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Activation of Expression of the *Escherichia coli cir* Gene by an Iron-Independent Regulatory Mechanism Involving Cyclic AMP-Cyclic AMP Receptor Protein Complex

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Synthesis of the colicin I receptor protein, encoded by the *cir* gene, was determined to be sensitive to control by the catabolite repression regulatory system. Under both high- and low-iron conditions for growth, mutants unable to produce cyclic AMP (cAMP) (*cya*) or functional cAMP receptor protein (*crp*) exhibited decreased membrane levels of the receptor relative to those of the wild-type strain. Exogenous addition of cAMP to the *cya* mutant restored maximal expression. cAMP-dependent changes in steady-state levels of *cir* mRNA suggested that the effect is mediated by control of transcript synthesis or stability. Potential mechanisms for regulation were examined by deletion and sequence analysis.

A primary feature of most microbial high-affinity iron uptake systems is the specific recognition and binding of nonporphyrin, nonprotein iron-chelating molecules called siderophores by protein receptors located in the outer membrane (8, 24, 25). In *Escherichia coli*, several of these receptors are also required for specific binding of a number of colicins and bacteriophages (8, 17, 24, 25). A role in iron acquisition or metabolism for the 74,000-dalton colicin I receptor, which is encoded by the *cir* gene (7, 14), has not been determined; mutations in *cir* do not appear to affect growth of otherwise isogenic strains in either iron-replete or iron-deficient laboratory media (unpublished results). However, indirect evidence exists which suggests that the natural ligand may be a siderophore with a catechol-type structure (9). The pattern of regulation of the *cir* gene by iron and the apparent evolutionary relationship of this gene to genes encoding siderophore receptors (23) support this hypothesis.

The promoter region of the *cir* gene has been cloned and analyzed (14), and the molecular mechanism responsible for its regulation by iron has been determined (13). Deletion analysis allowed delineation of sequences necessary for control of transcription initiating at two overlapping promoters, P1 and P2. Analogous to the mechanism described for regulation of the operon encoding the proteins required for transport and biosynthesis of the siderophore aerobactin (12), a protein known as Fur (ferric uptake regulation) was determined to act as a repressor of initiation of *cir* transcription by specifically binding in a metal-dependent manner to the region encompassing the −10 and −35 sequences of both promoters.

In contrast to what has been learned about regulation of the *cir* gene by iron, very little is known about the process by which another environmental factor, elevated growth temperature, decreases *cir* activity. The effect of temperature was found to be independent of the state of the *fur* gene and appeared to be at least partly mediated by changes at the transcriptional level (27).

In this communication we report yet another factor capable of modulating synthesis of Cir: the intracellular level of cyclic 3′,5′-AMP (cAMP). After comparing the results of various unrelated experiments carried out in our laboratory over several years, we noted that levels of β-galactosidase produced by strains with *cir-lac* fusions were consistently lower in glucose-grown cultures (minimal glucose or LB broth with 0.2% glucose) than in cultures grown in equivalent media with other carbon sources (minimal glycerol or LB broth) (unpublished data). The mechanism by which glucose affects synthesis of cAMP and imparts a repressive effect on the expression of a large number of genes as part of a global regulatory system known as catabolite repression has been well documented (for reviews, see references 1 and 10). The results presented here confirm that maximal expression of *cir* in vivo is dependent on a critical concentration of cAMP, irrespective of the concurrent state of repression of the gene by iron.

cAMP-dependent regulation of Cir synthesis. To determine whether synthesis of the colicin I receptor was subject to catabolite repression, receptor levels in a wild-type strain (JK213; *trpR55 trpA9605 his-85 metE*), a *cya* derivative defective in cAMP production (JK214), and a *crp* derivative unable to produce functional cAMP receptor protein (CRP) (JK215) were measured. Under both high- and low-iron conditions, the *cya* and *crp* mutants exhibited reduced levels of binding of radiolabeled colicin Ia relative to the wild-type strain (Fig. 1). Furthermore, exogenous addition of cAMP restored synthesis in the *cya* strain to near-wild-type levels, whereas it had no effect on the *crp* strain. Analogous results were obtained by measuring β-galactosidase activity in derivatives of these strains which contained a single chromosomal copy of the *cir-lac* fusion Mu dII1734-18 (data not shown; Mu dII1734 is described in references 13 and 14). The results indicate that functional interaction of cAMP and CRP is required for maximal production of Cir and that in addition to regulation by iron and temperature, synthesis of the Cir protein is controlled either directly or indirectly by levels of cAMP.

Northern analysis. Northern (RNA) analysis was carried out to determine whether the observed regulation was reflected by changes in the steady-state level of *cir* mRNA in response to cAMP. The sources of RNA were cell extracts prepared from iron-limited cultures of the *cya* strain JK214 to which cAMP was added or from which cAMP was omitted. When an internal restriction fragment from the *cir* structural gene was used as a probe, a single major RNA

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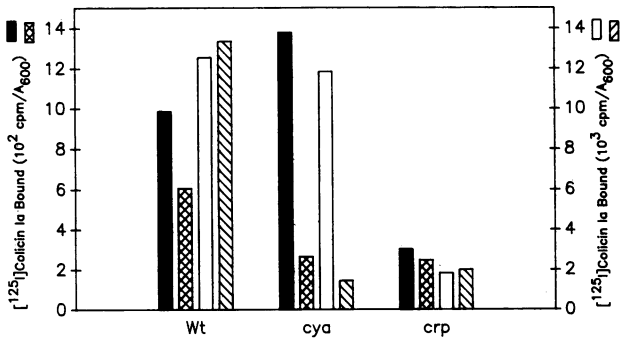

FIG. 1. Effect of cAMP on receptor synthesis during high- and low-iron growth. Strains were grown in LB broth (22) containing 0.2% glucose or in the same medium supplemented with 200 μM 2,2'-dipyridyl before inoculation to create conditions of iron starvation. During exponential growth, the cultures were split, and 20 mM cAMP was added to one set of flasks. After continued incubation for 1 h, the cultures were harvested and binding of radiolabeled colicin Ia to whole cells was determined by the filtration method (18). The values shown represent the amount of colicin Ia bound at saturation. Note that the scales are different for high-iron and low-iron conditions. Strains: JK213 (wild type [Wt]); JK214 (*cya*); JK215 (*crp*). Growth condition symbols: ■, high iron, with cAMP; , high iron, without cAMP; □, low iron, with cAMP; □, low iron, without cAMP.

species whose length corresponded to the expected length of the *cir* transcript (2.1 to 2.2 kilobases) was detected (Fig. 2). The apparently smaller mRNA species have calculated sizes which would be too small for translation of the complete Cir protein. These were concluded to be incompletely denatured forms of the large transcript, since the relative amount of these species was increased as the concentration of denaturant in the samples was decreased and the smaller bands were not observed in a *cir* mutant which did not exhibit the large transcript (data not shown). The presence of exogenous

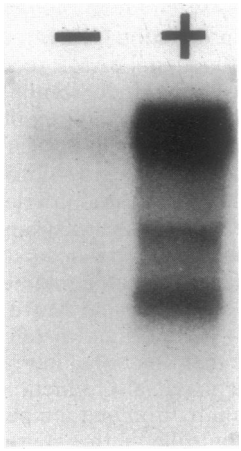

FIG. 2. Analysis of cAMP-induced changes in *cir*-specific mRNA levels. Strain JK214 was grown in LB broth containing 0.2% glucose and 200 μM 2,2'-dipyridyl. During logarithmic growth, the culture was split, and 20 mM cAMP was added to one flask. After continued incubation for 1 h, cell extracts were prepared and equal amounts of RNA were fractionated on a 0.8% agarose gel as described by Kornblum et al. (19). The *Sal*I-generated internal restriction fragment from the *cir* structural gene (14) was used as the ^{32}P-labeled probe for Northern hybridization by standard techniques. −, Absence of cAMP; +, presence of cAMP.

cAMP in the growth medium greatly increased the amount of *cir*-specific mRNA detected in the extracts. After normalization for variation in cell density at the end of the incubation period, the increase due to cAMP was calculated by densitometric analysis to be 11- to 13-fold. The increase in the level of induction observed by measuring receptor levels in the outer membrane of this strain grown in the same medium with and without cAMP was six- to eightfold (Fig. 1). Therefore, the regulatory effect of cAMP on synthesis of the colicin I receptor could be completely accounted for by an increase in transcription of the *cir* gene. However, we cannot exclude the possibility that cAMP functions in other ways, such as to prevent premature transcription termination or to protect the transcript from degradation. It is unlikely that cAMP affects translation of the *cir* structural gene, which in turn could affect mRNA stability, because regulation was still observed with the *cir-lac* fusion Mu dII1734-18 (described above), which contains only the first 36 nucleotides of *cir* (data not shown).

Effect of upstream deletions on activity and regulation. The sequence of the 525-base-pair (bp) *Hae*III fragment, which was cloned as previously described (14) into the vector pMC1871 to form plasmid pH3, which contains a hybrid *cir-lacZ* gene, is shown in Fig. 3. The promoters in this sequence overlap with a region to which the Fur protein was shown to be capable of binding in vitro, and expression of the hybrid gene was regulated normally in vivo by iron availability (14). To identify *cis*-acting sequences in the *cir* promoter region which might be required for cAMP-CRP complex to stimulate transcription, we examined the effect of deletions extending downstream through the left *Hae*III site (Fig. 3) on regulation. The deletion Δ15 was created by *Bal* 31 exonuclease digestion as reported previously (13). The ΔP3 deletion was constructed by subcloning the 177-bp *Sau*3AI-*Hae*III fragment from the insert in plasmid pH3 (Fig. 3) into the *Sma*I site of pMC1871 to form the same junction with the *lac* DNA sequences that exists in parent plasmid pH3. Because the plasmids containing these deletions were originally constructed as derivatives of the multicopy plasmid pH3, stable cointegration with a low-copy, modified F factor (pMH15) was carried out as previously described (13). The host strain for these plasmids, DG194 (*polA lac trp*::Tn*9*), contained a *polA* mutation to prevent replication originating from the ColE1 origin. This was necessary because replication of ColE1 replicons such as pMC1871 has been reported to be catabolite repressed (16), making it difficult to distinguish *cir*-specific regulatory processes from changes due to variations in the copy number of the plasmid under different conditions.

The deletion mutation Δ15 resulted in the loss of all natural DNA sequences upstream of the −35 region of the P2 promoter (Fig. 3). Expression and regulation by iron were not significantly affected, since the promoters and the sequences which compose the binding site for the Fur protein were retained (13). Furthermore, catabolite activation was still observed in this derivative (Table 1), indicating that the sequences upstream of the two *cir* promoters, P1 and P2, were not required for the mechanism of transcriptional stimulation involving cAMP-CRP complex.

The plasmid containing deletion ΔP3 lacked a larger segment of DNA which included the transcriptional start sites for both iron-regulated promoters, P1 and P2, and the entire binding site for the Fur protein (Fig. 3). The strain exhibited an extremely low level of gene activity (Table 1). This slight activity is likely a result of readthrough transcription from an upstream promoter located in the vector. It is

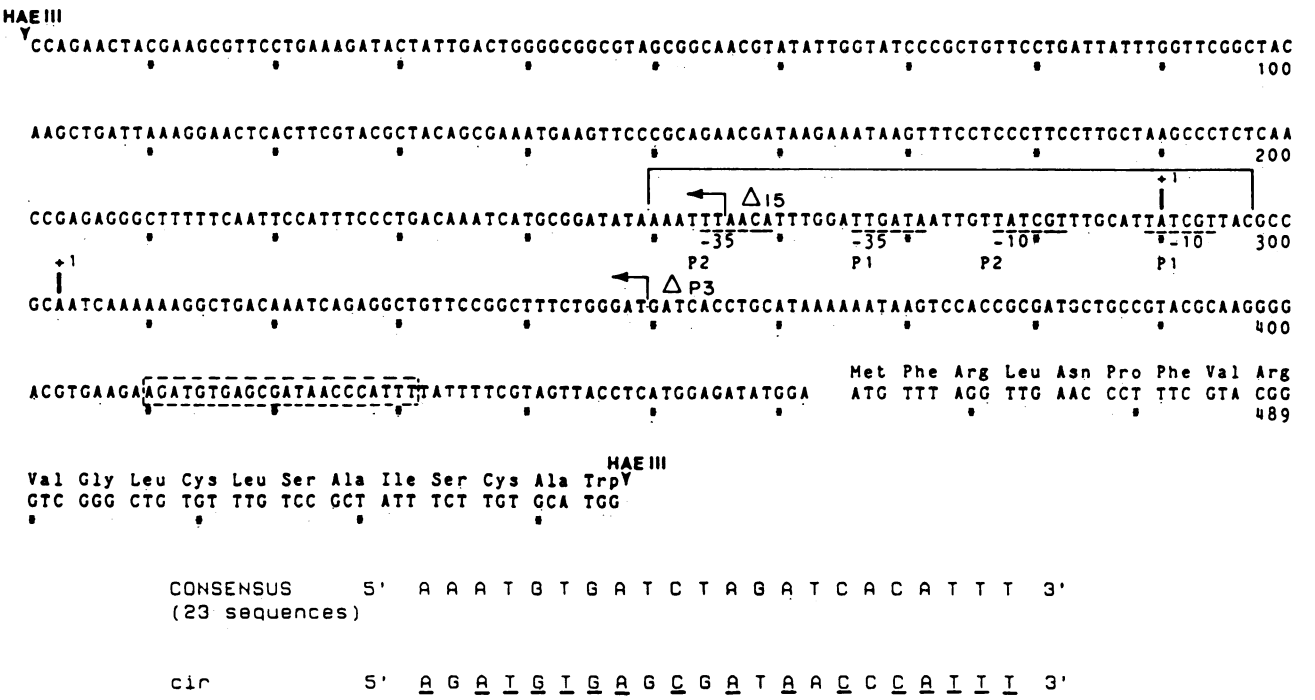

FIG. 3. Nucleotide sequence of the promoter-regulatory region of *cir*. The sequence shown represents the cloned insert contained in the previously described plasmid pH3 (13). The two sites for initiation of transcription are indicated (+1), and the corresponding −10 and −35 regions for the promoters are underlined. The brackets enclose the area which footprinting analysis has shown to be bound by the Fur repressor protein under high-iron conditions. The sequences lying upstream of the bent arrows at nucleotide positions 256 and 350 (*Sau*3AI site) were deleted to create the plasmid derivatives pH3F-Δ15 and pH3F-ΔP3, respectively, as described in the text. The location of the potential recognition site for cAMP-CRP complex is marked by the boxed sequence and is compared to the consensus cAMP-CRP binding site (5) at the bottom of the figure. These data have been submitted to GenBank under accession no. J04229.

not clear whether the apparent increase in enzyme activity in the presence of cAMP is significant, because of the difficulty of accurately assaying very low levels of β-galactosidase. In any case, the result provides evidence against a model for regulation in which a previously undetected third *cir* promoter located in the region between the *Sau*3AI site and the start site of translation would promote high-level expression after binding of cAMP-CRP complex upstream.

Discussion. At least three models can be postulated to describe the mechanism by which cAMP and CRP affect expression of *cir*. (i) Regulation could be mediated by binding of cAMP-CRP complex directly to the *cir* promoter region. (ii) Regulation could be mediated indirectly by influencing the amount of activity of another protein, such as Fur, which in turn affects *cir* expression. (iii) Alteration of cellular metabolism in the catabolite-repressed state could affect the amount or ability of iron in the cell to act as a corepressor with Fur. The last two models, at least to the extent to which they involve Fur, seem less likely than the first, since the effect of cAMP on *cir* expression in the *cya* mutant was nearly the same regardless of whether the cells were grown under iron-replete or iron-deficient conditions (Fig. 1). Therefore, whether the Fur-iron complex is bound to the promoter region or not, expression can be stimulated by cAMP, and catabolite repression appears to be an independent regulatory circuit superimposed on that responsible for regulation by iron. Further evidence that Fur does not mediate the effect of cAMP on *cir* expression can be derived from a study of the regulation of the *fur* gene itself (11). Like *cir*, maximal expression of *fur* in a *cya* strain required exogenously added cAMP. However, since high levels of cAMP resulted in increased synthesis of the Fur repressor, it was expected that the activity of genes such as *cir* would be decreased, if affected at all, under these conditions. Contrarily, the expression of *cir* was increased under the same conditions as is the expression of *fur*.

TABLE 1. Effect of deletions on regulation of *cir-lac* by cAMP

Plasmid	β-Galactosidase activity (U)[a]	
	Absence of cAMP	Presence of cAMP
None	0.0	0.1
pH3F	58	332
pH3F-Δ15	66	208
pH3F-ΔP3	0.5	3.0

[a] Two cultures containing 2× YT broth were inoculated with strain DG194 containing the indicated plasmid. During exponential growth at 37°C, 20 mM cAMP was added to one set of cultures. After continued incubation for 1 h, enzyme activity was determined. The activities shown were calculated as the averages of two independent assays.

A computer-assisted analysis of the DNA sequence of the *Hae*III-generated restriction fragment shown in Fig. 3 revealed only one site which was significantly similar (16 out of 22 bp; 73% similarity) to a proposed consensus sequence for recognition and binding of cAMP-CRP complex (Fig. 3, bottom). Berg and von Hippel have developed a statistical-mechanical selection theory to analyze families of protein-binding sites which allows one to calculate a λE, or discrimination energy, value for any given sequence (4, 5). This is a statistical measure of the deviation of a nucleotide sequence from the consensus, taking into account the level of variability that occurs at each individual position in the population

of sites being analyzed. Experimental data for binding of CRP to DNA in vitro has shown that a correlation exists between the measured ratio of specific to nonspecific binding and the calculated λE value for a sequence (5). The value we have calculated for the potential binding site in the *cir* promoter region (λE = 8.1) is similar those calculated for sites in other genes to which binding of CRP protein has actually been demonstrated (e.g., *gal*, λE = 7.7; *ilvB*, λE = 8.9; *crp*, λE = 8.1; values from reference 5), suggesting that the site in *cir* may also be functional.

If the cAMP-CRP complex does bind to the site between the promoters and the amino-terminal-coding region of the receptor (shown in Fig. 3) in vivo, this raises the question of how such binding might mediate regulation. In operons such as *lac*, *gal*, *ara*, and many others in which transcription is activated by cAMP-CRP, binding occurs at sites upstream of the promoters, although the distance between the binding site and the start site for transcription may vary widely in different genes (10). In the case of *fur*, a proposed cAMP-CRP binding site is situated about 60 bp upstream from the transcriptional start sites (11). Although much remains to be learned about the molecular processes which occur during activation, it appears that binding of cAMP-CRP results in activation in most systems by increasing the affinity of RNA polymerase for the promoter or by blocking RNA polymerase from binding to competing, overlapping sites (10). However, unlike the situation in the genes described above, in which the cAMP-CRP complex binds in the region upstream of the promoters, the proposed binding site in *cir* lies far downstream (over 100 bp) from the start sites of transcription for promoters P1 and P2 (Fig. 3). The results of the deletion analysis showed that no sequences in the region upstream of promoters P1 and P2 were required for catabolite repression and that there was no indication of another promoter in the region downstream of the potential binding site. Precedence does exist for a binding site located downstream from the site of transcription initiation in at least one other gene (*crp*), but binding inhibits expression of that gene by activating synthesis of an interfering divergent transcript (26). Perhaps looping of the DNA occurs in *cir* to allow contact between CRP bound at the downstream site and RNA polymerase. Another possibility is that binding of cAMP-CRP affects the formation of secondary structures in the unusually long leader region so that transcription initiation, elongation, or termination is affected in such a way as to increase gene activity.

Several studies have indicated that the protein composition of the outer membrane is affected either by growth on glucose or, more specifically, by levels of cAMP and CRP (2, 3, 6, 20, 21). The affected proteins include LamB (maltodextrin-specific pore), OmpA (possible roles in maintaining membrane structure; conjugation), Tsx (nucleoside transport; receptor for phage T6 and colicin K), and protein III (major outer membrane protein; function unknown). Our results constitute the first evidence for cAMP-dependent regulation of the synthesis of a TonB-dependent outer membrane protein. It is difficult to speculate about the physiological significance of this mode of genetic control, especially since *fur*, the repressor of *cir* transcription, is activated by cAMP under the same conditions as is *cir*. Interestingly, it has been observed that *fur* mutants are not able to grow on succinate, fumarate, or acetate (15), suggesting that the role of Fur protein may not be confined to regulation of iron uptake but may also provide a link between iron metabolism and the general catabolic status of the cell. In the case of *cir*, the existence of multiple independent regulatory pathways specific for a variety of environmental effectors suggests a potential for great sensitivity and flexibility in controlling the outer membrane levels of the Cir protein.

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