JOURNAL OF BACTERIOLOGY, Sept. 1982, p. 1346–1357
0021-9193/82/091346-12$02.00/0

Vol. 151, No. 3

Regulation of Adenylate Cyclase Synthesis in *Escherichia coli*: Studies with *cya-lac* Operon and Protein Fusion Strains

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Received 19 April 1982/Accepted 28 May 1982

We have isolated *cya-lac* operon and protein fusions in *Escherichia coli* K-12, and we used these to study the regulation of *cya*, the structural gene for adenylate cyclase. Data obtained from these fusion strains suggest that neither cyclic AMP (cAMP) nor the cAMP receptor protein plays a major role in transcriptional or translational regulation of *cya* expression. Modulation of intracellular cAMP concentrations elicited only weak repression of *cya-lac* fusion activity under conditions of high intracellular cAMP, relative to fusion activity under conditions of low intracellular cAMP. The functional cAMP receptor protein was required for this effect. Incorporation of Δ*crp* into *cya-lac* fusion strains did not affect fusion expression in glucose-grown cells as compared with similarly cultured isogenic *crp*$^+$ strains. Furthermore, 20 independently obtained mutants derived from a *cya-lacZ* protein fusion strain exhibiting a weak Lac$^+$ phenotype were isolated, and it was determined that the mutants had β-galactosidase activities ranging from 2- to 77-fold greater than those of the parental strain. None of the mutations responsible for this increase in fusion activity map in the *crp* locus. We used these mutants to aid in the identification of a 160,000-dalton *cya-lacZ* hybrid protein. Finally, chromosome mobilization experiments, using *cya-lac* fusion strains, allowed us to infer a clockwise direction of transcription for the *cya* gene relative to the standard *E. coli* genetic map.

Glucose and its non-metabolizable analogs, such as α-methylglucoside and 2-deoxyglucose, elicit repression of catabolic enzyme synthesis in the bacterium *Escherichia coli*. This phenomenon is termed catabolite repression. The initial insight into the mechanism of catabolite repression correlated low intracellular cyclic AMP (cAMP) concentrations with severe reduction in synthesis of catabolic enzymes (21). Furthermore, the repression could be relieved by adding this cyclic nucleotide to the growth medium (24). It is now recognized that cAMP and its receptor protein (CRP) comprise a general positive control complex required for efficient induction of catabolite-sensitive genetic systems. Such systems include those involved in maltose, ribose, L-arabinose, and glycerol metabolism (25, 26).

Although the precise role for cAMP-CRP in mediating catabolite repression remains controversial (12, 15, 38), there can be no doubt of the important function served by cAMP as a regulatory entity in *E. coli* (6, 23). Certain aspects concerning regulation of cAMP biosynthesis are becoming clear. There exists compelling evidence for involvement of phosphotransferase carbohydrate transport components in post-translational regulation of adenylate cyclase (AC), the enzyme product of the *cya* locus that is responsible for cAMP synthesis (26, 28, 34). AC activity in whole cells has also been reported to require an established proton electrochemical gradient to be regulated by transport of non-phosphotransferase carbohydrate transport substrates (27).

Little is currently known about transcriptional regulation of *cya*. Indeed, the genetic structure of *cya* also remains largely undefined (32). Using the gene fusion techniques described by Casadaban (8, 9), we generated *cya-lac* operon and protein fusions. We utilized these fusions as probes to study transcriptional and translational regulation of *cya* expression. We also initiated studies on the genetic structure of *cya* by determining the orientation of *cya* relative to its promoter on the *E. coli* genetic map.

MATERIALS AND METHODS

Bacterial and phage strains, media and chemicals, genetic techniques. The bacterial and bacteriophage strains employed in this study are described in Tables 1 and 2. Complex media included LB medium and triphenyltetrazolium chloride agar (14, 22). The minimal base medium was M63 (22) supplemented with appropriate carbon sources and amino acids to final

TABLE 1. Bacterial strains

Strain	Genotype	Source
E. coli K-12		
CA8404	HfrH Δ*cya crp* rpsL thi*	J. Beckwith
CA8439	HfrH Δ*cya* Δ*crp-39 rpsL thi*	(33)
CA8445	HfrH Δ*cya* Δ*crp-45 rpsL thi*	(33)
CH50	F⁻ *gal araD139* Δ*lacU169 zab*::Tn*5*	This study
CH58	CH50 *rpsL*	This study
CH62.19-1	CH50 λp1(209) ΔMu Φ(*cya-lac*⁺)19-1[a]	This study
CH62.53-1	CH50 λp1(209) ΔMu Φ(*cya-lac*⁺)53-1[a]	This study
CH62.55-1	CH50 λp1(209) ΔMu Φ(*cya-lac*⁺)55-1[a]	This study
CH54.42-2	CH50 λp1(209) ΔMu Φ(*cya-lacZ*)$_{hyb}$42-2[a]	This study
CH150	CH54.42-2 *rpsL*	This study
CH151	CH54.42-2 *rpsL crp**	This study
CH153	CH58 λp42-2	This study
CH155	CH50 λp42-2	This study
CH156	CH54.42-2 *rpsL* Δ*crp-39*	This study
CH158	CH155 *rpsL* Δ*crp-39*	This study
CH200	CH62.19-1 *rpsL*	This study
CH201	CH62.19-1 *rpsL crp**	This study
CH203	CH58 λp19-1	This study
CH205	CH50 λp19-1	This study
CH206	CH62.19-1 *rpsL* Δ*crp-39*	This study
CH208	CH205 *rpsL* Δ*crp-39*	This study
SE4050	F⁻ *lamBS60 rbs*::Tn*10 araD139 thi relA rpsL* Δ*lacU169*	Scott Emr
7005.11	F⁻ Δ*lacU169 trp*(Am) *araD araC*(Am) *rpsL relA tyrT*	M. Berman
MC4100	F⁻ *araD139 lacU169 rpsL relA thi*	(8)
RK1041	F⁻ *ilv argH metB his pyrE60 cysE lac mtl rpsL*	R. Kadner
S. typhimurium TT629	F′ (Ts)114 *lac*⁺ *zzf*::Tn*10*/*rpsL1 pyrC1*	R. Kadner

[a] The scheme for nomenclature and designation of operon and protein fusions used here is the same as that described in reference 3.

concentrations of 0.2% and 50 μg/ml, respectively. When included, kanamycin sulfate was added at 30 μg/ml, streptomycin sulfate at 150 μg/ml, tetracycline at 20 μg/ml, ampicillin at 25 μg/ml, uracil (free base) at 40 μg/ml, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XG) at 40 μg/ml. All of the antibiotics, carbohydrates, cAMP (free acid), *ortho*-nitrophenylgalactoside, and uracil were obtained from Sigma Chemical Co.; XG was purchased from Bachem, Inc.; and 2,3,5-triphenyltetrazolium chloride (TTC) was purchased from Difco Laboratories. Generalized transduction with phage P1 *vir* was performed as described by Miller (22). Additional bacterial and phage genetic techniques have been described (3, 22, 37).

β-galactosidase assays. β-galactosidase activity was determined by the method of Miller (22). Phenotypically characterized strains were grown for at least eight generations in the appropriate minimal medium at 30°C and a setting of 7.0 in a New Brunswick Scientific Gyrotory shaking water bath (model G76). Assay values represent averages of duplicate determinations performed on three independent cultures for any given strain cultured under a given set of conditions. Equal portions of bacterial cultures to be assayed were streaked on glucose-XG agar to ascertain the curing frequency for λ p*cya-lac* lysogens. In all cases, this was negligible.

Isolation of *cya*::Mu *c*(Ts) mutants. Strain CH50 is sensitive to phage λ *vir* (λ^s) and also sensitive to L-arabinose due to a mutation in *araD*. Isogenic *cya* or *crp* derivatives of strain CH50 are resistant to both λ *vir* and L-arabinose. These phenotypes provide the basis for a positive selection for Cya⁻ mutants. An LB top agar lawn of strain CH50 was spotted with a fresh lysate of phage Mu *c*(Ts) as described previously (37). Survivors from each spot were suspended in 0.5 ml of LB medium, challenged with 10^{10} PFU of λ *vir*, diluted 10-fold in LB medium containing 1% L-arabinose, and grown to heavy turbidity at 30°C with aeration. Equal portions of each culture were streaked for isolated colonies onto ribose-glycerol-triphenyltetrazolium chloride agar. One red colony (i.e., Rbs⁻ Gly⁻) was selected per culture to insure independent lysogenic events and was purified. Lysogeny with Mu *c*(Ts) was confirmed for each isolate by resistance to phage Mu *c* and by temperature sensitivity (at 42°C). The Cya⁻ phenotype was demonstrated for each isolate by the pleiotropic inability to utilize ribose, glycerol, and maltose in the absence of exogenously supplied cAMP. (Since this selection does not initially discriminate between Cya⁻ and Crp⁻ lysogens, we expected to frequently detect lysogens of the latter class. However, only 2 of 66 lysogens screened in this manner proved to be Crp⁻.) Single Mu *c*(Ts) lysogens with *cya* as the site of insertion [i.e., *cya*::Mu *c*(Ts)] were demonstrated by transduction of the Cya⁻ lysogen to Gly⁺ Tet^r, using phage P1 *vir* propagated on strain SE4050 (*rbs*::Tn*10*). Lysogens yielding Gly⁺ Tet^r transductants that subsequently scored as Rbs⁻ Mal⁺ Mu *c*^{s} were considered *cya*::Mu *c*(Ts). Of 64 independently obtained, putative *cya* insertions analyzed, 23 behaved as *cya*::Mu *c*(Ts) by these criteria.

TABLE 2. Bacteriophage strains

Strain	Bacterial genes carried	Source
P1 *vir*		Laboratory stock
Mu *c*(Ts)		Laboratory stock
Mu *c62*		Laboratory stock
Mu *dl*(Ap^r *lac*)	trp'BA'-W209 *lac'*OZYA'	J. Beckwith
λ *vir*		Laboratory stock
λp1(209)	::(+Mu')*trp'BA'*-W209 *lac'OZYA'*	Laboratory stock
λp1(209,118)	::(+Mu')*trp'BA'*-W209 *lac'OZU118YA'*	Laboratory stock
λp19-1	Φ(*cya-lac*$^+$)19-1	This study
λp42-2	Φ(*cya-lacZ*)$_{hyb}$42-2	This study
λ *cI h80*		Laboratory stock
λ *cI hPA-2*		This study
Φ80 d*tyr*T	*tyrT*	Laboratory stock

Isolation of *cya*(Am) mutants. Strain CH50 was mutagenized with 2-aminopurine as described previously (22). Survivors were selected and scored for Cya$^-$ as described above. Cya$^-$ isolates were cross-streaked against phage φ80 d*tyrT* (SuIII$^+$) on glycerol minimal agar. Strains suppressed to Gly$^+$ were similarly tested for suppression to Mal$^+$, and mutants exhibiting suppression to growth on both carbon sources were considered to have amber nonsense mutations in *cya*. This was further confirmed by mapping these mutations to the *cya* locus, also as described above.

Isolation of *cya-lac* fusions. To obtain operon fusions, Mu d(Ap^r *lac*) lysogens of strain CH50 were obtained as described by Casadaban and Cohen (9). Subsequently, insertions of Mu d(Ap^r *lac*) into *cya* were selected as above and screened for a blue reaction, indicating a Lac$^+$ phenotype, on glucose-XG minimal agar. A λ transducing phage was obtained for each putative *cya-lac* operon fusion exactly as described by Komeda and Iino (20). Fusion of *lac* to *cya* was unambiguously demonstrated as described below.

Potential *cya-lacZ* protein fusions were obtained by selection for thermoresistant Lac$^+$ survivors derived from single λp1(209,118) lysogens of *cya*::Mu *c*(Ts) insertion strains, as described before (8). Lac$^+$ colonies began to appear on selection agar some 12 days after plating and were obtained at a frequency of approximately one for every 10^9 cells plated. A Lac$^+$ transducing phage was isolated from each potential *cya-lacZ* protein fusion strain, as described previously (8, 37). Insertion of λ *lac* into the *cya* locus was determined as described above for *cya*::Mu *c*(Ts).

Direction of transcription. The direction of Φ(*cya-lac*) transcription was determined on the basis of F'(Ts)*lac*-mediated chromosome mobilization (10, 35). *Salmonella typhimurium* strain TT629 was mated with strains CH62.19-1 and CH54.42-2 for 30 min at 30°C in LB medium. Transconjugants were selected on lactose-tetracycline-kanamycin sulfate minimal agar supplemented with a few crystals of cAMP, which were placed in the center of the plate. Several large colonies located near the center of each selection plate were purified and scored for a cAMP-dependent Lac$^+$ phenotype. Both CH62.19-1 and CH54.42-2 grow poorly on lactose minimal agar, and *cya*-directed *lac* expression is not cAMP dependent. Transconjugants exhibiting cAMP-dependent Lac$^+$ phenotypes were thought to possess F'(Ts)*lac*. Integration of the episome into the chromosome was selected for by growth on lactose-tetracycline minimal agar, supplemented with 2 mM cAMP, at 42°C. The resulting Hfr strains were cultured in lactose-tetracycline liquid medium and mated with strain RK1041 for 30 min at 37°C. Recombinants were selected for Met$^+$ Arg$^+$ or Pyr$^+$ Ilv$^+$ Cys$^+$. Streptomycin was used to counterselect donor strains.

Construction of phage λ *cI hPA-2*. To facilitate selection for Cya$^-$ lysogens of phage λ, it was desirable to possess a phage strain, in addition to λ *cI h80*, exhibiting λ *cI* immunity and a plating efficiency independent of *cya* expression in the host bacterium. Phage λ *cI hPA-2* was constructed in the manner described for phage Hy2 (2), except that λ *cI* was used instead of λ *vir* in the genetic cross. The identity of the phage was confirmed by its inability to plate on either λ$^+$ lysogens or *ompC* λ$^-$ strains (which lack a functional phage PA-2 receptor).

Immune precipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fusion strains were grown at 30°C with good aeration in glucose minimal medium into mid-log phase (optical density at 600 nm of 0.5). Aliquots (1 ml) were incubated with 5 μCi of ^{14}C-uniformly -labeled amino acids (ICN) for 1 min and then placed on ice. Cells were washed once with 50 mM Tris-hydrochloride (pH 8.0) and then solubilized in 100 μl of the same buffer containing 1% sodium dodecyl sulfate (SDS)–1 mM EDTA by heating in a boiling water bath for 2 min. Operon and protein fusion products were immune precipitated from this extract with anti-*E. coli* β-galactosidase serum and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography, as described previously (19).

RESULTS

Construction of *cya-lac* fusion strains. For the purposes of studying regulation of the *cya* locus, we obtained both *cya-lac* operon fusions and *cya-lacZ* protein fusions. Operon fusions place the *lacZY* genes under the control of the promoter for the *cya* gene (*cyap*). In strains harboring *cya-lac* operon fusions, one can conveniently monitor transcriptional regulation exerted at *cyap* by measuring levels of the enzyme β-galactosidase, the *lacZ* gene product. In generating protein fusions, a hybrid *cya-lacZ* gene results, encoding a hybrid protein in which the amino terminus of β-galactosidase has been replaced by an amino-terminal portion of AC, the *cya* gene product. Such hybrid proteins retain β-galactosidase activity. Since the translational

start sequences at the 5′ end of the *cya* message are preserved in the *cya-lacZ* fusion, β-galactosidase activity in these protein fusion strains is an indicator of both transcriptional regulation exerted at *cyap* and any additional regulatory influences that may be exerted at the level of translation initiation. Several different techniques have been described by Casadaban (8, 9) for generating such fusions in vivo in *E. coli*. These techniques have been widely used and will not be described in detail here.

To obtain *cya-lac* operon fusions, phage Mu d(Ap, *lac*) insertions into the *cya* locus were selected in strain CH50 (see above). We obtained 61 independent Mu d(Ap, *lac*) lysogens that exhibited the phenotype Cya$^-$ Lac$^+$ Ap^r. Presumably, in at least some of these strains, the desired event had occurred whereby the Mu prophage had inserted into the *cya* gene in the correct orientation, generating a *cya-lac* operon fusion. To simultaneously stabilize each putative fusion in the chromosome and to facilitate future genetic manipulations, the integrated Mu sequences were replaced with a λ prophage, a technique originally described by Komeda and Iino (20). Each of the 61 putative operon fusion strains was lysogenized with λp1(209). Insertion of this phage into the chromosome occurs primarily via Mu or *lac* DNA sequence homology present on both the λ genome and the previously integrated Mu d(Ap, *lac*) genome (Fig. 1). Since the Mu d(Ap, *lac*) prophage harbors a temperature-sensitive repressor mutation, selection for thermoresistant survivors of the double lysogens yields strains that have lost the Mu prophage, thereby placing the λ prophage adjacent to the putative *cya-lac* operon fusion. These strains are phenotypically Cya$^-$ Lac$^+$ Ap^s and express λ immunity (λ$^+$). At this point, a bonafide *cya-lac* operon fusion strain would be expected to harbor the λ prophage integrated within the *cya* locus. To confirm this, each of the 61 strains in which Mu had been replaced with λ was transduced to Tet^r, using phage P1 *vir* grown on strain SE4050. This particular donor strain provided the means of introducing the *cya*$^+$ allele into each of our putative fusion strains as an unselected marker approximately 50% cotransducible with *rbs*::Tn*10*. If the recipient strain harbors a *cya-lac* fusion, it should become phenotypically Lac$^-$ and lose λ immunity in every instance the unselected *cya*$^+$ allele is cotransduced with Tn*10*. Likewise, Tet^r transductants scoring as Cya$^-$ are expected, in every instance, to remain Lac$^+$ λ$^+$. We found that 58 of our 61 strains did not behave as predicted, suggesting (i) that Mu d(Ap, *lac*) had not inserted into the *cya* gene during our initial selection or (ii) that Mu d(Ap, *lac*) had inserted into *cya* but, perhaps as a result of transposition events, resided at multiple sites

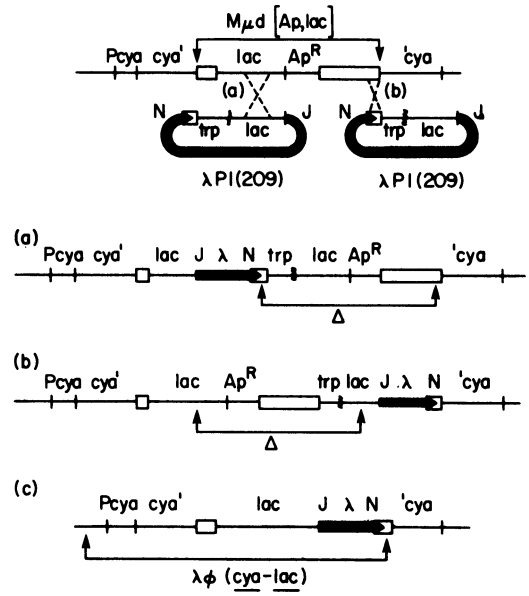

FIG. 1. Replacement of Mu d with λ. Integration of λp1(209) into the bacterial chromosome preferentially occurs via (a) *lac* homology or (b) Mu homology. Selection for thermoresistant survivors of these lysogens (c) yields deletion mutants, cured of Mu d via excision by homologous recombination, exhibiting a Cya$^-$ Ap^s λ$^+$ phenotype (see reference 20).

on the bacterial chromosome. However, we did have three putative *cya-lac* operon fusion strains that met all of our criteria. For each of these, at least 15 Tet^r Cya$^+$ and 15 Tet^r Cya$^-$ transductants were scored for their Lac phenotype and λ immunity.

Protein fusions were obtained by a different procedure. A number of independently isolated Mu *c*(Ts) insertions in the *cya* gene (see above) were lysogenized with phage λp1(209,118), and thermoresistant Lac$^+$ survivors were selected as described by Casadaban (8). Putative *cya-lacZ* protein fusions were obtained, starting with six different Mu insertions. We failed to obtain any Lac$^+$ colonies, starting with a number of Mu *c*(Ts) insertion strains; presumably, the insertions in these strains are in the wrong orientation with regard to *cyap*. For 13 putative *cya-lacZ* protein fusions, we applied the same mapping strategy given above for our operon fusions. For each of the 13 strains, both the fusion and the λ immunity were 100% linked to the *cya* locus.

Genetic proof for fusion of *lac* to *cya*. The technique used to genetically confirm that we had obtained *cya-lacZ* protein fusions is shown in Fig. 2. We induced a λp transducing phage carrying the putative *cya-lacZ* protein fusion (see above) from each of five different strains. These phages were used to lysogenize deriva-

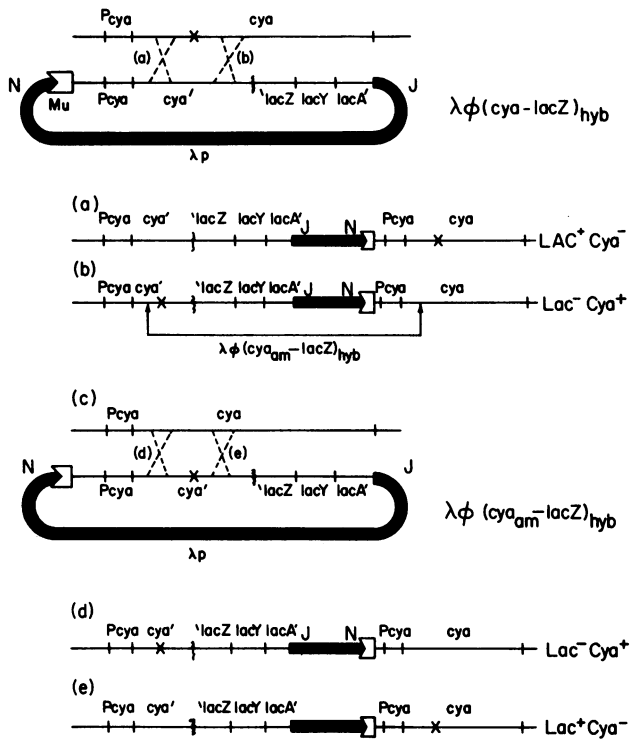

FIG. 2. Genetic proof for *cya-lacZ* protein fusion. Integration of a putative λΦ(*cya-lacZ*)$_{hyb}$ transducing phage into the genome of a strain carrying a *cya*(Am) mutation (X) occurs primarily via homology in or adjacent to the *cya* locus. An integration event promoter proximal to the *cya*(Am) mutation will yield a Lac$^+$ Cya$^-$ lysogen (a), whereas an integration event promoter distal to the amber mutation will generate a Lac$^-$ Cya$^+$ lysogen (b). Fusion phages reisolated from this latter class of lysogens will now most likely carry the *cya*(Am) mutation (c). When a *cya*$^+$ strain is infected with such a Lac$^-$ phage, two classes of lysogens result, depending upon where recombination between the bacterial and phage chromosome occurs. Recombination via homology promoter proximal to the *cya*(Am) mutation results in a Lac$^-$ Cya$^+$ lysogen (d). On the other hand, integration occurring by homologous recombination promoter distal to *cya*(Am) yields a Lac$^+$ Cya$^-$ lysogen (e). The ability to demonstrate the Lac$^-$ Cya$^+$ class of lysogens described in (b) and then to recombine the *cya*(Am) mutation from a λ phage obtained from such a lysogen into a *cya*$^+$ gene, as shown in (e), conclusively demonstrates that the original transducing phage does indeed carry a *cya-lacZ* protein fusion. Note: If (X) were to represent a Mu insertion in *cya*, this can also be recombined into a *cya-lac*$^+$ operon fusion in an identical manner. In this instance, the Mu insertion will be polar on *lac* expression, and the lysogen will be Lac$^-$ Cya$^+$. However, since Mu is too large to be picked up on a λ phage by excision (as shown in [b]), only Lac$^+$ phages should be obtained upon induction, these being generated by excision in essentially the reverse manner as the original integration event.

tives of strain CH50 carrying amber nonsense mutations in *cya* (obtained as described above). These amber mutants are λ^r but can be rendered λ^s by growth in maltose minimal medium containing 2 mM cAMP. Integration of the fusion phage into the *E. coli* chromosome was expected to occur via homology in the *cya* region. If the *cya*(Am) mutation in the host strain is promoter proximal to the fusion joint, two classes of lysogens are predicted. Integration promoter proximal to the *cya*(Am) mutation results in a Lac$^+$ Cya$^-$ lysogen. Integration of the λ prophage promoter distal to the *cya*(Am) mutation results in a Lac$^-$ Cya$^+$ lysogen. Furthermore, the Lac$^-$ phenotype of this latter class of lysogens should be suppressed to Lac$^+$ by introducing the amber suppressor mutation *tyrT* into these strains. The Lac$^-$ Cya$^+$ class of lysogens can only be obtained if the transducing phage carries a *cya-lacZ* protein fusion. The identity of *malT-lacZ* protein fusions, using *malT*(Am) mutations, was recently confirmed in a similar manner (11).

We infected four independently isolated *cya*(Am) mutants with each of the five different λ transducing phage carrying presumed *cya-lacZ* protein fusions. We were unable to detect Lac$^-$ Cya$^+$ lysogens for four of the five phages.

However, the λ phage isolated from the presumed protein fusion strain CH54.42-2 (designated λp42-2) yielded the predicted class of Lac$^-$ Cya$^+$ lysogens with each of the four *cya*(Am) mutants. For example, 10% of the lysogens obtained after infection of strain AP9.1 (*cya2091*(Am)) with λp42-2 were Lac$^-$ Cya$^+$. The remaining 90% were Lac$^+$ Cya$^-$. As predicted, the Lac$^-$ phenotype of the minor class of lysogens was suppressible to Lac$^+$ by *tyrT*. The genetic proof for this one protein fusion was extended by inducing the fusion phage from several of the Lac$^-$ Cya$^+$ lysogens. On XG agar, these phage formed colorless plaques on strain MC4100 (Su$^-$) and blue (i.e., Lac$^+$) plaques on strain 7005.11 (SuIII$^+$). Furthermore, two classes of lysogens of the parental strain CH50 (*cya*$^+$) were obtained with these λ phage (see Fig. 2). The majority of the lysogens (again, about 90%) were phenotypically Lac$^-$ Cya$^+$; a minor class (10%) was Lac$^+$ Cya$^-$, with the Cya$^-$ phenotype being suppressible by *tyrT*. Our observation that an amber mutation can be crossed from the chromosomal *cya* locus into the *cya-lacZ* hybrid gene and subsequently from the hybrid gene back into the wild-type *cya*$^+$ allele conclusively demonstrates that strain CH54.42-2 harbors a *cya-lacZ* protein fusion.

To genetically confirm the identity of *cya-lac* operon fusions, we chose a strategy that previously had been used to confirm operon fusions of *lac* to the *tyrT* and *ompC* genes (5, 17). Five independently isolated *cya*::Mu *c*(Ts) insertion strains were infected with λp transducing phages induced from the three candidate *cya-lac* operon fusion strains described above. Cya$^+$ λ lysogens were selected on glycerol minimal agar plates seeded with 10^9 PFU each of phages λ *c*I *h*80 and λ *c*I *h*PA-2. If the phage carries a *cya-lac* operon fusion, then integration of the phage into the chromosome of the *cya*::Mu *c*(Ts) insertion mutant in such a way to regenerate *cya*$^+$ must position the Mu *c*(Ts) prophage at a site that will prevent expression of the *lac* genes initiated at *cyap* (see legend to Fig. 2). With each of the three putative operon fusion phages, we obtained Lac$^-$ Cya$^+$ lysogens for at least two of the five *cya*::Mu *c*(Ts) insertion mutants tested. The Lac$^-$ Cya$^+$ lysogens, as would be expected, liberated almost exclusively Lac$^+$ phages (i.e., blue plaques with the appropriate indicator strain on XG agar) upon induction with UV irradiation. In addition, the two *cya*::Mu *c*(Ts) insertion mutants that yielded Lac$^-$ Cya$^+$ lysogens with each of the three operon fusion phages also yielded Lac$^-$ Cya$^+$ lysogens when infected with λp42-2, the phage bearing the proven *cya-lacZ* protein fusion. Our ability to recombine the two Mu *c*(Ts) insertions into the *cya-lacZ* protein fusion identifies these mutations as true *cya*::Mu *c*(Ts) insertions. This is an important point since insertions of Tn*5* into *cya* appear to be unstable (32).

Effect of cAMP on expression of *cya-lac* fusions. Strains of *E. coli* and *S. typhimurium* lacking functional CRP have been reported to overproduce cAMP when growing in glucose, suggesting a possible role for this protein as a repressor of *cya* expression (7, 29, 30, 40). Botsford and Drexler (7) have presented evidence implicating the cAMP-CRP complex as the entity responsible for *cya* repression. The *cya-lac* fusion strains described above provide the means to directly test these hypotheses. We initially investigated the effect that modulation of intracellular cAMP concentrations had on the β-galactosidase activity of the fusion strains. To accomplish this, two different methods were employed.

(i) Fusion strains were cultured in glucose minimal medium either without cAMP or supplemented with 5 mM cAMP. As determined by measuring β-galactosidase activity, the three operon fusion strains, CH62.19-2, CH62.53-1, and CH62.55-1, exhibited cAMP-mediated repression ratios of 1.8, 2.2, and 1.5, respectively (Table 3). The protein fusion strain CH54.42-2 was similarly repressed. Exogenous cAMP concentrations of 2 mM yielded similar results (not shown). Similar repression ratios were also observed with strains CH205 and CH155, the *cya*$^+$ lysogens of λp19-1 (operon fusion phage derived from CH62.19-1) and λp42-2 (protein fusion phage), respectively.

(ii) Cellular levels of cAMP are known to fluctuate as a function of carbon source (13, 40). The *cya*$^+$ operon fusion strain CH205 was grown in minimal medium supplemented with several different carbon sources, and again, β-galactosidase activity was determined (Table 4). The greatest enzyme activity was observed when strain CH205 was grown on glucose, a condition of low intracellular cAMP concentrations. Glycerol- or maltose-grown cells contain elevated cAMP levels. With these carbon sources, strain CH205 reproducibly exhibited slightly repressed levels of β-galactosidase activity relative to glucose-grown cells. The lowest enzyme activity was noted in succinate-grown cells which, again, is consistent with the higher cAMP level associated with this carbon source. The repression ratios obtained with the same carbon sources for the *cya*$^+$ protein fusion strain CH155 were of a similar magnitude, although with this strain, the data are more difficult to evaluate owing to its low β-galactosidase activity. For both operon and protein fusion strains, the repression ratios we observed (1.8 at most) were modest and correlated well with those measured when the original Cya$^-$ fusion strains were grown in glucose minimal medium with or

TABLE 3. Effect of cAMP on expression of *cya-lac* fusions

Strain	Relevant genotype	Units of β-galactosidase		Repression ratio[b]
		Without cAMP	With cAMP[a]	
CH62.19-1	Φ(*cya-lac*$^+$)19-1	405	225	1.8
CH62.53-1	Φ(*cya-lac*$^+$)53-1	1,239	574	2.2
CH62.55-1	Φ(*cya-lac*$^+$)55-1	1,017	692	1.5
CH54.42-2	Φ(*cya-lacZ*)$_{hyb}$42-2	19	12	1.6
CH205	*cya*$^+$ λp19-1	398	204	2.0
CH155	*cya*$^+$ λp42-2	25	15	1.7
CH206[c]	Φ(*cya-lac*$^+$)19-1 Δ*crp-39*	387	395	1.0
CH208[c]	*cya*$^+$ λp19-1 Δ*crp-39*	395	416	0.9
CH156[c]	Φ(*cya-lacZ*)$_{hyb}$42-2 Δ*crp-39*	19	18	1.1
CH158[c]	*cya*$^+$ λp42-2 Δ*crp-39*	18	18	1.0

[a] cAMP (5 mM) was included in the growth medium.
[b] Units of β-galactosidase activity of strain grown in glucose minimal medium without cAMP/units of β-galactosidase activity of strains grown in the same medium with cAMP.
[c] The *rpsL* allele used to construct the Δ*crp* strains had no effect on fusion activity.

without cAMP (Table 3). These data do not indicate a major role for cAMP in regulating expression of the *cya* locus.

Fusion activity in *crp* strains. If CRP is involved in repressing transcription from *cyap*, then β-galactosidase activity in *cya-lac* fusion strains should be enhanced upon the introduction of a Δ*crp* mutation. We incorporated the Δ*crp-39* mutation (33) into a number of fusion strains by cotransduction with *rpsL*. β-galactosidase activity was determined for isogenic *crp*$^+$ and Δ*crp-39* strains grown in glucose minimal medium, with or without functional AC present (Table 5). For both an operon fusion and a protein fusion, no significant effect of the Δ*crp* mutation on expression of β-galactosidase activity was discerned. Similar results were also obtained when isogenic Δ*crp-45* (33) derivatives of these same fusion strains were analyzed (data not shown).

We note above that 5 mM cAMP elicits weak repression of *cya-lac* operon and protein fusion strains (all *cya crp*$^+$) (Table 3). This weak repression was not exhibited by isogenic Δ*crp-39* derivatives of these fusion strains (Table 3), indicating that this effect must be mediated by the cAMP-CRP complex. Hence, we studied the effect of an altered CRP on β-galactosidase activity in two of these fusion strains. We introduced the *crp** allele of Sabourin and Beckwith (33) into strains CH62.19-1 and CH54.42-2 by cotransduction with *rpsL*. The *crp** mutation phenotypically suppresses *cya* mutations, presumably by encoding an altered CRP that does not require cAMP for activity (33). This mutant CRP* protein, however, exhibits positive control functions that are not sensitive to catabolite repression. The *crp** operon fusion strain CH201 grown in glucose minimal medium exhibited β-galactosidase activity that was depressed 1.4-fold relative to that found in its isogenic *crp*$^+$ strain under the same conditions (Table 5). This result is consistent with our earlier observations when cAMP concentrations were modulated in isogenic *crp*$^+$ strains (Tables 3 and 4). As expected, in the *crp** strain, regulation of fusion expression by variation of the carbon source was not observed (Table 5).

TABLE 4. Effect of carbon source on expression of *cya-lac* fusions

Strain	Relevant genotype	Carbon source	Units of β-galactosidase	Repression ratio[a]
CH62.19-1	Φ(*cya-lac*$^+$)19-1	Glucose	370	
CH205	*cya*$^+$ λp19-1	Glucose	355	1.0
		Maltose	252	1.4
		Glycerol	241	1.5
		Succinate	199	1.8
CH54.42-2	Φ(*cya-lacZ*)$_{hyb}$42-2	Glucose	22	
CH155	*cya*$^+$ λp42-2	Glucose	25	1.0
		Maltose	22	1.1
		Glycerol	25	1.0
		Succinate	18	1.4

[a] Units of β-galactosidase activity of strains grown in minimal medium with glucose as the carbon source/units of β-galactosidase activity of strains grown in minimal medium with alternate carbon source.

TABLE 5. Regulation of *cya-lac* expression by CRP

Strain	Relevant genotype	Carbon source	Units of β-galactosidase
CH200	Φ(*cya-lac*$^+$)19-1	Glucose	380
CH206	Φ(*cya-lac*$^+$)19-1 Δ*crp-39*	Glucose	349
CH203	*cya*$^+$ λp19-1	Glucose	340
CH208	*cya*$^+$ λp19-1 Δ*crp-39*	Glucose	343
CH201	Φ(*cya-lac*$^+$)19-1 *crp**	Glucose	272
		Maltose	282
		Glycerol	285
		Succinate	312
CH150	Φ(*cya-lacZ*)$_{hyb}$42-2	Glucose	18
CH156	Φ(*cya-lacZ*)$_{hyb}$42-2 Δ*crp-39*	Glucose	18
CH153	*cya*$^+$ λp42-2	Glucose	19
CH158	*cya*$^+$ λp42-2 Δ*crp-39*	Glucose	21
CH151	Φ(*cya-lacZ*)$_{hyb}$42-2 *crp**	Glucose	16
		Maltose	14
		Glycerol	18
		Succinate	17

Direction of transcription of *cya* locus. The homology provided by *lac* DNA sequences in a *cya-lac* fusion strain and on F′(Ts)*lac* can be utilized to direct the insertion of the episome into the chromosome. The direction of transfer of chromosomal genes by the resultant Hfr strain is dependent upon the orientation of the fusion in the chromosome and can be used to infer direction of *cya-lac* transcription. This rationale has been given previously (10, 35). Chromosomal transfer by four independently obtained Hfr strains generated upon integration of F′(Ts)114 *lac* into either strain CH62.19-1 or CH54.42-2 was essentially unidirectional. In time-interrupted or spot mating experiments with strain RK1041 as the recipient, we observed early and efficient transfer of the donor *metB*$^+$ and *argH*$^+$ alleles. These two genes are located clockwise to the *cya* gene on the *E. coli* genetic map (1). In marked contrast, the *ilv*$^+$, *pyrE*$^+$, and *cysE*$^+$ donor alleles, located counterclockwise from *cya*, were transferred very poorly. Typically, we counted greater than 300 *metB*$^+$ *argH*$^+$ recombinants, as compared with 15 or fewer *ilv*$^+$ *pyrE*$^+$ *cysE*$^+$ recombinants, after a 30-min liquid mating. The orientation of *lac* relative to *oriT* on F′(Ts)114 *lac* is such that genomic integration of the episome via *lac* homology results in Hfr strains transferring only those genes that lie promoter distal to the *cya-lac* fusion early and efficiently. These results indicate that *cyap* is *cya* distal to *metB* and *argH*. Therefore, the *cya* locus must be transcribed in a clockwise direction on the *E. coli* chromosome (Fig. 3).

Isolation of regulatory mutants. The low β-galactosidase activity of the protein fusion strain CH54.42-2 (ca. 19 U) presented a direct strategy for isolating regulatory mutants exhibiting enhanced fusion activities. This strain grows very slowly on lactose minimal medium, and faster growing (i.e., Lac/up) mutants are easily obtained. We isolated 20 spontaneous, independent, Lac/up mutants and categorized these into three classes based on the magnitude of stimulation of fusion activity relative to the parental strain. Class I mutants (17 of 20) exhibited two- to fourfold enhancement of β-galactosidase activity. The three remaining mutants showed significantly stronger stimulation of activity. The two class II mutants, strains CH112 and CH128, exhibited activities 13- and 17-fold greater, respectively, than that of the parental strain. The single class III mutant, strain CH129, had a level of enzyme activity some 77-fold greater than that of the parent.

Each of the 20 Lac/up mutants remained phenotypically Cya$^-$ Crp$^+$, i.e., they could still be induced to utilize maltose and glycerol as sole carbon sources in the presence of exogenous cAMP. This result suggested that *crp* was not the site of the regulatory mutations, since all *crp* mutations known to result in altered regulation of AC activity are phenotypically Crp$^-$ (29, 30, 40) or are capable of suppressing *cya* mutations (16). We confirmed this genetically by transducing the *crp* locus from the 20 Lac/up mutants into a Δ*crp-45* derivative of the parental strain CH54.42-2. For each donor strain, several Crp$^+$ transductants were isolated, and in all cases, these transductants exhibited the very low β-galactosidase activity characteristic of the parent. Furthermore, *cya-lac* fusion activity in the class II and class III mutants remained unaffected in Δ*crp-39* derivative strains (data not shown). Our preliminary data indicate that at least the class II and class III mutations responsible for the Lac/up phenotype are approximately 50% cotransducible with *rbs*::Tn*10*, indicating linkage of these regulatory mutations to *cya*.

Since these mutations affect expression of the *cya-lacZ* protein fusion, it was of interest to determine whether these Lac/up mutants still exhibit the weak cAMP-CRP-mediated regulation observed in the parental strain. We found that exogenously supplied 5 mM cAMP elicited repression ratios of 1.2 to 2.4 for fusion activity in these strains (data not shown). These values are of the same magnitude as that observed for the parental strain CH54.42-2.

Identification of the *cya-lacZ* hybrid protein.

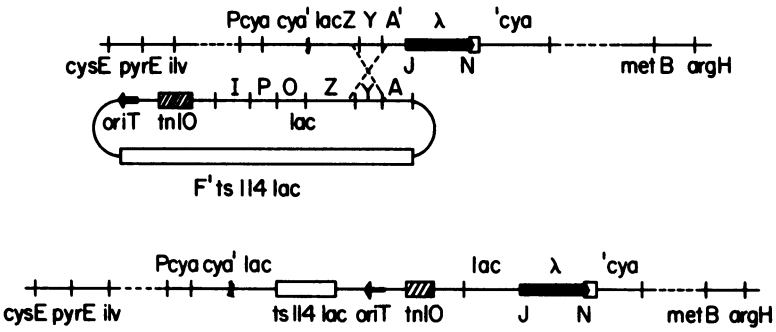

FIG. 3. Direction of *cya* transcription. Recombination of F'(Ts)114 *lac* into the chromosome is directed by *lac* homology present in the resident *cya-lac* fusion. Since orientation of *oriT* is opposite relative to the direction of *lac* transcription on the episome, integration of the F' via *lac* homology generates an Hfr strain, transferring those genes located promoter distal to the *cya-lac* fusion as early markers and at high frequencies in mating experiments.

We identified the *cya-lacZ* hybrid protein synthesized by strain CH54.42-2 by immune precipitation with anti-β-galactosidase serum, followed by SDS-PAGE and autoradiography (Fig. 4). The hybrid protein has an apparent molecular weight of approximately 160,000, significantly greater than that of the monomer subunit of native *E. coli* β-galactosidase (116,000). We had anticipated finding a rather large hybrid protein, since we were able to recombine four of four independently obtained *cya*(Am) mutations into the *cya-lacZ* hybrid gene (as described earlier). We also identified the hybrid protein synthesized in representative Lac/up mutants (Fig. 4). These strains synthesized considerably more hybrid protein, as indicated by the amount of protein precipitated from similar amounts of cell extract. The hybrid proteins from each of the Lac/up mutants analyzed migrated identically by SDS-PAGE as the hybrid protein from the parental strain, indicating that the Lac/up phenotype did not result from the deletion of a portion of the *cya-lacZ* hybrid gene or by fusion to a second structural gene. Also, we observed by SDS-PAGE several lower-molecular-weight protein bands of the immune precipitates that may be degradation products of the hybrid protein. These hybrid proteins are abnormal proteins, and proteolytic degradation has been observed with other hybrid proteins having a β-galactosidase moiety (3, 18).

DISCUSSION

The most complete model for transcriptional regulation of the *cya* locus has been provided by Botsford and Drexler (7). This model proposes a negative control function for the cAMP-CRP complex in regulating AC activity at the transcriptional level and is based on the following observations. (i) *crp* mutants overproduce cAMP (29, 30, 40), and (ii) the number of AC molecules within a cell, as determined by differential rates of cAMP synthesis, varies as a function of the available carbon source (7). Carbon sources eliciting high intracellular cAMP are suggested to exert minimal, transport-related, post-translational inhibition of AC activity while precipitating maximal repressing conditions for *cya* transcription. These formulations were derived from a series of well-conceived experiments that involved the following assumptions. (i) Carbon source transport was the only means for post-translational regulation of AC; therefore, rates of cAMP synthesis in the absence of transport directly reflected the number of AC molecules; and (ii) AC regulation by the cAMP-CRP complex occurred at the level of transcription (7). Direct proof of this model required a system with which *cyap* activity could be measured directly.

We investigated regulation of *cya* by constructing *cya-lac* operon and protein fusion strains, using stringent genetic criteria to confirm their identity. These fusion strains provided us with an in vivo system for directly monitoring regulatory signals affecting expression of *cya* at the levels of transcription and translation initiation. The Botsford and Drexler model predicts *cya-lac* fusion activity will be inversely related to intracellular cAMP concentrations. Furthermore, these fusions are predicted to exhibit maximum expression in *crp* strains. We found that modulation of intracellular cAMP levels, either by providing this cyclic nucleotide exogenously or by growing cells in various carbon sources, resulted in *cya-lac* fusion activities that behaved consistently with the former prediction (Tables 3 and 4). However, the observed regulatory effects were extremely weak and were

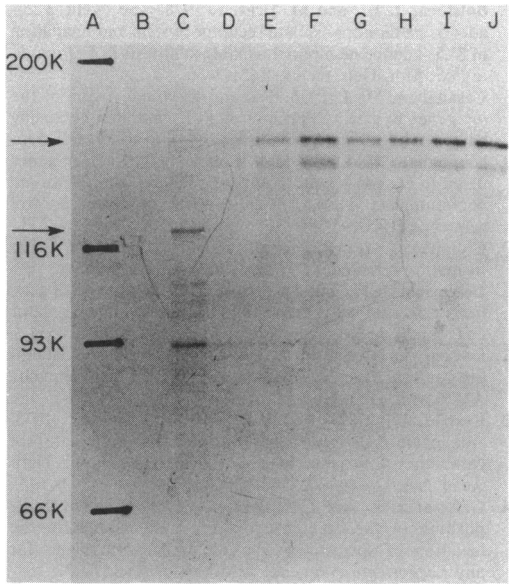

FIG. 4. Identification of the *cya-lacZ* hybrid protein. Fusion strains were grown and radiolabeled, and the fusion products were immune precipitated and analyzed by SDS-PAGE and autoradiography as described in the text (gel length, 16 cm; acrylamide concentration, 7.5%). The positions of unlabeled known protein molecular weight standards are shown in lane A. The immune precipitates obtained from various cell extracts with anti-β-galactosidase serum are shown in lanes B through J. (B) *cya*$^+$ Δ*lac* parental strain CH50; (C) *cya-lac*$^+$ operon fusion strain CH62.19-1; (D) original *cya-lacZ* protein fusion strain CH54.42-2; (E) Lac/up strain CH111; (F) Lac/up strain CH112; (G) Lac/up strain CH121; (H) Lac/up strain CH127; (I) Lac/up strain CH128; (J) Lac/up strain CH129. The precipitate obtained from an equal number of cells (with similar incorporation of radiolabel) was loaded onto the gel in every case except for the class III Lac/up mutant strain CH129. In this instance, only one-third of the precipitate obtained was used. The upper arrow designates the position of the *cya-lacZ* hybrid protein, and the lower arrow designates the position of the *cya-lac*$^+$ operon fusion product.

probably not physiologically significant. Since both the *cya-lac* operon and the protein fusions exhibited similar responses to cAMP, we infer that the weak repression occurs at the level of transcription. Functional CRP was required for the elaboration of these weak regulatory signals as evidenced by the insensitivity of *cya-lac* fusion activity to cAMP in a Δ*crp* genetic background. Furthermore, incorporation of the *crp** allele into *cya* fusion strains resulted in β-galactosidase levels similar to those observed in isogenic *crp*$^+$ strains under conditions of elevated intracellular cAMP. Fusion activities in the *cya crp** strains were independent of modulation by carbon sources (Tables 3–5). We were also unable to demonstrate a functional role for CRP in *cya* regulation at the level of transcription or translation initiation since *cya-lac* operon or protein fusion activities were identical in isogenic *crp*$^+$ and Δ*crp* strains (Table 5). These results were supported by our analysis of regulatory mutations lending 2- to 77-fold enhancements in *cya-lacZ* protein fusion activity. Of the 20 mutants studied, none were Crp$^-$ and none of these 20 independently obtained mutations mapped in the *crp* locus. Furthermore, introduction of Δ*crp-39* into the class II and class III mutant strains had no effect on the mutant Lac phenotypes.

From these data, we conclude that the cAMP-CRP complex does not play a significant role in transcriptional or translational (or both) regulation of AC. The weak regulatory effects exerted at *cyap* by this complex may reflect a nonspecific metabolic regulation (31). The simplest explanation required to reconcile our results with the Botsford and Drexler model is to propose that the cAMP-CRP complex is involved, directly or indirectly, as a negative element in post-translational regulation of AC. For instance, cAMP-CRP could bind directly to AC and thus mediate allosteric inhibition of the enzyme. Alternatively, this complex could interact with, and thereby deactivate, a regulatory subunit required for AC activity. The concept of regulatory proteins interacting with AC has been suggested previously (16, 27, 30, 34). An attractive feature to this hypothesis is that it provides a more rapid and sensitive mechanism for regulating AC activity in response to fluctuating intracellular cAMP levels than that offered by the transcriptional control model. Considering the central regulatory role played by cAMP in *E. coli* (6, 23), such an advantage would not be trivial. Our data do not eliminate the possibility that cAMP-CRP elicits a negative control function directed at transcription of an AC-activating regulatory protein. In this case, the cAMP-CRP complex would play an indirect role in post-translational regulation of the enzyme. We favor the direct involvement interpretation, since Wang et al. (39) found that strains overproducing AC do not proportionately overproduce cAMP.

Although we have not identified any obvious regulation at the level of *cyap*, detailed analysis of the regulatory mutants obtained in this study should aid in elucidating the *cya* regulation scheme. Preliminary data suggest that the strong mutations conferring a Lac/up phenotype to the parental *cya-lacZ* protein fusion are linked to the *rbs* locus and therefore presumably to *cya*. We have established that all of the 20 independently obtained Lac/up mutants exhibit cAMP-CRP-mediated repression for β-galactosidase at magnitudes comparable to the repression ratio ob-

served for the parental strain. This, coupled with the observation that the hybrid *cya-lacZ* proteins in all 20 mutant strains are identical in size to the hybrid protein synthesized by the parental strain CH54.42-2, argues strongly that the *cya-lacZ* structural gene remains intact, thus facilitating recombination of these mutations into a *cya*$^+$ genetic background.

A potentially powerful tool for extending our *cya* studies is strain CH129, the regulatory mutant exhibiting some 77-fold enhancement of protein fusion activity. We are interested in purifying the *cya-lacZ* hybrid protein from CH129 by taking advantage of the β-galactosidase character of the protein in the hope of raising antibody directed against AC. An analogous strategy has facilitated the raising of antisera directed against another *E. coli* protein to which β-galactosidase was attached (36). Antiserum capable of precipitating *E. coli* AC may prove useful in approaching solutions to problems of localization, turnover, and post-translational regulation of this protein, about which little is currently known (6).

Finally, using *cya-lac* fusions, we have in this paper inferred the direction of *cya* transcription to be clockwise on the *E. coli* genetic map. This particular piece of information is proving useful in devising strategies for recombining the Lac/up mutations we have obtained into a wild-type *cya*$^+$ locus. We believe that the genetic approach espoused by Beckwith (4) and undertaken here will facilitate elucidation of the structure and regulation of *cya* and AC, a most important component in the regulatory scheme of *E. coli*.

ACKNOWLEDGMENTS

This research was supported by a Public Health Service grant from the National Institute of Allergy and Infectious Diseases (AI17292). V.A.B. is a predoctoral fellow of The Humphrey Foundation.

We thank R. Kadner, R. Ebright, and S. Emr for bacterial strains, G. Stewart for critical reading of the manuscript, and special thanks to Beth Ann Rasmussen for the anti-β-galactosidase serum.

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