+3) are shown by arrows. The −10 and −35 region of the P1 promoter is underlined and that of P2 is indicated with a line over
the sequence. The known consensus −10 and −35 sequence recognized by E.
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repressed by CRP and Mlc is shown as box. The start codon of mlc, GTP, is also indicated. All of the numbering of this sequence is based on the transcription start point of the P1 promoter.

FIG. 2. The effect of CRP-CAMP on mlc transcription in vitro.

The supercoiled DNA template, pMX, was used for the in vitro transcription
by E.
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repressed by E.
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(lanes 1 and 2) and E.
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( lanes 3 and 4) in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of the CRP-CAMP complex. The transcriptions from the plasmid origin of replication (106/107 nucleo-
tides) are marked as rep. The 132-nucleotide transcript from P1 and the 120-nucleotide transcript from P2 are indicated.
MC4100 and its isogenic mutant strains were grown at 30 °C in the presence or absence of glucose then total RNA was extracted as described under “Experimental Procedures.” Transcription from both P1 and P2 promoter was detected and the level of expression from two promoters was similar when the wild type cells were grown in the absence of glucose (Fig. 4A, lane 2). However, when cells were grown in the presence of glucose, transcription from P1 was increased slightly while that from P2 was not detectable (Fig. 4A, lane 1). These results establish that P1 and P2 promoter activity was dependent on CRP-cAMP in vivo in accordance with the in vitro transcription assay results as described above and that the overall expression level of mlc was changed less than 2-fold by glucose.

We analyzed the mlc expression in SR505 (MC4100, mlc::Tn10) to test the autoregulatory effect of Mlc in vivo. The level of transcription could be analyzed in SR505 because Tn10 was inserted in the C-terminal region of the mlc gene. Transcription from both promoters was increased clearly when the mlc::Tn10 strain was grown in the absence of glucose (Fig. 4A, compare lanes 2 and 4), suggesting that Mlc acts as a repressor of the mlc promoter. A strong stimulatory effect of CRP-cAMP on mlc P2 promoter activity could also be seen when we compared the P2 promoter activities in SR505 grown in the presence and absence of glucose (Fig. 4A, lanes 3 and 4).

The P2 Promoter of mlc Is a Bona Fide Heat Shock Promoter—In vitro transcription assay showed that the P2 promoter of mlc was recognized by Erp32 as well as Erp20. We analyzed expression of mlc in the ΔrpoH strain using primer extension assay. SR701 strain (MC4100, suxX1, ΔrpoH) can grow at 30 °C because it expresses a high level of GroELS due to an IS31 element inserted upstream of the groE gene (6, 26). Overexpression of GroELS did not affect mlc expression (data not shown). As shown on Fig. 4A (lanes 5 and 6), the expression level of mlc was decreased but the overall expression pattern was not changed in the ΔrpoH cell compared with wild type. However, P2 transcription was increased and its activity was not much affected by glucose in cells overexpressing σ32 (Fig. 4A, lanes 7 and 8). It is also interesting to note that even the activity of P1 that is not recognized by Erp32 in vitro was increased significantly when cells overexpressing Erp32 was grown in the presence of glucose (Fig. 4A, lane 7).

We also examined changes in the mlc expression upon heat shock by primer extension analysis. Growth temperature of a wild type strain, MC4100, was shifted from 30 to 42 °C, and cells were further incubated for the designated time as described under “Experimental Procedures.” As shown on Fig. 4B, temperature upshift caused dramatic changes in the mlc expression. The mlc expression reached the maximum level 5 min after heat shock (Fig. 4B, lanes 3 and 4), when the intracellular concentration of Erp32 is known to be at the highest level (20, 32). When cells were grown in the presence of glucose (Fig. 4B, odd lanes), expression of both P1 and P2 was increased transiently after heat shock as in the cells overexpressing σ32 (Fig. 4A). The increased level of mlc expression from both P1 and P2 upon heat shock was decreased sharply after 15 min at 42 °C in the presence of glucose. Activation of mlc by heat shock was not dependent on the presence of glucose. The cells grown in the absence of glucose showed relatively little change in the P1 transcription but a high level of activation in the P2 transcription in response to heat shock (Fig. 4B, even lanes). However, heat shock effect was not observed in the ΔrpoH strain (data not shown), confirming that activation of mlc transcription by heat shock was dependent on Erp32. These results show that Erp32 can recognize the P2 promoter in vivo and Erp32-directed transcription from P2 becomes more prominent when cells were grown at a high temperature or overexpressing σ32.

Transcription of ptsG Is Increased by Heat Shock and Glucose Induction of ptsG Requires Erp32—How does the highly increased level of mlc expression in heat-shocked cells affect glucose induction of Mlc regulon? To answer this question, we analyzed changes in the ptsG transcription upon heat shock. It has been known that transcription of the ptsG gene in E. coli encoding the major membrane-bound glucose transporter, EIICB^Glc, is initiated from a major promoter, P1, and a minor
promoter, P2, and that both P1 and P2 transcription is regulated negatively by Mlc (3, 5). There were no changes in P2 transcription of ptsG by heat shock (data not shown). But P1 transcription of ptsG was increased 5 min after heat shock when wild type cells were grown in the presence of glucose (Fig. 5A, compare lanes 1 and 3) despite that mlc expression was also increased at this condition as shown in Fig. 4B. However, ptsG P1 transcription was not changed by heat shock when cells were grown in the absence of glucose. Activation of ptsG P1 transcription by heat shock was also seen in the Δmlc strain (Fig. 5A, lane 7), suggesting the possibility that Mlc was not involved in the activation of ptsG P1 by heat shock. In that expression of both EIICB\[^{\text{Glc}}\] and its negative regulator Mlc was increased by heat shock, it seems that there is another mechanism(s) to maintain glucose induction of the Mlc regulon under the heat shock condition.

As shown on Fig. 5A, induction of ptsG P1 transcription by glucose was reduced significantly in the ΔrpoH strain. From these results, together with our previous data (9), it could be assumed that the Mlc regulon could not be induced by glucose because not enough EIICB\[^{\text{Glc}}\] was available to sequester Mlc in the ΔrpoH strain. As expected, pts P0, one of several genes known to be under Mlc control (6–8), was not induced in the ΔrpoH strain (Fig. 5B). These results support the view that the expression of EIICB\[^{\text{Glc}}\] and Mlc is balanced in response to glucose uptake even when the cells were heat-shocked and that Er\[^{32}\] plays an important role in this regulation. However, it should be noted that heat shock still increased P1 transcription of ptsG in the ΔrpoH strain albeit to a lower degree when cells were grown in the presence of glucose (Fig. 5A).

**DISCUSSION**

It has been suggested that the mlc gene has two promoters, P1 and P2, which are separated by 12 bases and autoregulated by its product (2). Here, we report that the transcription of the mlc gene is regulated in a highly sophisticated manner and that heat shock α factor, α\[^{32}\], is involved in its transcription.

It is known that the expression level of several genes encoding transcriptional repressors such as galS (33), nagC (34), purR (35), and trpR (36) of E. coli is low and that their expression level is not modulated much in various growth conditions. It seems likely that both CRP-cAMP and Mlc work together in E. coli to maintain the level of Mlc optimum in response to availability of glucose. All genes known to be regulated negatively by Mlc, such as manXYZ, malT, ptsG, and pts, are also regulated positively by CRP-cAMP (2–8). *In vitro* transcription assay with Er\[^{32}\] and CRP-cAMP showed two opposite effects on each promoter of mlc, that is the positive effect on P2 and the negative effect on P1 (Fig. 2). This can be explained based on the fact that the CRP-binding site of P2 centered at −71.5 to the transcription start site is more compatible for a functional CRP site (37) compared with that of P1 centered at −58.5 to the transcription start site. However, Er\[^{32}\]-directed P2 transcription was insensitive to CRP-cAMP. The P2 promoter of mlc should be a good model system to assess the effect of σ factor on transcription activation by CRP-cAMP because it has been known that CRP-cAMP activates transcription by direct protein-protein interaction with the α-subunit of RNA polymerase (38).

Repression of the P2 transcription when cells were grown in the presence of glucose implies that the action of CRP-cAMP is dominant over the self-repression by Mlc in the regulation of the mlc P2 promoter. The low binding affinity of Mlc to its own promoter that is 10 times weaker than that to ptsG or pts P0 promoters (9) seems to be a major reason for the low influence of Mlc on regulation of its own gene. *In vitro* transcription assay revealed that each promoter of mlc has a different sensitivity to Mlc (Fig. 3). When cells were grown in the absence of glucose, a similar level of expression from both P1 and P2 was observed even though the P2 transcription is more sensitive to Mlc repression probably because the intracellular concentration of Mlc is limiting in *E. coli* (3). The condition seems to be similar to the *in vitro* transcription condition where both CRP-cAMP and a small amount of Mlc were present as shown in lane 6 of Fig. 3A. However, P1 was as active as P2 when the mlc strain was grown in the absence of glucose. These results imply that the concentration of intracellular CRP-cAMP is lower than that of CRP-cAMP used for *in vitro* transcription reactions (40 nM) (39). When Mlc was induced and the concentration of CRP-cAMP was lowered by the addition of glucose in the growth medium, the P1 promoter was activated slightly while the P2 promoter was repressed because the P2 promoter is active only in the presence of CRP-cAMP. This situation is similar to the *in vitro* transcription condition where neither CRP-cAMP nor Mlc were present (lane 1 of Fig. 3A). Therefore, the addition of glucose in the growth medium resulted in the reduction of mlc expression by about half. These results also agree with the previous report by Decker et al. (2) that expression of mlc is reduced by half when cells were grown in the presence of glucose by measuring the β-galactosidase activity of the mlc-lacZ fusion. Level of Mlc expression can vary precisely in response to the available sugars but the variation range is less than 2-fold in that the availability of unphosphorylated EIICB\[^{\text{Glc}}\] may be more critical than the intracellular level of Mlc for induction of the Mlc regulon by glucose as shown in our previous report (9). Er\[^{32}\] is involved in the transcription of the mlc gene. *In vitro* transcription assay with Er\[^{32}\] showed that Er\[^{32}\] could recognize the P2 promoter of the mlc gene. Transcription of P2 was increased when σ\[^{32}\] was overexpressed (Fig. 4A). Moreover, mlc expression was increased upon heat shock. It is known that the intracellular concentration of σ\[^{32}\] in *E. coli* increases from 15–20-fold within 5 min then declines to a new steady-state level severalfold higher than the preshift level in response to tem-
temperature shift from 30 to 42 °C (20, 32). Transcription from P2 recognized by Eo32 was induced transiently to an extraordinary level upon heat shock when cells were grown in the absence of glucose (Fig. 4B, lane 4). The level of mle transcription was changed parallel to the changes in intracellular concentration of o32. In addition, P2 transcription was activated upon heat shock even when cells were grown in the presence of glucose. These results imply that the major RNA polymerase which activated the P2 transcription upon heat shock was Eo32 because Eo32 was less sensitive to Mlc than Eo70 and the P2 transcription by Eo32 was not dependent on CRPcAMP as revealed by the in vitro transcription assay (Fig. 3). It is not clear why the P1 transcription was reduced in the ΔrpoH strain and activated by heat shock or when cells overexpressing o32 were grown in the presence of glucose even though P1 promoter was not recognized by Eo32 in vitro. Because heat shock should exert pleiotropic effects by regulating transcription of various genes (21), further study on the mechanism of heat shock is needed for a better understanding of these phenomena.

To investigate whether the increased level of mle expression resulting from heat shock can influence Mlc-dependent gene expression, we analyzed changes in ptsG expression by heat shock. The P1 expression of ptsG was increased significantly by heat shock only when cells were grown in the presence of glucose regardless of the presence of Mlc (Fig. 5A). These results suggest that activation of ptsG P1 by heat shock was not mediated by Mlc or CRPcAMP even though Mlc repression might be dominant over activation of ptsG P1 by heat shock. We have reported that the unphosphorylated form of EIICBG3e sequesters Mlc from its target promoters upon glucose uptake by direct protein-protein interaction (9). Therefore, glucose is required to maximize the level of dephosphorylated EIICBG3e necessary to sequester Mlc that is increased by heat shock. However, contrary to the case of mle P2 transcription in which Eo32 plays a major role in its regulation, it is likely that additional factors independent of Eo32 are involved in regulation of the ptsG expression because ptsG P1 expression was increased partially upon heat shock even in the ΔrpoH strain grown in the presence of glucose. It means that two separate mechanisms involving Eo32-dependent and Eo32-independent activation of ptsG P1 may work additively for full activation of ptsG P1 by glucose when cells were heat-shocked. The importance of Eo32 in glucose induction of the Mlc regulon was manifested by the fact that glucose induction of ptsG could not be observed in the ΔrpoH strain. The inability of glucose to activate ptsG expression resulted in an insensitivity of the pts P0 promoter to glucose in ΔrpoH strain. We are trying to elucidate the mechanism of activation of ptsG transcription by heat shock in the absence of Eo32 in order to understand the general role of Eo32 in regulation of genes involving carbohydrate metabolism.

Acknowledgments—We are grateful to Dr. C. Park for providing KD413 and Dr. T. Yura for providing bacterial strains and a pKV10.

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doi: 10.1074/jbc.M101757200 originally published online May 4, 2001

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