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Regulation of a *cya-lac* Fusion by Cyclic AMP in *Salmonella typhimurium*

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cya-lac and _crp-lac_ operon fusions were isolated in _Salmonella typhimurium_ by using the phage Mu d1(_lac_ cts Ap^r). Both transduction and reversion analyses have indicated that _lac_ expression is controlled by the appropriate promoter, e.g., either _crpp_ or _cyap_. By using chromosomal mobilization techniques, we found that _cya_ had a clockwise direction of transcription on the standard _S. typhimurium_ map. The _cya-lac_ fusions could be complemented by _Escherichia coli_ F′133, which covers _cya_, with a resultant 17 to 38% decrease in _cya_ expression. Cyclic AMP was found to be able to repress the expression of the _cya-lac_ fusion ninefold when present at 25 mM. This repression was not seen in _crp_ backgrounds, and hence is mediated by the cAMP receptor protein. Repression of _cya_ was also found upon growth on carbon sources known to elicit high cyclic AMP levels.

The global regulation by cyclic AMP (cAMP)-cAMP activator protein (CAP) is one of the best understood of all the cellular control systems. The physiology of cAMP production has been extensively studied, in a number of genetic backgrounds and under many different growth conditions (for reviews, see references 7, 30, 33, 36, 44). The cAMP activator protein, through which cAMP exerts its effect in bacteria (31, 47), has been purified to homogeneity (4, 37), crystallized, and studied with X-ray crystallography (22, 23), and DNA sequences that the cAMP-CAP complex binds to have been determined (14, 16, 26, 43, 44). Many mutants in both *cya*, which encodes adenylate cyclase which in turn produces cAMP, and *crp*, which encodes CAP, have been isolated (3, 32, 40). Mutations in *cpd*, which codes for cAMP phosphodiesterase (28), have also been found in *Salmonella typhimurium* (2).

In the midst of this well characterized system, many questions and apparent anomalies remain. The molecular mode of action of the cAMP-CAP complex is unclear, as is the extent of the interaction of CAP with RNA polymerase. There is a report that cAMP and CAP act as an anti-terminator (45), and studies on cAMP excretion have shown that most of the cAMP made is excreted (9, 15). No explanation of this seeming waste of energy has yet been found. It has also been found that *crp* strains overproduce and then excrete cAMP at 32 times the rate found with wild-type strains (15).

The phenomenon of cAMP overproduction in *crp* strains has lead investigators to explore whether the cAMP overproduction was an effect due to the overproduction of adenylate cyclase or to an increase in the activity of preexisting adenylate cyclase (8, 17, 20). An early report of an in vivo aeration assay of cAMP with cells suspended without a carbon source led to the conclusion that the amount of adenylate cyclase varied depending upon the carbon source and whether a functional CAP was present (8). Majerfeld et al. (20) subsequently used both an in vivo and an in vitro assay of cAMP production to show that *crp* strains appear to overproduce cAMP by two to four times the rate seen in isogenic *crp*$^+$ strains. This overproduction in cAMP was shown by a mixing experiment not to be due to a soluble functional activator of adenylate cyclase. The authors concluded that whereas *cya* was repressed by up to twofold by cAMP, cAMP regulation was primarily at the level of adenylate cyclase activity and not of adenylate cyclase production.

A report by Bankaitis and Bassford (5) described the isolation of fusions of *cya* to *lac* in *Escherichia coli*. They presented genetic evidence confirming that they had indeed isolated *cya-lac* fusions and physiological evidence that the addition of 5 mM cAMP to minimal medium repressed *cya* expression by twofold in *E. coli*. They concluded that repression of *cya* by the cAMP-CAP complex is not physiologically significant because of their observed maximum effect of only twofold.

The present study reports the isolation of *cya-lac* fusions in *S. typhimurium*. Fusions of *lac* to both *cya* and *crp* were made in *S. typhimurium* by using Mu d1(*lac* cts Ap^r). Genetic evidence confirmed the insertion of Mu d1 into *cya* or *crp*. The direction of transcription of *cya* was shown to be in the clockwise direction on the standard *S. typhimurium* (39) map by use of chromosomal mobilization techniques (29). The basic physiology of *cya* was studied under different growth conditions and in *crp*$^+$ and *crp* backgrounds (S. Jovanovich, L. Couper, and S. Artz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, H2, p. 106). *cya* was shown to have decreased expression when studied in a complemented strain or under conditions known to increase cAMP levels, or when cAMP was added externally. Using higher cAMP concentrations than Bankaitis et al. (5), we found a ninefold repression of *cya*, indicating that the regulation might be of physiological importance. The results are discussed with respect to the problem of control of cAMP levels and transcriptional regulation of *cya* by the cAMP-CAP complex.

MATERIALS AND METHODS

Bacterial and phage strains, media, and chemicals. Bacterial and phage strains used, along with their sources, are listed in Table 1. Luria-Bertani broth (LB), MacConkey agar, and nutrient broth (NB) were all made by the method of Miller (24); M56 medium and green plates were made as

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TABLE 1. Strain list

Strain	Genotype	Source
AZ105	F'128/*proA15*	D. Palmer
JL2554	*argG*::Tn*10*	J. Ingraham
JL3047	F'133/*hisD2421 metE338 ilvC401 ara-9*	J. Ingraham
JL3082	*metE863*::Tn*10*-219	J. Ingraham
JL3102	*cysB14 rpsL201*	J. Ingraham
JL3521	*ara-9 ilv-8 pyrB647 metA309*	J. Ingraham
JV114	*crp-404 rpsL201*	TA3335 transduced by JL3102
JV143	*crp rpsL201*	LT2, selected fosfomycin resistant, transduced by JL3102
JV145	*ara-9 ilv-8 pyrB647 proAB47 argG*::Tn*10 metA309* Mu d(cts Ap^r)	JL3521 transduced by JL2554, then by JV324, selecting for ampicillin resistance
JV146	*ara-9 ilv-8 pyrB647 proAB47 argG*::Tn*10* Mu d(cts Ap^r)	JV145 transduced by LT2 to Met^+
JV304	Φ(*crp-lac*)*1* Mu d(cts Ap^r) *cysB12 trpD10 ara-1*	Selection of L1119, cured of episome
JV324	Φ(*crp-lac*)*3* Mu d(cts Ap^r) *cysB12 trpD10 ara-1*	Selection of L1119, cured of episome
JV342	Φ(*cya-lac*)*1* Mu d(cts Ap^r *X*::Tn*9*) *cysB12 trpD10 ara-1*	JV344 transduced by RB430
JV344	Φ(*cya-lac*)*1* Mu d(cts Ap^r) *cysB12 trpD10 ara-1*	Selection of L1119, cured of episome
JV347	Φ(*cya-lac*)*1* Mu d(cts Ap^r) *cysB12 trpD10 ara-1 metE863*::Tn*10*-219	JV344 transduced by JL3082
JV350	F'133/Φ(*cya-lac*)*1* Mu d(cts Ap^r) *cysB12 trpD10 ara-1 metE863*::Tn*10*-219	JV347 × JL3047
JV358	F'tss114/Φ(*cya-lac*)*1* Mu d(cts Ap^r *X*::Tn*9*) *cysB12 trpD10 ara-1*	JV342 × NK1017
JV364	Φ(*cya-lac*)2 Mu d(cts Ap^r) *cysB12 trpD10 ara-1*	Selection of L1119, cured of episome
JV367	Φ(*cya-lac*)2 Mu d(cts Ap^r) *cysB12 trpD10 ara-1 metE863*::Tn*10*-29	JV364 transduced by JL3082
JV370	F'133/Φ(*cya-lac*)2, Mu d(cts Ap^r) *cysB12 trpD10 ara-1 metE863*::Tn*10*-29	JV367 × JL3047
JV387	Φ(*cya-lac*)*1* Mu d(cts Ap^r *X*::Tn*9*) *cysB12 trpD10 ara-1 rpsL201*	JV342 transduced by JV143
JV388	Φ(*cya-lac*)*1* Mu d(cts Ap^r *X*::Tn*9*) *cysB12 trpD10 ara-1 rpsL201 crp*$^-$	JV342 transduced by JV143
JV399	F'tss114 *lac*$^+$ *zzz*::Tn*10*/Φ(*cya-lac*)6 Mu d(cts Ap^r) *cysB12 trpD10 ara-1*	Selection of L1119 (Lac^-)
JV402	F'tss114 *lac*$^+$ *zzz*::Tn*10*/Φ(*glnA-lac*) Mu d(Ap^r cts) *hisF695*	SK1019 × NK1017
L1119	F^+ Mu d(*lac* cts Ap^r)/*cysB12 trpD10 ara-1*	G. Wilcox
LT2	Wild type	B. Ames
NK1017	F'tss114 *zzz*::Tn*10*/*pyrC7* Str^r	L. Blaha
RB430	F^+::Mu *d*::Tn*9*/*pyrF146*, Δ*trp-43 leu-500*	P. Blum
SK1019	Φ(*glnA-lac*) Mu d(Ap^r cts) *hisF695*	S. Kustu
P22HT	*int-201*	S. Kustu

described previously (25, 42). VB medium is identical to the E medium of Vogel and Bonner (46). C-medium contains (per liter) K_2SO_4 (1.0 g), K_2HPO_4 (13.5 g), KH_2PO_4 (4.7 g), NaCl (2.5 g) and 2% $MgSO_4 \cdot 7H_2O$ (5 ml). PC medium contains (per liter) nutrient broth (8 g) and cysteine (50 mg), to which, after autoclaving, 30 ml of sterile 40% glucose was added, along with 15 ml of 50× concentrated VB salts. The solid media contained Difco agar at 1.5% (wt/vol). Carbon sources were added to 0.4% (wt/vol) unless otherwise noted. All minimal media used with strain L1119 and its derivatives contains L(−)cysteine and L(−)tryptophan at 50 μg/ml each. Antibiotics were added to the following concentrations (in micrograms per milliliter): ampicillin, 50; chloramphenicol, 12.5; streptomycin, 200; and tetracycline, 10. cAMP was added to 1 mM when used as a growth supplement. Unless otherwise noted, all chemicals were obtained from Sigma Chemical Co. Fosfomycin (phosphonomycin) was a gift from Merck & Co., Inc. 3-Chloro-4-bromo-5-indoyl-β-D-galactoside (X-Gal) was purchased from Bachem, and was added to 40 μg/ml after being dissolved in *N*,*N*-dimethylformamide.

Genetic techniques. Transductions were performed with P22HT *int-201* phage as described previously (41). Transductants were purified three times on green indicator plates before being finally tested for the appropriate markers. When the marker selected was Str^r, the transduction was performed on nutrient agar and then after 4 h of phenotypic expression was replica plated onto MacConkey agar containing 1% (wt/vol) glycerol and 1% (wt/vol) ribose with streptomycin added. The use of this media allowed the simultaneous selection of Str^r and screening for utilization of the carbon sources.

Strains to be conjugated were grown overnight, and then the donor strains were diluted 1:20 and the recipient strains were diluted 1:40 in NB. After 2 h of growth, 0.1-ml samples of each culture and of a mixture were plated onto media selective for the desired exconjugants. The plates were incubated for 3 to 5 days, and then colonies were purified and the phenotype was tested.

Selection for *cya-lac* or *crp-lac* fusions. The F^+ Mu d(*lac*$^-$ cts Ap^r)-containing *S. typhimurium* strain L1119, isolated by Lee et al. (18), was used to select the fusions. Overnight cultures were pregrown at 28°C in liquid VB medium supplemented with 10 mM glucose and 20 mM glucose-6-phosphate. To induce Mu d, the overnight cultures were diluted 1:20 into fresh media and incubated at 37°C for 8 h. Samples (10 to 100 μl) were plated onto MacConkey agar plates containing 0.4% glycerol (wt/vol), 0.4% ribose (wt/vol) 0.2 mg of fosfomycin, and 40 μg of X-Gal per ml. Plates were incubated at 30°C for 4 days. Blue colonies were possible *cya-lac* or *crp-lac* isolates and were marked after 2 days. Isolates were purified three times before phenotypic testing.

Selection of precise eductants. Precise eductants were selected by three methods: the plate method, the liquid method, and from *X*::Tn*9*-containing strains. The liquid and the plate methods essentially involved a selection of heat-resistant mutants, which in the case of the plate method were then pooled. The *X*::Tn*9* was used by transducing the desired strain to chloramphenicol resistance with phage grown upon an *X*::Tn*9*-containing strain, RB430, followed by screening of the isolates for heat resistance (P. Blum, L. Blaha, R. Shand, D. Holzschu, and S. Artz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, H128, p. 134). In all methods, the precise eductants were selected on C-plates containing glycerol and X-Gal, which allowed for screening for the loss of the Lac^+ phenotype on the selection plates.

White (Lac^-) colonies were either purified or replica plated onto MacConkey ribose. Possible precise eductants were purified before the appropriate phenotypes were tested. The protocol for each of these methods will be published elsewhere (P. Blum, L. Blaha, S. Jovanovich, and S. Artz, manuscript in preparation).

Assay of β-galactosidase activity in *cya-lac* fusion strains. Cultures to be assayed were inoculated 1:100 from fresh overnight cultures of growth medium into 25 ml of growth medium in 125-ml Erlenmeyer flasks. The cultures were grown in a New Brunswick rotary shaking water bath that was shaking at about 250 rpm, and growth was monitored by the optical density at 650 nm (OD_{650}) on a Gilford model 250 spectrophotometer. Usually, at each growth point 0.5 ml of culture was withdrawn, iced, and used for the β-galactosidase assay. β-Galactosidase assays were performed by the method of Miller (24). All cultures were followed either to an OD_{650} of 0.6 or until they reached stationary phase. Differential rate determinations were made with five or more samples from log-phase cultures in most cases. All cultures were checked after growth and found to be both uncontaminated and unchanged in their phenotype.

RESULTS

Isolation of *cya-lac* and *crp-lac* fusions. To select for *crp-lac* or *cya-lac* fusions, we modified the procedure of Alper and Ames (3) for the selection of *crp* and *cya* mutants. Their procedure entailed growth of an overnight culture in minimal media with glucose-6-phosphate, which induced *uhp* genes, and then plating the culture onto MacConkey agar with glycerol and ribose and the antibiotic fosfomycin. Fosfomycin-resistant colonies were found at a frequency of 10^{-5} to 10^{-6} per cell in this study. A class of the fosfomycin-resistant colonies is *crp* or *cya*, since uptake of fosfomycin is under cAMP control, as is the uptake of both glycerol and ribose. White, fosfomycin-resistant colonies are frequently *crp* or *cya* (3; unpublished observations). The modifications made to tailor the Alper and Ames procedure to the selection of *crp-lac* and *cya-lac* fusions were first, the use of strain L1119, which carries Mu d(*lac* cts Ap^r) on an F^+; second, the induction of Mu d by growth at 37°C for 8 h before plating; and third, plating of the Mu d-induced cultures on MacConkey plates containing glycerol, ribose, fosfomycin, and X-Gal.

The selection plates were used in an attempt to select fosfomycin-resistant colonies while screening for loss of both glycerol and ribose utilization and the acquisition of the Lac^+ phenotype. The rationale was that MacConkey indicators are pH dependent and give a red color from the acid produced when a carbon source is fermented; the X-Gal, in contrast, is histological and not pH dependent. If the two reactions did not interfere with each other, it would be possible to use them in conjunction and greatly reduce the task of screening the colonies. The selection plates contained colonies of one of four different colors (data not shown). The colors for glycerol and ribose nonfermenters, which are white on MacConkey plates with glycerol and ribose, were white for Lac^- colonies and blue for Lac^+ colonies, whereas for glycerol or ribose utilizers, which are red on MacConkey plates with glycerol and ribose, the Lac^- colonies were red and the Lac^+ colonies were purple. This screen is dependent on the strength of the blue color, which must not overwhelm the MacConkey red color, as might happen if *lac* were fused to a very strongly expressed promoter.

An average of five blue single colonies were found per plate, for a frequency of about 10^{-7} per cell. These were usually small colonies, typical of crp^- or cya^- colonies. A total of 102 blue colonies were picked from 20 selection-screening plates, purified, and tested. Four of the 102 blue colonies were found to be unable to use glycerol or ribose and were Lac^+, temperature sensitive, and ampicillin resistant and therefore had the phenotype expected for *crp-lac* or *cya-lac* fusions. The other 98 were usually ampicillin resistant, Lac^+, and were able to ferment glycerol and ribose. These 98 colonies therefore corresponded to spontaneous fosfomycin-resistant mutants which had also acquired a fusion of *lac* to genes other than *cya* or *crp*. The expression of *lac* in these cases had masked the MacConkey fermentation indicator on the selection plate. Two of the four putative fusions appeared crp^- and the other two were cya^-, since added cAMP had no effect on the fermentation of catabolite-sensitive compounds in the former but did have an effect in the latter. These four fusion strains were cured of their F^+ containing Mu d by the use of acridine orange and were named JV304, JV324, JV344, and JV364 (Table 1).

Genetic characterization of the putative fusions. For the putative fusions to be of interest, two criteria had to be satisfied. First, the Lac^+ phenotype and the lack of both glycerol and ribose fermentation had to be shown to be due to one event, and second, the insertion of Mu d must not have created a deletion in the region of DNA between the *cya* or *crp* promoter and the fusion juncture with *lac*. If the second condition were not satisfied, it could mean that a portion or all of the appropriate promoter might be deleted and some of the regulatory regions might be lost, even though transcription and translation still occurred.

To confirm that the fusion event and the lack of carbon source fermentation were at least physically closely related events, the four putative fusions JV304, JV324, JV344, and JV364 were transduced on glycerol plates with P22 grown on LT2. Transductants were purified and tested for carbon source utilization, the Lac phenotype, ampicillin resistance, and heat resistance. From the four presumed fusion strains, of 64 transductants tested (16 per strain), 62 simultaneously regained the ability to ferment glycerol and ribose and the ability to grow at 43°C, while losing the Lac^+ phenotype and ampicillin resistance. Although the other two, one each from JV324 and JV344, had regained the ability to ferment glycerol, they retained the ampicillin resistance, heat sensitivity, and Lac^+ phenotypes and thus appeared to contain Mu d, which had hopped to a new location. Therefore, for 62 of 64 transductants, the transduction to growth on glycerol had resulted in the loss of all the markers carried by Mu d along with a restoration of the ability to ferment ribose. As such, they displayed the phenotype expected if the Lac^+ and carbon source utilization phenotype were transduced out by P22 in one event. This leads to the conclusion that the Lac^+ phenotype and the carbon source utilization phenotype are within less than 1 map unit of each other and is consistent with their being caused by the same event. However, this result does not prove that the fusion is to the correct promoter, nor does it eliminate the possibility that a deletion of part of the host promoter has occurred upon the insertion of Mu d.

To establish the map position of the fusion, the presumed *cya* fusions JV344 and JV364 were transduced with phage grown on a *metE*::Tn*10* strain, JL3082. Tet^r colonies were selected, and the carbon source utilization was scored; 40% of the Tet^r transductants were found to have regained the ability to ferment ribose and glycerol. Similarly, the pre-

sumed *crp* fusions JV304 and JV324 were transduced with phage grown on a streptomycin-resistant (*rpsL*) strain, JL3102. Streptomycin resistance was selected, and 38% of the transductants were found to have regained the ability to ferment ribose and glycerol. *metE* and *rpsL* are known to be 10 and 40% cotransducable with *cya* and *crp*, respectively (6, 12). The finding that selection for markers at *metE* or *rpsL* nonselectively repaired the *cya* or *crp* loci, respectively, is strong evidence that the fusions indeed map at or near *cya* or *crp*.

Selection of precise eductants. To further confirm that the fusions were insertions of *lac* into *cya* or *crp* and that deletions of the chromosome had not occurred upon insertion of Mu d, precise eductants of Mu d were sought. Mu d insertions will not ordinarily revert, but the introduction of an *X* mutation into Mu d will render the fusion revertible (10). After selection of *X* mutants by heat resistance, precise eductants were selected by being plated onto C-glycerol, which is selective for revertants, glycerol promoter mutants, and *alt*s (unpublished observations). These three classes of mutants are easily distinguished by scoring the available phenotypic markers.

Precise eductants were selected by three different methods (see above). The methods and their effects upon reversion frequencies will be published elsewhere (Blum et al., in preparation). The basic data relevant to this report may be summarized as follows: JV304, JV324, and JV344 (or their derivatives) all gave precise eductants by any of the methods, whereas JV364 (or its derivatives) did not yield any precise eductants by any of the methods. We therefore believe that JV364 contains a deletion in *cya* and the other three strains have no detectable deletions.

Complementation of the *cya* fusions with an episome. To observe the regulation of *cya* in a wild-type background and to be able to detect the effects of adenylate cyclase or cAMP upon *cya* expression (see below), it was necessary to complement the fusion strains. This was accomplished by introducing a *metE*::Tn*10* into the strains containing Φ(*cya-lac*) by selection for Tet^r and scoring for Met^- colonies. The two strains constructed, JV347 and JV367, were then mated with the F′133-containing strain, JL3047, with selection for Met^+ exconjugants. F′133 is known to complement *cya* and *metE* (19). All of the Met^+ Tet^r isolates were found to be able to grow on catabolite-sensitive carbon sources and were Lac^+ Ap^r and temperature sensitive; therefore, F′133 complemented the cya^- phenotype. The complemented strains, JV350 and JV370, were also competent as donors of F′133, thereby confirming its presence.

Direction of transcription of *cya*. To further substantiate the fact that the presumed *cya-lac* fusions were indeed fusions of *lac* to the *cya* promoter and not perhaps insertions of Mu d into *cya* with a fusion to a promoter which is transcribed in the opposite direction of *cya*, we decided to isolate Lac^- insertions of Mu d into *cya*. The prediction was that Lac^- insertions of Mu d into *cya* which could give precise eductants would be in the opposite orientation to the Lac^+ presumptive *cya-lac* fusion, Φ(*cya-lac*)*1*.

A Lac^- cya^- strain was first isolated by our standard selection procedure, but this time the white colonies on the plates were chosen. One such isolate, no. 28, was shown by reversion analysis and transductional analysis to have Mu d inserted into *cya* and was capable of precise excision; no. 28 was Lac^- and therefore did not have *lac* fused to a promoter but presumably inserted into *cya* in a reverse orientation.

To show that the Lac^- Mu d insertion, no. 28, was in the opposite orientation to the Lac^+ insertions and to determine the direction of transcription of *cya*, we used chromosomal mobilization. An F′ *lac* is able to mobilize the chromosome by recombination through the homology present, since both the F′ and the chromosome contain *lac* and no other homologous markers (13, 29). Selection for wild-type recombinants at one of the auxotrophic markers of a multiply marked recipient strain will give exconjugants (or revertants which are readily distinguishable by scoring the unselected markers). Since the two *lac*s will recombine in the donor in only one orientation, the part of the chromosome on one side of the Mu d will be donated early, whereas that on the other side will be donated very late.

To orient *cya*, an F′114 which contains *lac* and Tn*10* was moved from NK1017 into no. 28 and into a Φ(*cya-lac*)*1*-containing strain, JV342, by a plate mating with selection for tetracycline resistance. A strain with a *glnA-lac* fusion, SK1019, was similarly constructed for use as a control for the orientation of *lac* on the F′. *glnA* is known to be transcribed in a counterclockwise direction (29; L. McCarter, personal communication). The F′114-containing derivatives of no. 28, JV342, and SK1019 were JV399, JV358, and JV402, respectively.

The resultant Mu d fusion strains containing an F′ *lac* were then mated with the multiply marked recipient strain, JV146, and markers on either side of the fusion were selected (Fig. 1). JV358, which contains the Φ(*cya-lac*)*1*, donated *pyrB* as an early marker (Table 2). When JV399 was used as a donor, *ilv* and *argG*, which are on the opposite side of *cya* from *pyrB*, were donated as early markers. The number of Ura^+ ($pyrB^+$) exconjugates for JV358 was almost 100 times the number of Ilv^+ exconjugants. In contrast, JV399 gave a 500-fold excess of Ilv^+ exconjugants compared with Ura^+ exconjugants. When JV402, which contains a *glnA-lac* fusion, was used, it was found that *ilv* was donated at 10 times the frequency of *pyrB*. This is in agreement with the known orientation of *glnA* and with a published report of the direction mobilization of *glnA* with F′114 Tn*10* in *E. coli* (29).

These data indicate that *cya* is transcribed in a clockwise direction (Fig. 1), opposite to that of *glnA*. Since *glnA* is transcribed in a counterclockwise direction and produces recombinants at *ilv* at a significantly higher frequency than at *pyrB*, our control tested as predicted. The chromosomal mobilization data also indicate that JV358 and JV399 are insertions of Mu d in opposite directions into *cya*. Since JV358 is Lac^+ and JV399 is Lac^-, these data, along with the precise excision and transduction data, convincingly argue that Φ(*cya-lac*)*1* is both an insertion into *cya* and actually a fusion of *lac* to the *cya* promoter.

Effect of growth media and carbon source. Since the regulation of *cya* in *S. typhimurium* was unknown, it was of interest to investigate the expression of *cya* in various growth media and on various carbon sources. To also see any effects which might only be present in a cya^+ strain, we used both strains carrying the fusion alone and those carrying *cya-lac*/F′ cya^+ heterozygotes. The merodiploid strain, JV350, had similar generation times on rich media and shorter generation times on minimal media than did the uncomplemented strain, JV344. The differential rate of synthesis of β-galactosidase under the control of the *cya* promoter generally decreased as the generation time increased for a given strain, although exceptions to this trend can be seen (Table 3). Comparison of the two strains in a given medium shows that JV350 had a consistently lower differential rate than JV344 in all of the media. This inhibition of expression in JV350 ranges from 17 to 38% of the level of

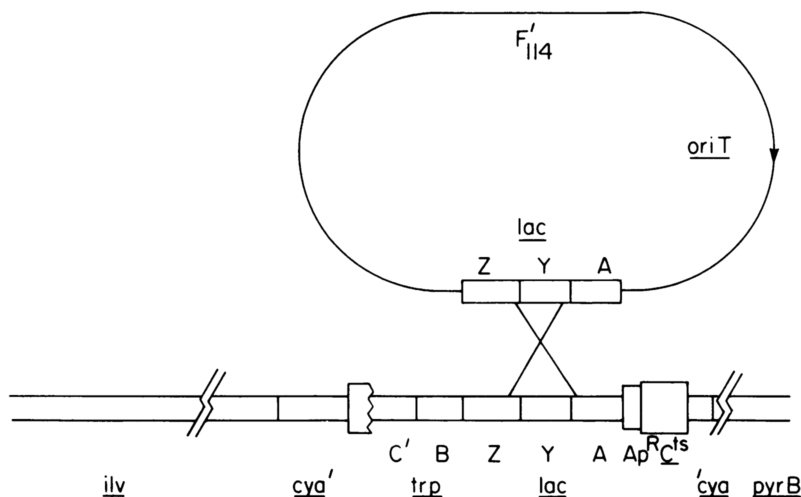

FIG. 1. Orientation of Φ(*cya-lac*)*1*. Orientation was determined by chromosomal mobilization which used homologous recombination between the *lac* genes inserted in *cya* and those carried by F'114. Conjugal transfer of F'114 is initiated at *oriT* and results in *pyrB*'s being donated as an early marker. The zigzags represent the presence of intervening genes. The figure is not drawn to scale.

expression in JV344. The expression of *cya* is therefore slightly but consistently repressed by the presence of the episome: the most obvious possibility is that cAMP may be repressing the expression of *cya*.

Table 3 also shows that the differential rate falls steadily as the cultures are grown on progressively poorer carbon sources. This direct relationship of the quality of the carbon source and the level of *cya* expression could be the result of the increased generation times on the poorer carbon sources or an effect of the increased cAMP levels found as the cells grow on the poorer carbon sources (see below).

Table 3 also contains, as a control of the methods, the results of the effects of the catabolite-sensitive carbon sources on the *S. typhimurium* strain AZ105, which carries *lac* on an episome, F'128. This strain is seen to have a similar growth response to the carbon sources as does the *cya* fusion strain but to have an opposite response of *lac* expression. In strain AZ105, *lac* is derepressed as the quality of the carbon source is decreased, whereas in strain JV350, *cya* expression is repressed. Therefore, the decreased expression by *cya* on poor carbon sources is promoter specific and not a generalized effect.

Effect of cAMP on expression of Φ(*cya-lac*)*1*. The isolation of *cya-lac* fusions allowed us to study the effects of cAMP and *crp* mutations on *cya* expression and to separate the transcription of *cya* from any effects of cAMP and *crp* upon adenylate cyclase activity. To determine whether the regulation of *cya* expression on the catabolite-sensitive carbon sources seen in Table 3 was due to cAMP-mediated effects, JV387 and JV388, a pair of *cya* fusions isogenic except for *crp*, were grown at cAMP concentrations up to 25 mM. cAMP had very little effect on the generation time of JV388 (crp^-), but for JV387 (crp^+), cAMP concentrations from 5 to 25 mM increased the generation times (Fig. 2). The maximal effect was a 180% increase in the generation time of JV387 when the concentration of cAMP was increased from 0 to 25 mM. When a cya^+ crp^+ strain, AZ105, containing an F'*lac* was grown under the same conditions, the generation time also increased with increasing cAMP concentration, but to a much lesser extent than for JV387 (Fig. 2).

Figure 3 shows the differential rate of synthesis plotted against the concentration of cAMP in the growth media. cAMP was found to strongly inhibit the expression of β-galactosidase in strain JV387 but not in strain JV388. The inhibition of *cya* expression in JV387 seemed to have two ranges of action. In the first range, up to 10 mM, cAMP rapidly decreased expression (down to 18% at 10 mM). At cAMP concentrations from 10 to 25 mM, expression slowly declined to 11% of the maximum. The expression of $lacp^+$ in AZ105 was rapidly derepressed as the cAMP concentration was increased from 0 to 2 mM and was saturated by the range of concentrations from 10 to 25 mM. Therefore, the inhibition of *cya* seen in Fig. 3 is promoter specific. The approximately ninefold repression of *cya* by 25 mM cAMP demonstrates that *cya* can be greatly repressed in vivo by added cAMP, and the lack of effect of added cAMP on the crp^- strain JV388 demonstrates that the effect of cAMP on *cya* expression is mediated via CAP.

DISCUSSION

A method for the isolation of *crp-lac* and *cya-lac* fusions in *S. typhimurium* was developed. The method combined the use of Mu d to generate mutants (11) with the fosfomycin selection of Alper and Ames for *crp* and *cya* mutants (3) and with a screening for Lac^+ isolates on the selection plates. The simultaneous selection and screening for both the Lac^+ and the carbon source utilization phenotypes should work

TABLE 2. Chromosomal mobilization[a]

Donor	No. of JV146 exconjugants on selective media for selected marker:		
	argG	*ilv*	*pyrB*
—[b]	0	0	2
JV358 Φ(*cya-lac*)*1*	ND[c]	16	1,482
JV402 Φ(*glnA-lac*)	ND	108	10
JV399 Φ(*cya*-Mu d) (Lac^-)	213	≥5,000	12

[a] Overnight cultures were diluted 1:20 for the donor and 1:40 for the recipient, JV146, into PC and grown for 2 h. A 100-μl portion of either donor or recipient or both was then spread on media selective for one marker, and the plates were incubated for 3 days at 30°C.

[b] —, Recipient control.

[c] ND, Not done.

TABLE 3. Effect of growth media on *cya* expression[a]

Medium	Effect on strain:							
	JV344 Φ(*cya-lac*)*1*			JV350 F′*cya*/Φ(*cya-lac*)*1*			AZ105 F′ *lac*	
	g (min)[b]	Sp act[c]	DR[d]	*g* (min)	Sp act	DR	*g* (min)	DR
LB	50	659	880	48	554	550	—[e]	—
PC	58	777	744	54	576	599	—	—
NB	68	572	632	79	429	421	51.1	4,667
C-glucose + Casamino Acids	64	534	642	52	394	425	—	—
C-glucose	142	297	307	114	235	257	129	3,260
C-glycerol	—	—	—	120	157	135	138	7,154
C-ribose	—	—	—	127	112	105	210	12,786

[a] All cultures were grown at 30°C in a shaking water bath. All C-media was supplemented with cysteine and tryptophan (at 50 μg/ml). When AZ105 was grown, all media were supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside. Cultures were inoculated 1:100 into 20 ml of medium in a 125-ml flask.

[b] *g*, Generation time.

[c] Specific activity (U/OD_{650}) at an OD_{650} of 0.6.

[d] DR, differential rate (U/OD_{650}).

[e] —, Not done.

for generating *lac* fusions in any selection in which MacConkey indicator plates can be used.

The genetic evidence that the isolated fusions are to *cya* and *crp* is as follows. First, the *crp* and *cya* fusions were shown by transduction to map at or near *crp* and *cya*, respectively. Second, the *cya* fusions were also complemented by an *E. coli* episome F′133, known to complement *cya*. Third, both of the *crp* fusions and one of the *cya* fusions yielded precise eductants and therefore do not have detectable deletions of the native gene. This reaffirms that Mu d is actually inserted into the *crp* or *cya* genes.

The fact that the *crp* and *cya* fusions map at the appropriate position and can precisely excise strongly argues that Mu d is inserted into *crp* and *cya* and that no deletion of host DNA occurred on insertion. Since the fusions are Lac^+, the *lac* carried by Mu d has been fused to some promoter. Although it is unlikely that Mu d is inserted in the reverse

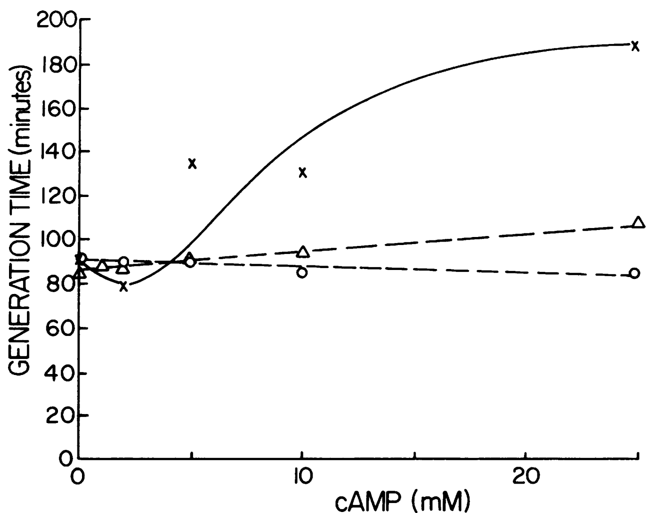

FIG. 2. Effect of cAMP on generation time. A 25-ml portion of M56 media supplemented with 10 mM glucose and L(−)cysteine and L(−)tryptophan was inoculated with 0.25 ml of an overnight culture. AZ105 cultures also contained 1 mM isopropyl-β-D-thiogalactopyranoside. The culture was grown at 37°C in a New Brunswick rotary shaker; 0.5-ml samples were withdrawn periodically and either iced for β-galactosidase assays or the OD_{650} was determined. ×, JV387 [Φ(*cya-lac*)*1* crp^+]; ○, JV388 [Φ(*cya-lac*)*1* crp^-]; △, AZ105 (F′ *lac*/cya^+).

orientation and fused to an adjacent promoter, the precise excision and transductional data do not formally exclude this possibility. For the *cya* fusion, the isolation of a Lac^- cya^- strain, JV399, which gives precise eductants and is oriented in an opposite direction from the Lac^+ *cya* fusions strongly argues that for Φ(*cya-lac*)*1* the *cya* promoter is indeed intact and fused to *lac*.

The orientation of *cya* was determined by the use of chromosomal mobilization. The data indicate that strains containing Φ(*cya-lac*)*1* have *lac* located between *cyap* and *pyrB*. Therefore, *cya* is transcribed in a clockwise direction. This is the same orientation as that reported by Bankaitis et al. (5) for *E. coli*.

The expression of the *cya* gene has been studied by using a gene fusion of *cya* to *lac*. The effects of growth media and cAMP addition in crp^+ crp^- backgrounds were examined. They lead to three major conclusions: first, *cya* is not expressed constitutively; second, *cya* is negatively regulated by cAMP; and third, the negative regulation by cAMP is dependent on a functional CAP. The repression of *cya* by cAMP was found to give up to a ninefold decrease in *cya* expression. The effect was dependent upon a functional CAP, since three independently isolated crp^- mutations all relieved the cAMP-mediated repression (Jovanovich, Ph.D. thesis).

The repression of *cya* in crp^+ strains could be elicited by direct addition of cAMP, by introduction of an F′ carrying *cya*, or by growth of a strain complemented for *cya* on catabolite-sensitive carbon sources. In the same order that the carbon sources derepressed *lac*, when controlled by the *lac* promoter, the carbon sources repressed expression from Φ(*cya-lac*)*1*. When compared with growth on a rich media, the maximal level of repression by growth in minimal media with a poor carbon source was comparable to that caused by cAMP addition.

The decrease in *cya* expression when CAP and cAMP are present is consistent with the idea that *cya* is regulated by CAP and cAMP. However, it does not eliminate the possibility that this may be an indirect effect based on changes in the overall metabolism of the cells or via some specific intermediary. Even if the repression of *cya* by cAMP proves to be indirect, it should still be of interest, since it is a large effect and *lac* is derepressed under the same conditions. To answer the question whether the CAP and cAMP effect is direct, experiments using an in vitro system made in a crp^- strain and using Φ(*cya-lac*)*1* DNA with CAP or cAMP, or both, added will be necessary.

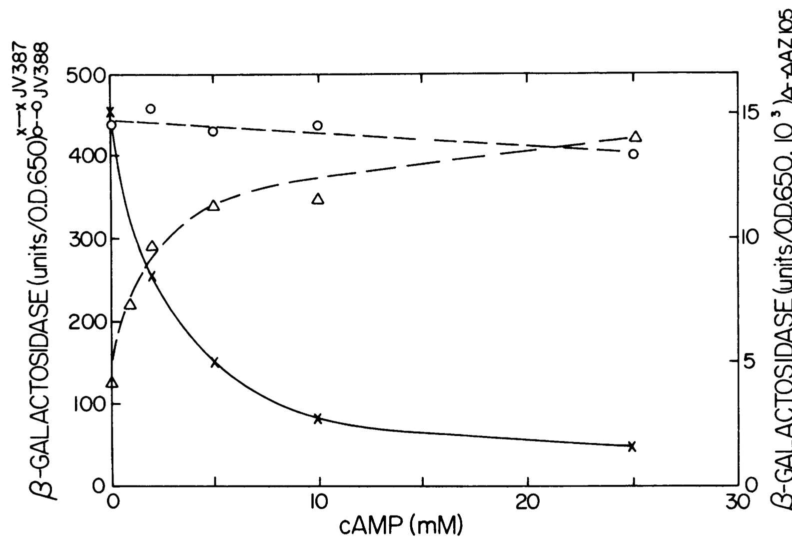

FIG. 3. Differential rate of synthesis of *lac* or Φ(*cya-lac*)*1* as a function of cAMP concentration. All conditions are the same as those described in Fig. 2. ×, JV387; ○, JV388; △, AZ105.

The results presented in Table 3 and Fig. 2 and 3 show a generalized trend of decreasing Φ(*cya-lac*)*1* expression with increasing generation time. Although this suggests a growth rate effect, we do not feel that the regulation that we are seeing is primarily a growth rate effect, for the following reasons. First, a reordering of Table 3 by growth rate shows multiple exceptions to the trend. Second, JV350 has both decreased expression and decreased generation times when compared to JV344. Third, comparison of Fig. 2 and 3 shows that the expression of Φ(*cya-lac*)*1* in JV387 steadily decreases as cAMP concentrations increase, whereas the generation times first decrease and then increase. Further experiments are necessary to explore whether *cya* is also regulated in response to the growth rate.

The relatively small change in *cya* expression upon complementation and on growth on poor carbon sources raises the question of the physiological importance of these changes. The finding that neither cAMP nor CAP can alone produce the repression of *cya* suggests that the repression is a specific effect; whether it is a direct effect awaits experimental testing as described above. If indeed the effect proves to be direct, the small change in *cya* expression under most physiological conditions may be due to its role as a "master control gene". Since it produces an enzyme which will in turn produce an alarmone, perhaps the normal range of its expression would be expected to be limited. The situation could be perhaps loosely likened to that of an amplifier, so that in this analogy *cya* expression would be the coarse-gain control and adenylate cyclase activity would be the finer-gain control; under conditions of balanced growth, major changes in the coarse control would be unnecessary and destabilizing. Only under conditions of dramatic and swift changes, such as carbon source shifts, would this control be expected to change greatly.

In their paper describing the regulation of *cya-lac* fusions in *E. coli*, Bankaitis and Bassford (5) observed up to a 2.2-fold repression of *cya-lac* expression by 5 mM cAMP in minimal medium, and a 1.8-fold repression by growth on succinate. As a result of the maximal, ca. twofold, repression, they concluded that cAMP weakly regulates *cya* and is of dubious physiological significance. Our results point out much larger ranges of regulation. We confirm their results under essentially the same conditions by using *cya-lac* fusions isolated in *S. typhimurium*. However, when conditions are extended to higher concentrations of cAMP, we find up to ninefold repression of *cya*, presumably by cAMP mediated via CAP. Regardless of the physiological meaning of the repression by 25 mM cAMP, the ninefold effect should be sufficient both for the selection of cAMP-independent *cya* promoter mutants and for *crp* mutants defective in negative control. In another in vivo study with plasmids containing in vitro constructed fusions of *cya* to *lacZ*, Roy et al. (38) obtained a stimulation of the rate of synthesis of β-galactosidase when 1 mM cAMP was added. The reasons for the apparent contradiction between their results and those presented by Bankaitis and Bassford (5) and in this study are not readily apparent.

The negative regulation of *cya* by cAMP has been previously proposed by Botsford and Drexler (8) on the basis of their physiological experiments involving an aeration assay with and without the presence of a carbon source. Our results definitely lend support to the negative regulation by cAMP and *crp*, but not of the magnitude seen by Botsford and Drexler. They saw up to a 30-fold increase in the capacity for cAMP synthesis in cells grown on glucose compared with succinate. Our results are reconcilable with theirs if the adenylate cyclase in their aeration assay is differentially activated in the absence of a carbon source. The carbon source on which they are grown may affect the activity of the adenylate cyclase through effects on the phosphotransferase system components, which are known to be differentially expressed according to the carbon source which is being used (35). These changes in the phosphotransferase system may lead to an amplified activation of adenylate cyclase activity when the cells are resuspended without a carbon source as in the aeration assay. Both Majerfeld et al. (20) and Joseph et al. (17) have interpreted their results of increased adenylate cyclase activity in crp^- strains and of decreased adenylate cyclase activity in strains grown on catabolite-sensitive carbon sources to mean that adenylate cyclase activity is primarily decreased by a CAP mediated adenylate cyclase interaction and not by repression of adenylate cyclase synthesis. Our results, as stated above, definitely support the idea of repression of *cya* by the

cAMP-CAP complex but do not exclude the possibility that CAP also negatively regulates the activity of adenylate cyclase.

The other examples of negative regulation by cAMP and *crp* are glutamate synthase (34), glutaminase A (34), outer membrane protein III (21), and the *gal* operon (27). All show a less than 10-fold repression by cAMP under the conditions studied. The concentration effect of cAMP is different in both these studies and the present one. In the case of outer membrane protein III, added cAMP inhibits by a concentration of 10 μM, and the maximum effect is seen by 100 μM. In the case of glutamate synthase and glutaminase A, no effect of cAMP is seen until the concentration exceeds 1 mM, and the concentration for 50% inhibition is at approximately 5 mM. This study indicates that inhibition for *cya* has occurred by 125 μM and continues to affect expression until approximately 10 mM (unpublished data).

The mechanism of negative control by cAMP and *crp* is unknown. Mallick et al. (21) proposed two conformations of *crp*, one present at low concentrations of cAMP (conformation A) and one at high concentrations (conformation B). They proposed that conformation A is saturated at low concentrations of cAMP and then binds DNA and exerts negative control, whereas conformation B is saturated with cAMP at high concentrations and exerts positive control. The present data show that the negative control is also exerted at levels of cAMP in which positive control is also functional. This evidence would seem to eliminate the usefulness of their model. The 100-fold range of cAMP concentrations necessary for repression of these various promoters may either be due to the difference in strain background in the studies or to a differential response of the promoters to CAP and cAMP. If true, the latter possibility would be analogous to the hierarchy of responses of positively regulated genes to cAMP.

The implication of this study is that *cya* appears to be autogeneously regulated by cAMP and CAP. If this regulation is direct, one would predict that the *cya* promoter region would contain a CAP binding site. The sequence of the putative promoter region of *cya* has been determined in *E. coli* by Aiba et al. (1). The authors show that the levels of adenylate cyclase and cAMP are not increased by the presence of a multicopy plasmid, implying some form of autoregulation. They have found two suspected CAP binding sites: one is adjacent to the presumed Pribnow box and the other is located at +120. The site located beside the Pribnow box might be expected to function as a classical repressor binding site, and when cAMP and CAP are bound at this site they would prevent binding and initiation by RNA polymerase. Since we saw a 90% repression of *cya*, if the binding site adjacent to the putative promoter is functional it must either be transiently unoccupied or else RNA polymerase must be able to displace the cAMP-CAP complex at a low frequency. It is interesting to note that the promoter regions of two other genes known to be negatively controlled by cAMP and CAP (*galP2* and *ompA*) have been sequenced and their CAP binding sites have been determined (26, 43). In both cases, the binding sites were located in the −35 region and were identical. These would also be expected to interfere with RNA polymerase binding.

Our experiments have demonstrated that *cya* is repressed by the cAMP-CAP complex, whether directly or indirectly. However, although these experiments shed light on how adenylate cyclase levels are controlled, they do not by themselves fully explain the increase in cAMP levels in crp^- strains. An important use of the complemented *cya* fusions will be in the study of the effects of growth conditions and of CAP and PTS mutations on Φ(*cya-lac*) expression, cAMP levels, and adenylate cyclase activity when measured simultaneously. These fusions should be an important key to dissecting the control of cAMP levels.

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