

Mechanism of the down-regulation of cAMP receptor protein by glucose in *Escherichia coli*: role of autoregulation of the *crp* gene

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Glucose causes catabolite repression by lowering the intracellular levels of both cAMP and cAMP receptor protein (CRP) in *Escherichia coli*. The molecular mechanism underlying the down-regulation of CRP by glucose has been investigated. We show that glucose lowers the level of *crp* mRNA without affecting its stability. Replacement of the *crp* promoter with the *bla* promoter almost completely abolishes the glucose-mediated regulation of *crp* expression. Only a slight reduction in the *crp* expression by glucose is observed in *cya*⁻ or *crp*⁻ strains, suggesting that a CRP–cAMP complex is needed for this regulation. We previously showed that transcription of the *crp* gene is regulated both negatively and positively. Positive autoregulation of *crp* is caused by the binding of CRP–cAMP to the CRP binding site II located upstream of the *crp* promoter. Here we show that disrupting the CRP binding site II essentially eliminates the down-regulation of *crp* expression by glucose. We conclude that the autoregulatory circuit of the *crp* gene plays a key role in the down-regulation of CRP by glucose.

Key words: autoregulation/catabolite repression/CRP–cAMP/*crp* expression/transcriptional activation

Introduction

The inhibition of gene expression by glucose in culture medium has been a paradigm for understanding how genes are regulated in response to nutrient status in bacteria. Glucose affects gene expression primarily at the transcriptional level by regulating the level or activity of transcriptional regulators (reviewed by Ullman and Danchin, 1983; Magasanik and Neidhardt, 1987; Kolb *et al.*, 1993).

In the case of the *Escherichia coli* lactose operon, glucose lowers transcription by increasing the level of free *lac* repressor and/or by decreasing the level of activator, CRP (cAMP receptor protein)–cAMP complex. First, glucose inhibits the uptake of inducer into the cell, which in turn increases the level of active free repressor. The inhibition by glucose of inducer uptake occurs by a mechanism called inducer exclusion whereby a dephosphorylated form of enzyme IIA (or enzyme III) specific for glucose, one of the components of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase

system (PTS), appears to play an essential role (reviewed by Postma *et al.*, 1993). Second, glucose represses *lac* transcription by lowering the concentration of the CRP–cAMP complex. This repression is independent of the repressor-mediated negative regulation, since it occurs both in the presence and absence of repressor (Ishizuka *et al.*, 1993). We showed previously that the major part of catabolite repression can be explained by the lowered concentration of the CRP–cAMP complex (Ishizuka *et al.*, 1993).

It had been believed that glucose lowers the level of CRP–cAMP complex only by reducing intracellular cAMP level (reviewed by Ullman and Danchin, 1983; Magasanik and Neidhardt, 1987; Kolb *et al.*, 1993). However, the actual reduction of cAMP level by glucose is too moderate to account for catabolite repression (Pastan and Adhya, 1976; Ishizuka *et al.*, 1993). Although the intracellular CRP level could be another determinant of the level of active CRP–cAMP complex, it had not been seriously tested whether the level of CRP is involved in the modulation of catabolite repression. Recently, we addressed this question by determining the intracellular CRP levels under various conditions. In the previous paper, we showed that the intracellular CRP level is markedly reduced by glucose and that this reduction in CRP level is an important determinant of catabolite repression (Ishizuka *et al.*, 1993). We proposed that glucose causes catabolite repression by reducing both intracellular CRP and cAMP levels.

In this paper we investigate the mechanism by which glucose lowers the intracellular CRP level. We found that glucose reduces the level of *crp* mRNA without affecting its stability. The *crp* promoter is needed for this down-regulation, suggesting that the regulation occurs at the step of transcription initiation. The effect of glucose on the *crp* transcription was mostly lost in *cya*⁻ or *crp*⁻ cells, indicating that CRP–cAMP is required for this regulation. Moreover, the disruption of the CRP binding site located upstream of the *crp* promoter essentially eliminated the effect of glucose. These data indicate that the autoregulatory loop of the *crp* gene plays a central role in the down-regulation of *crp* expression by glucose.

Results

Glucose lowers the expression of *crp* on a multicopy plasmid

We found recently that the presence of glucose in the culture medium lowers the CRP level 2- to 3-fold in exponentially growing wild-type cells (Ishizuka *et al.*, 1993). Here we tested the effect of glucose on the expression of *crp* in a multicopy plasmid pHA5 carrying the entire *crp* region in a *crp*⁻ strain PP47. The total proteins were prepared from cells growing exponentially

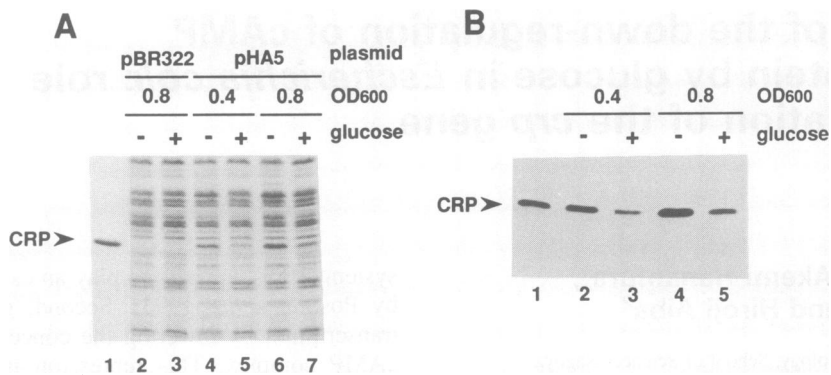


Fig. 1. Effect of glucose on the expression of *crp* in a multicopy plasmid. Total proteins from PP47 transformants grown to $OD_{600} = 0.4$ and 0.8 were analyzed. (A) SDS-PAGE of total protein (12 μ g). Lane 1 contains 0.5 μ g of purified CRP. The arrowhead indicates the CRP bands. (B) Western blotting of total protein (32 ng). Lane 1 contains 1 ng of purified CRP.

in LB medium with or without glucose and analyzed by SDS-PAGE. CRP was detected on a gel stained with Coomassie brilliant blue when cells harboring pHA5 were analyzed (Figure 1A, lanes 4–7). Densitometric scanning of the stained gel revealed that the relative level of CRP decreased 2- to 3-fold in the presence of glucose. Cells harboring a control plasmid pBR322 showed no detectable CRP band (Figure 1A, lanes 2 and 3). The reduction in CRP was also detected by Western blot analysis with the same samples (Figure 1B). The plasmid copy number was not affected by the presence of glucose in the medium (data not shown). Thus, glucose lowers the expression of *crp* in a multicopy plasmid to the same extent observed with the chromosomal *crp* gene (Ishizuka *et al.*, 1993).

Glucose lowers the *crp* mRNA level

The lowered level of CRP in cells grown on glucose-containing medium could be due to destabilization of the CRP protein and/or a decrease in the level of *crp* mRNA. To determine the steady-state levels of *crp* mRNA, total RNAs from cells harboring pHA5 grown on LB medium with or without glucose were analyzed by a quantitative S1 nuclease protection assay, using the lower strand of a 910 bp *Hpa*II fragment 32 P-labeled at its 5' end as a DNA probe (Figure 2B). An S1-resistant DNA band of 497 bases that corresponds to *crp* RNA was produced in this assay. When the RNA from cells grown in the presence of glucose was used, the S1-resistant band was markedly reduced relative to the band protected by RNA from cells grown in the absence of glucose (Figure 2A). The extent of the reduction in *crp* mRNA by glucose was essentially identical to that of the CRP protein. This suggests that the lowered level of CRP in glucose-containing medium is primarily due to the reduction in *crp* mRNA. We have not tested whether the stability of CRP protein is affected by glucose.

Glucose does not affect the stability of *crp* mRNA

How does glucose lower the steady-state level of *crp* mRNA? Glucose may destabilize the *crp* mRNA or it may affect the transcription of *crp*. To test the effect of glucose on the stability of *crp* mRNA, the rate of decay of *crp* mRNA was determined in exponential pHA5-containing cultures grown with or without glucose, after adding rifampicin to prevent further initiation of transcrip-

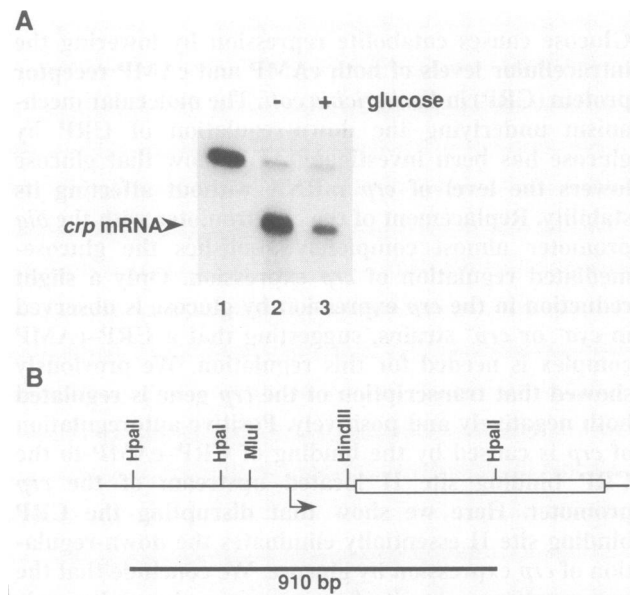


Fig. 2. Effect of glucose on steady-state level of *crp* mRNA. (A) Autoradiograph of S1 products. Total RNA (10 μ g) prepared from PP47 cells harboring pHA5 grown to an OD_{600} of 0.8 was subjected to S1 assay. The arrowhead indicates S1-resistant DNA bands. Lane 1, DNA probe without S1 treatment. (B) The upper line shows a restriction map of the *crp* region. The open bar indicates the coding region of *crp*. The arrow denotes the start and direction of *crp* transcription. The lower line shows the 910 bp *Hpa*II–*Hpa*II fragment used as a DNA probe.

tion. Total RNA was isolated at various times after the addition of rifampicin for the quantitative S1 assay (Figure 3A). The half-life of *crp* RNA was estimated to be 2.4 min both in the presence and absence of glucose by quantifying the intensity of the S1-resistant DNA (Figure 3B). Thus, glucose does not affect the stability of *crp* mRNA. This suggests that glucose may inhibit the transcription of *crp*.

The down-regulation of *crp* by glucose is dependent on the *crp* promoter

To gain further insight into the mechanism of *crp* down-regulation by glucose, we examined the effect of glucose on *crp* expression from the *bla* promoter in pHA7. Interestingly, the inhibitory effect of glucose on *crp* expression in cells harboring pHA7 was no longer observed

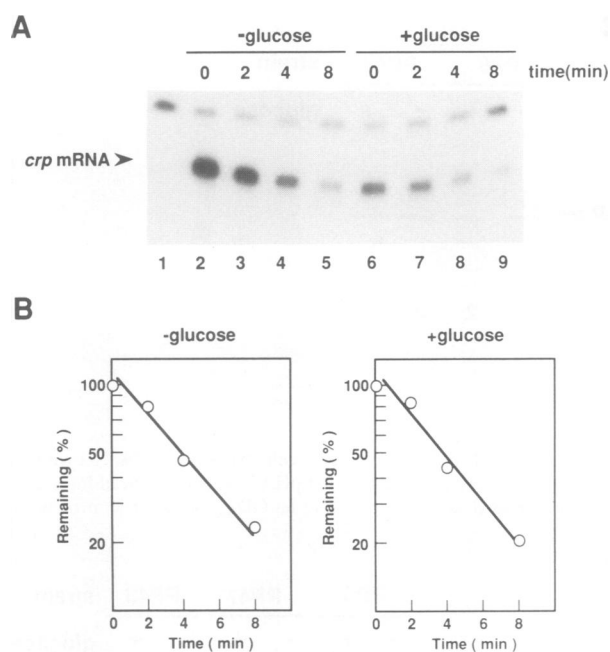


Fig. 3. Effect of glucose on the stability of *crp* mRNA. (A) Autoradiograph of S1 products. PP47 cells harboring pHA5 were grown to an OD_{600} of 0.8 and total RNA was extracted at the times indicated after the addition of rifampicin (50 μ g/ml). Total RNA (50 μ g) was subjected to S1 assay. The arrowhead indicates the S1-resistant DNA bands. Lane 1, DNA probe without S1 treatment. (B) Semilogarithmic plots of the radioactivity of S1-resistant DNA versus time. The DNA bands were quantified using a Bioimage Analyzer

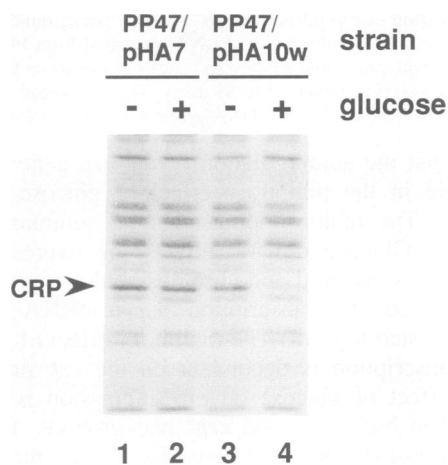


Fig. 4. Down-regulation of *crp* expression by glucose is dependent on the *crp* promoter. SDS-PAGE of total proteins from PP47 cells harboring the indicated plasmid grown to an OD_{600} of 0.8. The arrowhead indicates the CRP bands.

(Figure 4, lanes 1 and 2). This suggests that the down-regulation of *crp* by glucose occurs primarily at the initiation of transcription and that the *crp* promoter is needed. Further tests were made with a new plasmid, pHA10w (Figure 7A), in which the *bla* promoter in pHA7 was replaced by a 310 bp DNA fragment containing the wild-type *crp* promoter (see Materials and methods). The expression of CRP in cells harboring pHA10w was clearly repressed by glucose as in cells harboring pHA5 (Figure 4, lanes 3 and 4). Most of the down-regulation of *crp* by glucose is therefore dependent on the *crp* promoter.

However, experiments revealed that glucose reproducibly lowers the CRP level by ~10% even in pHA7 transformants. This small effect of glucose seems to play a role in the down-regulation of *crp* expression (see Discussion).

Functional CRP-cAMP mediates the inhibitory effect of glucose on *crp* expression

Having established the role of the *crp* promoter in the down-regulation of *crp* by glucose, it was interesting to examine the effect of cAMP on this regulation because the *crp* is autoregulated at the level of transcription (Aiba, 1983; Hanamura and Aiba, 1991, 1992). The effect of cAMP was tested by introducing pHA5 into a *cya*⁻ strain PP48 and analyzing total proteins by SDS-PAGE. The repressive effect of glucose on *crp* expression was lost mostly in the *cya* mutant (Figure 5A, lanes 3 and 4) while glucose again reduced the CRP level in *cya*⁺ cells (PP47) harboring pHA5 (Figure 5A, lanes 1 and 2). Western blot analysis confirmed that glucose represses expression of the chromosomal *crp* gene in the wild-type but not in the *cya* mutant (Figure 5B). This strongly indicates that cAMP plays a key role in the down-regulation of *crp* by glucose.

To explore the role of CRP itself in this regulation, the effect of glucose on the expression of *crp* mRNA in wild-type (PP6), *cya*⁻ (PP48) and *crp*⁻ (PP47) cells was examined by S1 assay. PP47 seems to have a point mutation in the *crp* structural gene and it produces a mutant *crp* mRNA that can be detected by S1 assay (Mori and Aiba, 1985). As shown in Figure 6, the reduction in *crp* mRNA by glucose was small in *crp*⁻, whilst glucose markedly reduced the *crp* mRNA in wild-type cells. As expected, the level of *crp* mRNA in *cya*⁻ cells was also slightly reduced by glucose (Figure 6, lanes 6 and 7). It is therefore concluded that functional CRP-cAMP complex mediates the repressive effect of glucose on *crp* expression. This strongly suggests that the autoregulatory mechanism controlling the *crp* gene is closely involved in the down-regulation of *crp* by glucose.

Mutations in the upstream CRP binding site in the *crp* promoter region abolish the effect of glucose

One simple explanation for the requirement for the *crp* promoter and CRP-cAMP for the effect of glucose on *crp* expression would be to propose that the activation of *crp* transcription by CRP-cAMP is reduced in the presence of glucose while the positive autoregulation of *crp* transcription is efficiently operating in the absence of glucose. If this were the case, the disruption of the CRP binding site II, which is located upstream of the *crp* promoter and responsible for the activation of *crp* transcription by CRP-cAMP (Hanamura and Aiba, 1992), should lower the expression of *crp* in the absence of glucose and eliminate the down-regulation of *crp* by glucose. To test this assumption, we have constructed deletion mutants in which the deletions extend into the CRP binding site II from upstream of the *crp* promoter (Figure 7). The effects of glucose on the expression of *crp* from the corresponding plasmids were analyzed in transformants of PP47. The repressive effect of glucose on *crp* expression was normally observed in cells harboring pHA100 that still retains the CRP binding site II (Figure 8A, lanes 3 and 4). When the deletion extends into the CRP binding site II, the *crp*

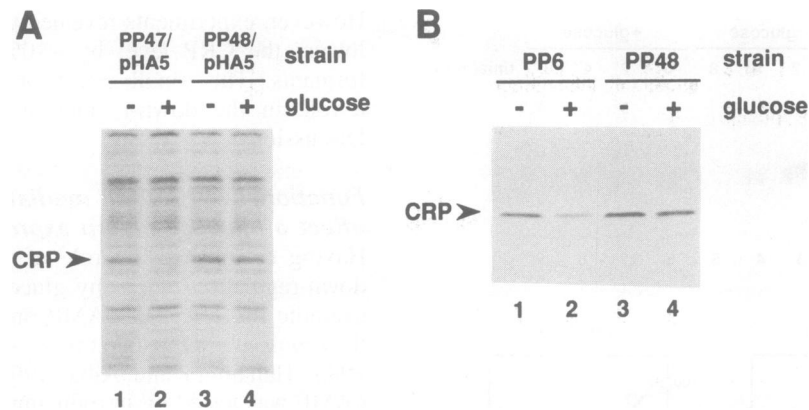


Fig. 5. Role of cAMP in the down-regulation of *crp* expression by glucose. (A) SDS-PAGE of total proteins from cells harboring pHA5 grown to an OD₆₀₀ of 0.8. Lanes 1 and 2, PP47 (*crp*⁻ *cya*⁺) cells harboring pHA5. Lanes 3 and 4, PP48 (*cya*⁻) cells harboring pHA5. The arrow head indicates CRP bands. (B) Western blotting of total proteins (0.63 µg) from PP6 (*crp*⁺ *cya*⁺) and PP48 (*cya*⁻) cells grown to an OD₆₀₀ of 0.8. The arrowhead indicates the CRP bands.

expression was significantly reduced even in the absence of glucose and the repressive effect of glucose on *crp* expression was almost eliminated (Figure 8A, lanes 5–8). With pHA10m, which carries point mutations (Figure 7B) in the CRP binding site II that prevent the transcriptional activation by CRP–cAMP (Hanamura and Aiba, 1992), the CRP level was clearly reduced relative to pHA10w in the absence of glucose (Figure 8B). Moreover, the repressive effect of glucose on *crp* expression was not observed (Figure 8B, lanes 3 and 4). All of these results are consistent with the view that the lowered expression of *crp* in the presence of glucose is due to the loss of activation of *crp* transcription by CRP–cAMP.

Discussion

In *E.coli*, glucose inhibits the synthesis of a number of proteins that are required for the metabolism of other carbon sources by several different mechanisms (reviewed by Ullman and Danchin, 1983; Magasanik and Neidhardt, 1987; Kolb *et al.*, 1993). One of the major effects of glucose is inducer exclusion whereby glucose prevents the uptake of inducer into the cell. The lowered concentration of inducer increases the level of active repressor leading in turn to greater repression of the target genes. Another moderate inhibitory effect of glucose is catabolite repression that is independent of the repressor/inducer system. The original model of catabolite repression, which has dominated many textbooks for a long time, argues that glucose causes catabolite repression by reducing intracellular cAMP level. However, this idea has been challenged by several reports, arguing that cAMP is not the sole mediator of catabolite repression (reviewed by Ullman and Danchin, 1983; Magasanik and Neidhardt, 1987). Our recent finding that glucose lowers the intracellular CRP level has settled this apparent controversy (Ishizuka *et al.*, 1993). In the previous study, we showed that glucose causes catabolite repression of the lactose operon by reducing both the CRP and cAMP levels. Thus, catabolite repression can be explained by a lowering of the concentration of the transcriptional activator, CRP–cAMP.

How does glucose reduce the CRP level? We have

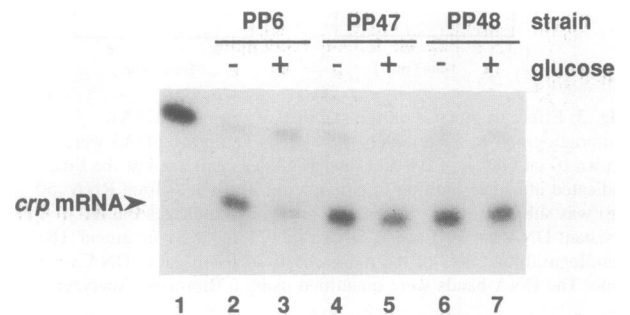


Fig. 6. Expression of *crp* mRNA in *cya*⁻ and *crp*⁻ backgrounds. Autoradiograph of S1 products. Total RNAs prepared from PP6 (*crp*⁺ *cya*⁺), PP47 (*crp*⁻ *cya*⁺) and PP48 (*crp*⁺ *cya*⁻) grown to an OD₆₀₀ of 0.8. RNA (80 µg) was subjected to S1 assay. The arrowhead indicates S1-resistant DNA bands. Lane 1, DNA probe without S1 treatment.

observed that the autoregulation of the *crp* gene plays a critical role in the inhibitory effect of glucose on *crp* expression. The major findings can be summarized as follows. (i) Glucose down-regulates the expression of both multicopy plasmid and chromosomal *crp* genes. (ii) Glucose reduces the transcription of *crp* mRNA without affecting its stability. (iii) The inhibitory effect of glucose on *crp* transcription is dependent on the *crp* promoter. (iv) The effect of glucose on *crp* expression is mostly eliminated in both *cya*⁻ and *crp*⁻ backgrounds. In other words, functional CRP–cAMP is needed for the down-regulation of the *crp* transcription by glucose. (v) Mutations in the CRP binding site II in the *crp* promoter region eliminate the effect of glucose.

We therefore propose the following mechanism for the lowering of CRP level by glucose (Figure 9). The addition of glucose to the culture medium causes a reduction in the intracellular cAMP level. This is presumably due to the decreased level of the phosphorylated enzyme IIA of PTS that appears to be an activator of adenylate cyclase (Postma *et al.*, 1993). The PTS-dependent transport of glucose is known to reduce the level of phosphorylated enzyme IIA (Postma *et al.*, 1993). In addition, the intracellular cAMP level is strongly reduced by CRP (Botsford and Drexler, 1978) due to the negative regulation of adenylate cyclase by CRP (Aiba, 1985; Mori and Aiba, 1985; Botsford and Harman, 1992; Postma *et al.*, 1993).

In addition, it has been shown that the intracellular cAMP level is inversely proportional to the CRP level (Ishizuka *et al.*, 1993; unpublished data). Therefore, in cells harboring a multicopy *crp* plasmid, the cAMP level is extremely low and the addition of glucose further reduces the cAMP level (Ishizuka *et al.*, 1993). The reduction in cAMP level by glucose should lead to the reduction in CRP-cAMP

level. This in turn reduces the extent of activation of the *crp* gene by CRP-cAMP. We believe that in the presence of glucose the level of CRP-cAMP complex is not sufficient to occupy the CRP binding site II, especially as we have previously shown that the CRP binding site II has a lower affinity for CRP-cAMP than the downstream CRP binding site I (Hanamura and Aiba, 1992). In such conditions, the transcription of *crp* may be moderately repressed by the binding of CRP-cAMP to the downstream CRP binding site I. Upon depletion of glucose, the cAMP level would increase resulting in the increased level of CRP-cAMP complex to allow the occupation of CRP-cAMP at the CRP binding site II, and the transcription of *crp* is activated. This autoregulatory loop can easily explain how glucose reduces the CRP level.

Although the down-regulation of *crp* expression by glucose can be explained by the autoregulatory loop, we also noticed that glucose slightly but consistently reduces the level of CRP independently of cAMP or the *crp* promoter. This small effect (estimated at ~10%) may act as an initial trigger along with the reduced cAMP level for the promoter-dependent down-regulation of *crp* by glucose. A possible mechanism for the additional effect of glucose would be a change in the helical density of DNA (Balke and Gralla, 1987).

In addition to the positive autoregulation, the *crp* gene is also negatively regulated by CRP-cAMP (Aiba, 1983). The negative regulation of *crp* transcription by CRP-cAMP is mediated by the CRP binding site I located 40 bp downstream from the *crp* start site. The binding of CRP-cAMP to CRP binding site I represses *crp* transcription by stimulating the binding of RNA polymerase to the divergent overlapping promoter and thus preventing the occupation of RNA polymerase at the *crp* promoter (Hanamura and Aiba, 1991). It should be noted that in the presence of glucose the levels of CRP and *crp* mRNA in *cya⁻* cells are significantly higher than those in wild-type cells (Figures 5 and 6). This suggests that in the presence of glucose the *crp* transcription is moderately repressed by CRP-cAMP in wild-type cells while the inhibition of *crp* transcription is eliminated in *cya⁻* cells. We believe that the lowered cAMP level in the presence of glucose may allow a significant repression of the *crp* gene but not the activation of this gene by CRP-cAMP. This argument is

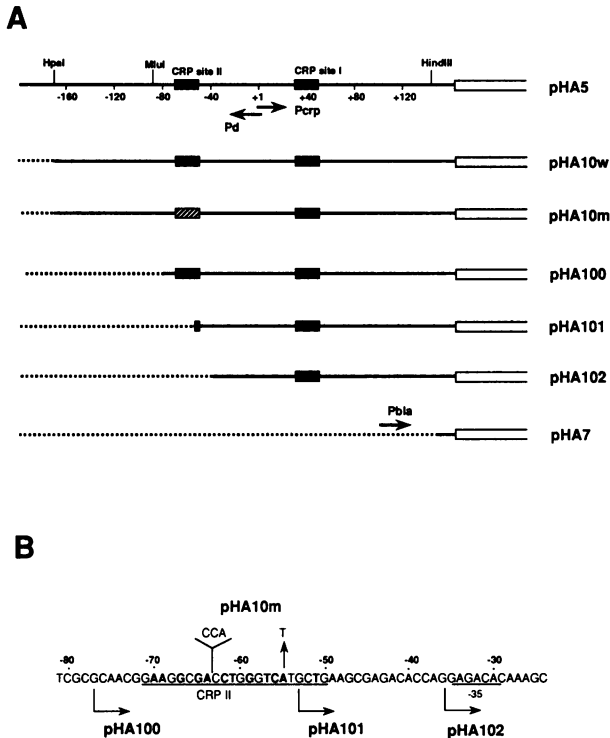


Fig. 7. The *crp* promoter and variants. (A) Diagram of the promoter variants used in this study. The solid lines correspond to the native *crp* regulatory region. The black boxes represent wild-type CRP binding sites. The hatched box indicates the mutated CRP binding site. The open boxes indicate the coding region of *crp*. The dotted lines are derived from the vector pBR322. Arrows indicate promoters. (B) Sequences of wild-type and mutant promoters. The DNA sequences around the CRP binding site II are shown. The CRP binding site II and -35 sequence are underlined. The bold letters indicate nucleotides that match the consensus sequence for CRP binding site. The horizontal arrows indicate the end points of deletions. The sequence of the CRP binding site II mutant (pHA10m) is shown at the top.

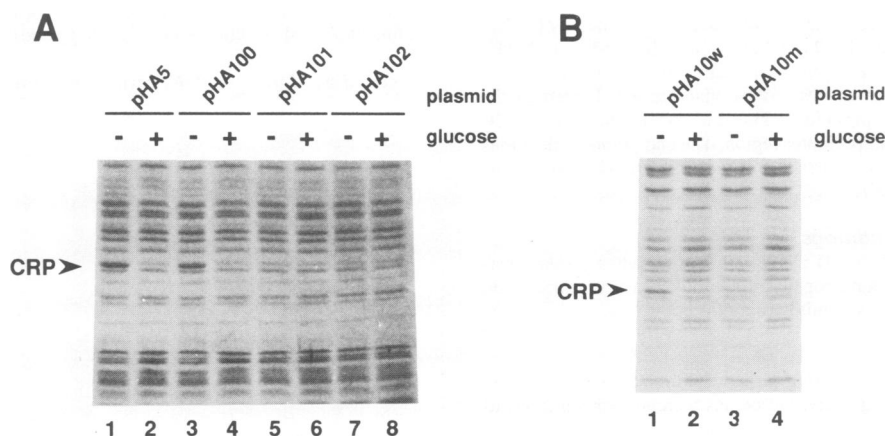


Fig. 8. Effects of mutations in CRP binding site II on the regulation of *crp* expression by glucose. SDS-PAGE of total protein from PP47 cells harboring the indicated plasmid grown to an OD₆₀₀ of 0.8. The arrowheads indicate the CRP bands.

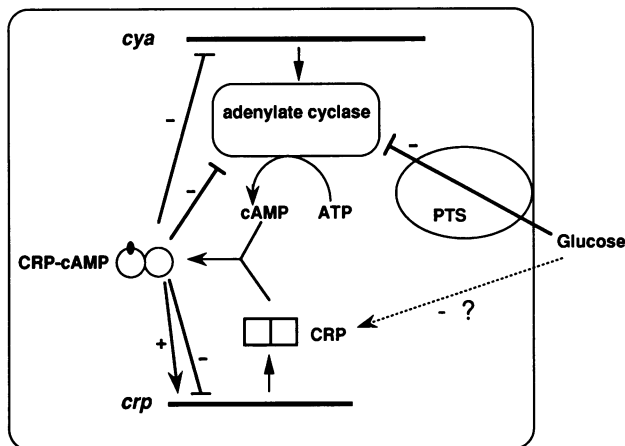


Fig. 9. Model for the down-regulation of *crp* expression by glucose. Glucose lowers the cAMP level by inhibiting adenylate cyclase activity through PTS. Glucose also lowers the CRP level slightly by an unknown mechanism (dotted arrow). The reduction in both cAMP and CRP leads to a decreased CRP-cAMP level. The initial reduction of CRP-cAMP affects the positive autoregulatory circuit of *crp* to cause a further reduction of *crp* expression.

reasonable since it is known that the cAMP concentrations needed for positive autoregulation are higher than those for negative autoregulation (Hanamura and Aiba, 1992). The present study has clarified the role of the positive and negative autoregulation of the *crp* gene. The down-regulation of CRP by glucose would be a consequence of this dual autoregulatory mechanism of the *crp* gene.

Materials and methods

Bacterial strains and plasmids

The *E. coli* K-12 strains used were PP6 (*crp*⁺ *cya*⁺), PP47 (*crp*⁻ *cya*⁺) and PP48 (*crp*⁺ *cya*⁻) (Aiba *et al.*, 1981). Plasmid pHA5 contains the complete *crp* gene while plasmid pHA7 contains the *crp* structural gene under the control of the *bla* promoter (Aiba *et al.*, 1982). Plasmids pHA10w and pHA10m contain the *crp* structural gene under the control of the wild-type and mutant *crp* promoters, respectively. To construct pHA10w, the 346 bp *Bam*HI–*Hind*III fragment of pHA7 containing the *bla* promoter region was first removed, and the 310 bp *Hpa*I–*Hind*III fragment containing the *crp* promoter from pHA5 was inserted after treating with Klenow fragment of DNA polymerase I. Similarly, pHA10m was constructed by using a 313 bp *Hpa*I–*Hind*III fragment containing the mutant *crp* promoter from pCR41 (Hanamura and Aiba, 1992). Deletion plasmids, pHA100, pHA101 and pHA102, were constructed as follows. pHA10w was digested with *Mlu*I and treated with nuclease *Bal*31. The fragments were repaired with Klenow fragment of DNA polymerase I, ligated to an 8 bp *Bam*HI linker, and digested with *Bam*HI and *Hind*III. The resulting *crp* promoter fragments carrying a series of deletions were eluted from an 8% polyacrylamide gel, ligated to the linearized pHA7 receptor plasmid lacking the 346 bp *Bam*HI–*Hind*III fragment containing the *bla* promoter region. The end points of deletions were determined by DNA sequence analysis. Deletions used in this study are shown in Figure 7.

Media and growth conditions

Cells were grown at 37°C in LB medium. Glucose, when present, was added at 0.8%. When cells harbor plasmid, ampicillin was added at 50 µg/ml. Bacterial growth was monitored at 600 nm.

Protein analyses

Cells were grown to mid- to late-log phase. Culture samples equivalent to 0.5 OD₆₀₀ were centrifuged and the pellets were suspended in 50 µl of 50 mM Tris–HCl (pH 8.0), 5 mM EDTA and 0.1 mM dithiothreitol. To solubilize cells, the cell suspensions were mixed with 50 µl of 2 × loading buffer (4% SDS, 10% 2-mercaptoethanol, 125 mM Tris–HCl, pH 8.0, 10% glycerol, 0.2% bromophenol blue) and heated for 10 min

at 90°C. The protein concentration in solubilized cells was determined by the method of Schaffner and Weissmann (1973) by using bovine serum albumin as standard. Total protein (12 µg) was loaded onto 0.1% SDS–13% polyacrylamide gels and electrophoresed. After electrophoresis, the gels were stained with Coomassie brilliant blue. To determine the amount of CRP, the stained gels were scanned with an LKB Ultrascan XL laser densitometer. For the immunoblot analysis of CRP, the protein samples were diluted appropriately with distilled water. Diluted protein samples in 10 µl volume were mixed with 10 µl of 2 × loading buffer and subjected to electrophoresis as described above. Western blotting was performed as described previously (Ishizuka *et al.*, 1993) using polyclonal anti-CRP antibody and a horseradish peroxidase-conjugated secondary antibody.

S1 nuclease assay

A quantitative S1 assay was performed as described previously (Mori and Aiba, 1985). Total RNA was isolated from exponentially growing cultures. The lower strand of the 910 bp *Hpa*II–*Hpa*II fragment ³²P-labeled at its 5' end was used as the DNA probe for the *crp* mRNA. The DNA probe and total RNA were hybridized, and treated with 50 units of S1 nuclease at 37°C for 5 min. The reaction products were analyzed on a 6% polyacrylamide–8 M urea gel.

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