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Mechanism of CRP-Mediated *cya* Suppression in *Escherichia coli*†

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Escherichia coli strain NCR30 contains a *cya* lesion and a second-site *cya* suppressor mutation that lies in the *crp* gene. NCR30 shows a pleiotropic phenotypic reversion to the wild-type state in expressing many operons that require the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex for positive control. In vivo β-galactosidase synthesis in NCR30 was sensitive to glucose-mediated repression, which was relieved not only by cAMP but also by cyclic GMP and cyclic CMP. The CRP isolated from NCR30 differed from the protein isolated from wild-type *E. coli* in many respects. The mutant protein bound cAMP with four to five times greater affinity than wild-type CRP. Protease digestion studies indicated that native NCR30 CRP exists in the cAMP-CRP complex-like conformation. The protein conferred a degree of cAMP independence on the in vitro synthesis of β-galactosidase. In addition, the inherent positive control activity of the mutant protein in vitro was enhanced by those nucleotides that stimulate in vivo β-galactosidase synthesis in NCR30. The results of this study supported the conclusion that the *crp* allele of NCR30 codes for a protein having altered effector specificity yet capable of promoting positive control over catabolite-sensitive operons in the absence of an effector molecule.

The expression of catabolite-sensitive operons in *Escherichia coli* is dependent upon the positive control activity of the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex (24). Strains that lack a functional adenylate cyclase (i.e., *cya* mutants) cannot activate the CRP and are pleotropically affected in their ability to express catabolite-sensitive operons. A class of mutants has been described recently which contains a *cya* mutation and yet expresses many, if not all, operons requiring the cAMP-CRP complex for positive control (1, 5, 6, 21, 26). Genetic analysis showed that these *cya* suppressor strains contain a second-site mutation that lies in the *crp* gene (1, 5, 6, 21, 26). Takebe et al. (26) provided biochemical evidence that the *cya* suppressor mutation resulted in the production of an altered CRP.

Two mechanisms have been proposed for CRP-mediated *cya* suppression. First, the altered CRP could exert positive control over catabolite-sensitive operons in the absence of an effector molecule (i.e., cAMP) (1, 5, 6, 21, 26). Alternatively, the altered protein might be activated through the binding of naturally occurring metabolites other than cAMP (i.e., cyclic GMP [cGMP]) (1, 26). In this report, we present evidence for the former mechanism, namely, that an *E. coli cya* suppressor strain contains a CRP that can act alone in mediating positive control over catabolite-sensitive operons.

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MATERIALS AND METHODS

Chemicals. Common salts and buffer components were reagent grade. cAMP was obtained from the United States Biochemical Corp. GDP 3′-diphosphate and GDP 3′-phosphate were from P-L Biochemicals, Inc. All other nucleotides, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, subtilisin BPN′, chymotrypsin, dithiothreitol, *E. coli* W. tRNA, folinic acid, and cAMP-agarose (C-8 attached, hexane spacer) were obtained from Sigma Chemical Co. Trypsin was purchased from Worthington Diagnostics. Hydroxylapatite, BioRex-70, and chemicals used for performing gel electrophoresis were supplied by Bio-Rad Laboratories, Phosphocellulose (P-11) and DEAE-52 were purchased from Whatman. Sephadex G-100, Sephadex G-200, and DEAE-Sephadex A-50 were supplied by Pharmacia Fine Chemicals, Inc. NCS was supplied by Amersham Corp., and [^{3}H]cAMP (22 Ci/mmol) is a product of Moravek Biochemicals.

Bacterial strains. The *E. coli* strains used in this study are as follows: K-12 (wild type) (30) and its mutant derivatives; NCR31 (*cya-57*) (21); NCR30 (*cya-57 crp-57*) (21); and NCR307 (*cya-57 crp-57 lacI*). (The genotype of the *cya* suppressor mutant NCR30 was previously designated *csm* [21]. The work presented in this report confirms that the cAMP suppressor mutation does indeed lie in the *crp* gene. The genotype of

NCR30 has been changed to *crp-57* to reflect this confirmation.) NCR307 arose spontaneously from a culture of NCR30 initially identified as a *lac* constitutive strain capable of hydrolyzing *o*-nitrophenyl-β-D-galactopyranoside on minimal glucose agar (22). Verification of the *lacI* lesion was conducted in the following way. A culture of cured CSH44 (*tonA* Δ*lac thi*) (22) was transduced to Lac^+, using φP1kc grown on NCR307. Transductants, along with CSH36 (*lacI thi*) and CSH37 (*lacO^c thi*) (22), were streaked onto minimal glucose agar containing thiamine and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. The plates were incubated at 37°C for 24 to 30 h and scored. The transductants scored as *lacI*.

Strain SB7223 (Δ*lac* Δ*ara*) (31) was used as a source of in vitro synthesizing extract (S-30 fraction) prepared as described by Wilcox et al. (31). Strain CSH44 (*tonA* Δ*lac*|λ *c*I857 *S*t68 h80|*thi*|λ *c*I857 *S*t68 h80 d*lac*$^+$|) (22) was used as a source of *lac* template DNA.

Growth of cells, induction, and assay of β-galactosidase. Cells were grown overnight in minimal salts medium (7) containing 25 mM glycerol and 0.125% vitamin-free casein hydrolysate. The cultures were diluted to 50 μg of cell dry weight per ml in fresh medium and incubated at 37°C with vigorous shaking. Exponentially growing cultures were diluted 1:2 into prewarmed medium (37°C), 2.5 mM in isopropyl-β-D-thiogalactoside and containing additional compounds as dictated by the experiment. The cultures were incubated at 37°C with shaking. Samples (0.5 ml) were removed at prescribed intervals and diluted 1:10 in ice-cold sodium phosphate buffer (pH 7.5) containing 200 μg of chloramphenicol per ml. Cell dry weight and β-galactosidase activity were measured as described by Dobrogosz (7).

Cyclic nucleotide binding assay. During the course of CRP purification, cAMP binding activity was assayed by the ammonium sulfate precipitation procedure described by Emmer et al. (12). For the kinetic studies we used this same procedure with slight modification. The reactions were carried out in 1.5-ml Eppendorf centrifuge tubes. Reaction mixtures (0.1 ml) contained 1 μg of purified CRP, 1 mM potassium phosphate buffer (pH 7.7), 20 μg of casein, and various amounts of 50 μM [^{3}H]cAMP (2.0 Ci/mmol). Binding reactions were initiated with the addition of [^{3}H]cAMP, allowed to proceed for 15 min in an ice water bath, and terminated with the addition of 0.4 ml of cold saturated $(NH_4)_2SO_4$. Protein precipitates were collected by centrifugation at 12,000 × *g* and solubilized in 0.25 ml of NCS, and the radioactivity retained by the protein was measured by conventional liquid scintillation spectroscopy in 5 ml of scintillation cocktail containing toluene–Triton X-100 (2:1), 0.4% PPO (2,5-diphenyloxazole), and 0.1% POPOP [1,4-bis-(5-phenyloxazolyl)benzene].

Purification of CRP. The CRP from both strains K-12 and NCR30 was isolated on two separate occasions by a modification of the procedure described by Boone and Wilcox (3). On both occasions, bacterial cells were grown in medium which contained (per liter): K_2HPO_4, 28.9 g; KH_2PO_4, 5.6 g; yeast extract, 10 g; and 50 mM glucose added after autoclaving. The protein was isolated from approximately 200 g of wet packed cells. The first purification of CRP was performed with the following scheme. In step i, the crude cell-free extracts were loaded onto either a phosphocellulose column (NCR30) or a BioRex 70 column (K-12), and the protein was eluted in a linear phosphate-buffered KCl gradient as described by Boone and Wilcox (3). The cAMP binding fractions (active fractions) were pooled. In step ii, the protein solutions were diluted, loaded onto the hydroxylapatite column, and eluted in a linear phosphate gradient as described by Boone and Wilcox (3). The active fractions were pooled and dialyzed against 50 mM Tris-hydrochloride (pH 8.1)–1 mM EDTA (TE buffer) containing 7 mM 2-mercaptoethanol and 10% (vol/vol) glycerol. The soluble protein was precipitated by the addition of solid $(NH_4)_2SO_4$ to 60% saturation, and the precipitates were collected by centrifugation and dissolved in a small volume of TE buffer containing 0.1 M KCl (TEK buffer). In step iii (K-12), the K-12 CRP preparation was loaded onto a Sephadex G-200 column (1 by 85 cm) and eluted in TEK buffer. The active fractions were pooled, the protein was precipitated in 60% $(NH_4)_2SO_4$, and the preciptiate was dissolved in a small volume of TEK buffer. In step iv (K-12), the protein was loaded onto a cAMP-agarose column (1 by 5 cm), washed with TEK buffer, and eluted in TEK buffer containing 10 mM cAMP. The protein was precipitated in 60% $(NH_4)_2SO_4$, dissolved in a small volume of TEK buffer, dialyzed against several changes of TEK buffer, frozen in liquid N_2, and stored at −20°C. In step v (NCR30), to avoid the use of the cAMP-agarose column in purifying the NCR30 CRP, the protein solution from step ii was loaded onto a Sephadex G-100 column (1 by 75 cm) that had been overlaid with 10 cm of DEAE-Sephadex A50 and eluted in TEK buffer. The active fractions were pooled, the protein was precipitated in 60% $(NH_4)_2SO_4$, and the precipitate was dissolved in TE buffer containing 0.5 M KCl, dialyzed against TE buffer containing 0.5 M KCl, frozen in liquid N_2, and stored at −20°C. At this stage, the K-12 CRP preparation appeared to be homogeneous on 12.5% polyacrylamide–sodium dodecyl sulfate gels, whereas the NCR30 CRP preparation was judged to be >90% pure. The proteins were used without further purification.

The second purification of the CRP from both K-12 and NCR30 was performed exactly as described by Boone and Wilcox (3). The protein solutions obtained from the DEAE/DNA-cellulose column were found to be insufficiently pure, and an additional chromatography step was used in obtaining the final CRP preparations. The proteins were loaded onto a Sephadex G-100 column (2.4 by 85 cm) and eluted in TE buffer. The active fractions showing a constant specific activity were pooled, the proteins were precipitated in 60% $(NH_4)_2SO_4$, and the precipitates were dissolved in a small volume of TE buffer. At this stage, the CRP preparations were judged to be homogeneous on 12.5% polyacrylamide–NaDodSO$_4$ gels and were frozen and stored at −70°C.

The cyclic nucleotide binding data and the in vitro transcription-translation studies were performed with the K-12 and NCR30 CRP isolated by the first purification scheme described above. The proteins isolated by the second method were found to behave identically to those isolated by the first method in protease digestion studies. The protease digestion studies reported here were performed on the proteins isolated by the second purification method.

In vitro synthesis of β-galactosidase. The in vitro

system used was essentially that described by Zubay (32). Enzyme synthesis reactions were carried out in a total volume of 0.2 ml. Reaction mixtures contained the following: 8.5 μg of λ h80 d*lac*$^+$ DNA; 4.2 μmol of Na_3 phosphoenolpyruvate; 3.0 μmol of $Mg(OAc)_2$; 0.45 μmol of $Ca(OAc)_2$; 80 μl of a solution which contained (per milliliter) 128 μmol of Tris-OAc (pH 8.2), 4.45 μmol of dithiothreitol, 160 μmol of KOAc, 1 μmol each of 20 amino acids, 1.7 μmol each of CTP, UTP, and GTP, 6.6 μmol of ATP, 80 μmol of NH_4OAc, 320 μg of *E. coli* W tRNA, 80 μg of folinic acid, 400 μg of NAD, and 45 μg of polyethylene glycol 6000. Reaction mixtures were incubated at 37°C for 5 min before the addition of 1.1 mg of S-30 protein. The tubes were incubated at 37°C with gentle shaking for 60 min. The enzyme synthesis reaction was terminated by the addition of 1.4 ml of *o*-nitrophenyl-β-D-galactopyranoside solution which contained (per milliliter): 0.55 mg of *o*-nitrophenyl-β-D-galactopyranoside; 0.1 mmol of sodium phosphate (pH 7.1); 0.14 mmol of β-mercaptoethanol; 0.31 μmol of chloramphenicol. The tubes were incubated at 28°C for 5 h and treated as described by Zubay (32). The amount of enzyme found in each tube was expressed as units of β-galactosidase per milliliter per hour.

Protein assay. Protein determinations were performed with the protein assay kit supplied by Bio-Rad Laboratories.

Polyacrylamide gel electrophoresis. Samples for electrophoresis and 12.5% polyacrylamide–$NaDodSO_4$ slab gels were prepared as described by Laemmli (19). Standard running buffer was used in an open circulatory buffer chamber. Electrophoresis of protein samples was carried out at 30 mA per gel. Proteins were stained in a solution containing (per liter): methanol, 490 ml; glacial acetic acid, 100 ml; water, 410 ml; and Coomassie brilliant blue R-250, 1.25 g. After 1 h at 50°C, the gels were destained in a solution containing (per liter): methanol, 250 ml; glacial acetic acid, 75 ml; and water, 675 ml.

Protease digestion of CRP. Digestion mixtures (total volume, 20 μl in 50 mM Tris-hydrochloride [pH 8.2], 1 mM EDTA) contained 5 μg of CRP, 0.33 μg of subtilisin BPN′, trypsin, or chymotrypsin; and 6.4 μg of cAMP (where indicated). Digestion reactions were carried out at 37°C for 5 min, stopped by the addition of 5 μl of 5 mM phenylmethylsulfonylfluoride, and incubated for an additional 5 min at 37°C. After the addition of 12.5 μl of 3× sample buffer (19), the reaction mixtures were boiled for 5 min. Samples (25 μl) of the reaction mixtures were analyzed by polyacrylamide-$NaDodSO_4$ gel electrophoresis.

RESULTS

Characteristics of *cya* suppressor strain NCR30. *E. coli* strain NCR30 was derived from *cya* strain NCR31. The fermentation patterns of NCR30 and wild-type strain K-12 are very similar on eosin methylene blue indicator plates containing lactose, arabinose, galactose, maltose, melibiose, sorbitol, glycerol, ribose, xylose, or gluconate, in contrast to strain NCR31 which failed to ferment any of these compounds in the absence of cAMP. Both cAMP assays and transductional analysis showed that NCR30 synthesizes no detectable cAMP (<1 pmol/ml of culture fluid) and that NCR30 contains both the *cya-57* allele of NCR31 and a second-site mutation that lies in the *crp* gene (allele *crp-57*) (21). Detailed transductional analysis of this and several other *cya* suppressor strains has led to the conclusion that CRP-mediated *cya* suppression occurs independent of the nature of the *cya* lesion (e.g., point mutation or deletion) and is not a result of a leaky *cya* phenotype (21).

The data presented in Table 1 show that strain NCR30 synthesized β-galactosidase at about one-third the differential rate observed in strain K-12. The addition of cAMP to the culture medium stimulated the differential rate of enzyme synthesis in NCR30 to wild-type levels. Addition of glucose to the cultures repressed β-galactosidase synthesis in both strains, and repression was overcome by cAMP in both cases. The mechanism responsible for mediating catabolite repression of β-galactosidase synthesis in a strain which produces the enzyme in the absence of cAMP is unknown. Similar results have been reported by Alexander (1), Dessein et al. (6), Melton et al. (21), and Takebe et al. (26). We have assessed the effect of inducer exclusion on β-galactosidase synthesis in NCR30. Inducer exclusion does not play a role in decreasing the differential rate of β-galactosidase in NCR30 as evidenced by the fact that the synthesis of β-galactosidase remained sensitive to glucose repression in strain NCR307, a *lacI* derivative of NCR30 (Table 1).

TABLE 1. Effect of glucose and cAMP on the synthesis of β-galactosidase in cultues of K-12, NCR30, and NCR307[a]

Addition(s)	β-Galactosidase activity (%)		
	K-12	NCR30	NCR307
None	100	29	31
cAMP	123	117	104
Glucose	27	12	8
Glucose + cAMP	88	96	80

[a] Exponentially growing cultures were diluted 1:2 into prewarmed (37°C) medium, 25 mM in glycerol and 2.5 mM in isopropyl-β-D-thiogalactoside (IPTG). Where indicated, the following additions were made: cAMP to a final concentration of 5 mM, and glucose to a final concentration of 25 mM. Samples were removed at 20-min intervals over a period of 60 to 80 min, and cell dry weight and β-galactosidase activity were measured. The differential rate of β-galactosidase synthesis was calculated for each culture condition and expressed as a percentage of that value observed in the culture of K-12 grown in glycerol minimal medium containing inducer (Δ 0.48 U of β-galactosidase/Δ μg of cell dry weight). The values reported for strain NCR307 were identical to those obtained from cultures grown in the absence of IPTG.

An additional characteristic of NCR30 attributed to the *crp-57* allele is illustrated in Table 2. These data show that in addition to cAMP, cGMP, cyclic CMP (cCMP), and, to a lesser extent, cyclic IMP and cyclic UMP stimulated the differential rate of β-galactosidase synthesis in NCR30 grown on glucose. In contrast, β-galactosidase synthesis in the *cya* parental strain NCR31 was stimulated by the addition of cAMP alone, with only slight effects observed upon the addition of cGMP and cCMP. This suggests that the *crp-57* allele of NCR30 partially relieves the cAMP requirement for β-galactosidase synthesis under nonrepressing conditions and codes for a protein which exhibits altered effector specificity.

Several other nucleotides (see footnote to Table 2) were tested for their ability to stimulate β-galactosidase synthesis in NCR30 grown on glucose. None of these was found to elicit significant stimulation of β-galactosidase synthesis. From this it appears that the effector specificity of the NCR30 CRP, although altered, is restricted to cyclic 3′,5′-monophosphates, particularly cAMP, cGMP, and cCMP.

In vitro characteristics of CRP. To facilitate the study of the mechanism by which the *crp-57* allele of NCR30 suppresses a *cya* mutation, the CRP from strains K-12 and NCR30 was isolated.

TABLE 2. Effect of cyclic nucleotides on synthesis of β-galactosidase in NCR31 and NCR30[a]

Addition	β-Galactosidase activity (%)	
	NCR31	NCR30
None	1	11
cAMP	100	112
cGMP	8	129
cCMP	12	123
cIMP	1	26
cUMP	1	26
cTMP	1	9

[a] Exponentially growing cultures were diluted 1:2 into prewarmed (37°C) medium, 25 mM in glucose, 2.5 mM in isopropyl-β-D-thiogalactoside, and 5 mM in the indicated nucleotide. Samples were removed at 20-min intervals over a period of 1 h, and the cell dry weight and β-galactosidase activity were measured. The differential rate of β-galactosidase synthesis was calculated for each culture condition and expressed as a percentage of that value observed in the culture of NCR31 grown in glucose minimal medium containing inducer and 5 mM in cAMP (Δ 0.5 U of β-galactosidase/Δ μg of cell dry weight). Several other nucleotides were tested and found to be ineffective in stimulating β-galactosidase synthesis, including: adenine; adenosine; adenosine, guanosine, and cytidine 2′-, 3′- and 5′-monophosphates; cyclic adenosine, guanosine, cytidine, and uridine 2′,3′-monophosphates; guanosine; cytidine; cytosine; GDP 3′-phosphate; GDP 3′-diphosphate; and TMP.

The affinities of both CRP preparations for cAMP, cGMP, an cCMP were measured by the method of Emmer et al. (12). From Lineweaver-Burk analysis of the cAMP binding data (Fig. 1), the disassociation constants (K_d) for the wild-type and NCR30 CRP preparations were calculated to be 2.0 and 0.44 μM, respectively. The K_d for cAMP binding to the wild-type protein agrees with previously reported values (12, 26). The difference in K_d values between the wild-type and NCR30 CRP preparations indicates that the *crp-57* allele does indeed code for an altered CRP. This observation is similar to that reported by Takebe et al. (26), who found that CRP isolated from their *cya* suppressor strain had an affinity for cAMP that was 10 times greater than that observed with the wild-type protein.

Studies have shown that cGMP is a competitive inhibitor of cAMP binding to CRP, with an apparent inhibitor constant (K_i) of 10 to 20 μM in the ammonium sulfate precipitation assay (12). The data presented in Fig. 1A indicate that both cGMP and cCMP are competitive inhibitors of cAMP binding to wild-type CRP, with calculated K_i values of 19 and 60 μM, respectively. In contrast to its increased affinity for cAMP, NCR30 CRP displayed K_i values for cGMP and cCMP similar to those obtained with the wild-type protein, 30 and 52 μM, respectively. As with wild-type CRP, the binding of cGMP and cCMP to the mutant protein appears to be competitive with cAMP binding (Fig. 1B).

The ability of other components to compete with cAMP in binding to NCR30 CRP was tested. These included ribose; adenine; the adenosine, cytidine, and guanosine 3′ and 5′-monophosphates; the cyclic adenosine, cytidine, and guanosine 2′,3′-monophosphates; cytidine; and guanosine. None of these compounds, at 50 μM, elicited more than a 12% reduction of the radioactivity bound to the mutant protein in the presence of 0.5 μM [^{3}H]cAMP. Under these same conditions, 50 μM cAMP, cGMP, or cCMP reduced the amount of [^{3}H]cAMP bound to the protein by 98, 62, and 41%, respectively (data not shown). These results indicate that the nucleotide binding properties of NCR30 CRP remain specific for cyclic nucleotide 3′,5′-monophosphates, paralleling the effector specificity of NCR30 in vivo.

The binding of cAMP to CRP is accompanied by a conformational change in the protein that activates its potential as a positive control element. Eilen et al. (11) showed that the conformation of CRP isolated from *E. coli* K-12 was reflected in its sensitivity to protease digestion. Native CRP was relatively resistant to proteolysis by subtilisin, trypsin, or chymotrypsin. In the presence of cAMP the protein was rapidly

cleaved by the proteases to a protease-resistant α-core fragment (αCRP). With this in mind, we reasoned that if the CRP of *cya* suppressor strains function as a positive control element in the absence of an effector molecule, then it should exist in a conformation similar to the cAMP-CRP complex and be digested by subtilisin, trypsin, or chymotrypsin independent of cAMP. The results of such a proteolysis experiment are shown in Fig. 2. The untreated wild-type (Fig. 2A, lane 1) and mutant (Fig. 2B, lane 1) proteins comigrated on 12.5% polyacrylamide–$NaDodSO_4$ gels to a position corresponding to about 22,500 daltons. Figure 2A shows the results obtained when K-12 CRP was treated with subtilisin (lanes 2 and 3), trypsin (lanes 4 and 5), and chymotrypsin (lanes 6 and 7) in the absence (lanes 2, 4, and 6) or presence (lanes 3, 5, and 7) of 1 mM cAMP. The data clearly show that the wild-type CRP is more susceptible to

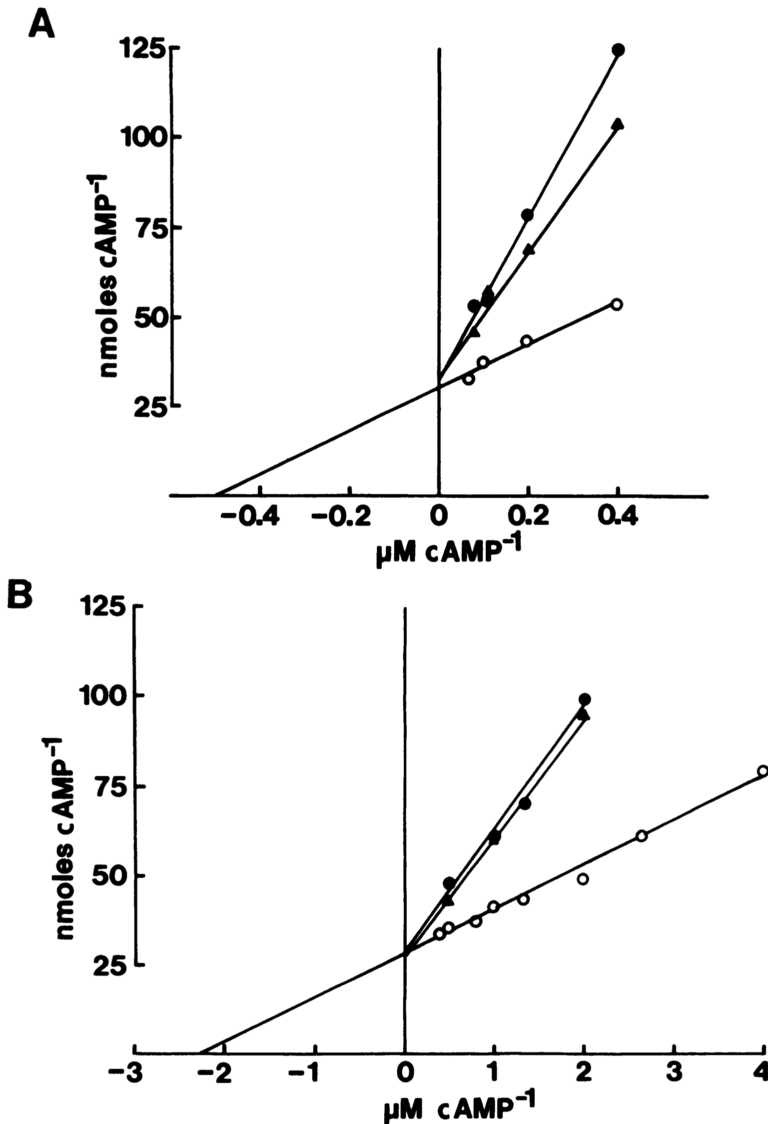

FIG. 1. Lineweaver-Burk analysis of cyclic nucleotide binding to CRP. The cAMP binding studies (○) were carried out in reaction mixtures (0.1 ml) containing 10 µg of CRP per ml and various amounts of [^{3}H]cAMP. These were repeated in the presence of 50 µM cGMP (●) or 100 µM cCMP (▲) to obtain the inhibitor constants for both cyclic nucleotides (A) K-12 CRP: K_d^{cAMP} = 2.0 µM; K_i^{cGMP} = 19 µM; K_i^{cCMP} = 60 µM. (B) NCR30 CRP; K_d^{cAMP} = 0.44 µM; K_i^{cGMP} = 30 µM; K_i^{cCMP} = 52 µM.

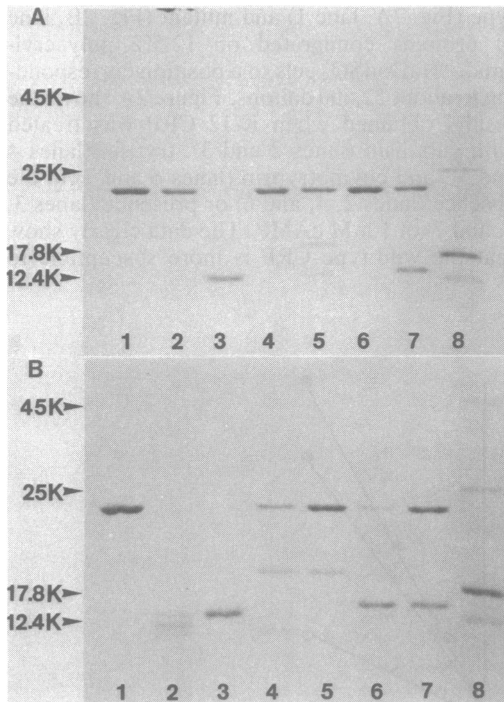

FIG. 2. Proteolytic probe of CRP conformation. (A) Molecular weight markers—cytochrome *c* (12,400), myoglobin (17,800), chymotrypsinogen (25,000), and ovalbumin (45,000) (lane 8); untreated K-12 CRP (lane 1); K-12 CRP incubated with subtilisin (lane 2), trypsin (lane 4), or chymotrypsin (lane 6) in the absence of cAMP; K-12 CRP incubated with subtilisin (lane 3), trypsin (lane 5), or chymotrypsin (lane 7) in the presence of 1.0 mM cAMP. (B) Same as (A) except that NCR30 CRP was used as a substrate for subtilisin (lanes 2 and 3), trypsin (lanes 4 and 5), and chymotrypsin (lanes 6 and 7) in the absence (lanes 2, 4, and 6) or presence (lanes 3, 5, and 7) of 1 mM cAMP.

protease digestion in the presence of cAMP. This is indicated by a decrease in the amount of protein in the monomer band and the appearance of lower-molecular-weight protein bands. In contrast to this, the data presented in Fig. 2B show that the mutant CRP was digested by the proteases in both the presence (lanes 3, 5, and 7) and the absence (lanes 2, 4, and 6) of cAMP. The results indicate that the CRP isolated from *cya* suppressor strain NCR30 has a native conformation similar to that of the cAMP-CRP complex.

It is apparent from the data presented in Fig. 2B that the extent to which NCR30 CRP was digested by the proteases was affected by cAMP as evidenced by the increased intensity of the CRP monomer bands in lanes 3, 5, and 7 compared with those in lanes 2, 4, and 6. This implies that the rate of CRP digestion was affected by cAMP, indicative of a cAMP-induced conformational change in the protein. Time course subtilisin digestion experiments support this conclusion (data not shown).

Eilen and Krakow (10) have shown that cAMP binds to and induces a conformational change in wild-type αCRP which results in increased resistance of the protein to digestion by subtilisin. Digestion of NCR30 CRP with subtilisin resulted in the production of three αCRP-like fragments (Fig. 2B, lane 2). The addition of cAMP to a parallel digestion mixture (lane 3) resulted in the production of a stable αCRP fragment which comigrated with the largest of the three fragments in lane 2. The cAMP-dependent production of a stable αCRP supports the conclusion that cAMP binds to and induces a conformational change in NCR30 CRP.

The ability of both the wild-type and mutant CRP to promote the synthesis of β-galactosidase in vitro has been examined (Fig. 3). The synthesis of β-galactosidase in reaction mixtures containing K-12 CRP was found to be strictly dependent upon the addition of cAMP. In contrast, the addition of NCR30 CRP to reaction mixtures resulted in the cAMP-independent synthesis of β-galactosidase. The synthesis of enzyme was found to be directly proportional to the amount

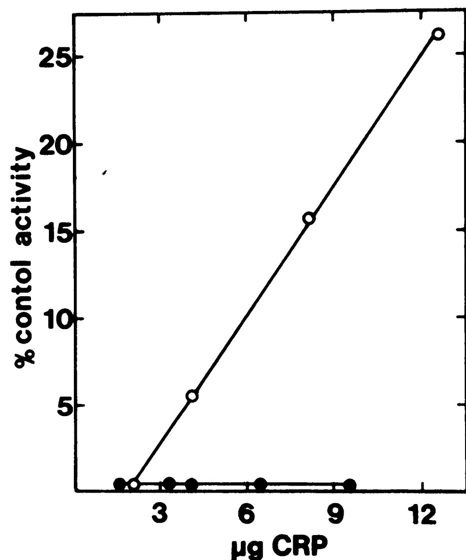

FIG. 3. In vitro synthesis of β-galactosidase. Reaction mixture received either K-12 (●) or NCR30 (○) CRP, and the synthesis reaction was allowed to proceed for 1 h at 37°C in the absence of cAMP. The data are expressed as a percentage of the activity observed in a reaction mixture which received 4.0 μg of K-12 CRP and was 0.5 mM in cAMP (2.0 U of β-galactosidase/ml per h). The CRP preparations were dialyzed against 50 mM Tris-hydrochloride (pH 8.2) before their addition to reaction mixtures.

of mutant CRP added to the reaction mixture. This indicates that the unique conformation of the native NCR30 CRP has physiological significance in that it enables the protein to suppress the cAMP requirement for *lac* operon expression.

The data presented in Table 3 summarize the effects of cyclic nucleotides on the in vitro system containing either wild-type or mutant CRP. The synthesis of β-galactosidase in reaction mixtures containing wild-type CRP was dependent upon cAMP and stimulated by cAMP alone. Reaction mixtures containing the mutant CRP synthesized β-galactosidase in the absence of cAMP and responded not only to the addition of cAMP but also to that of cGMP and cCMP. The results of these studies show that the characteristics of *lac* operon expression in NCR30 were reproduced in vitro by the addition of CRP isolated from the *cya* suppressor strain.

TABLE 3. Effect of cyclic nucleotides on in vitro synthesis of β-galactosidase[a]

Addition	β-Galactosidase activity (%)	
	K-12 CRP	NCR30 CRP
None	1	10
cAMP	100	103
cGMP	1	119
cCMP	4	129

[a] Reaction mixtures contained 4.0 μg of either K-12 CRP or NCR30 CRP with the indicated addition at 0.5 mM. The data represent the average of two independent trials and are expressed as a percentage of the activity observed in reaction mixtures 0.5 mM in cAMP containing 4.0 μg of wild-type CRP (trial 1, 2.0 U of β-galactosidase/ml per h; trial 2, 6.4 U of β-galactosidase/ml per h). The CRP preparations were dialyzed against 5 mM Tris-hydrochloride (pH 8.2) before their addition to reaction mixtures.

DISCUSSION

CRP mediates the cAMP-dependent expression of catabolite-sensitive operons in *E. coli*. The activity of CRP as a positive control element is allosterically regulated by the binding of cAMP (2, 9–11, 16–18). In its native form CRP isolated from the wild-type strain exists in a conformation that is relatively resistant to protease digestion (11, 16, 17; Fig. 1) and fails to promote the expression of catabolite-sensitive operons (31, 32; Fig. 3, Table 3). When modified by cAMP the protein exists in a conformation that is subject to proteolytic degradation (11, 16, 17; Fig. 2), binds to a unique site in catabolite-sensitive promoters (13, 20, 24), and stimulates the expression of catabolite-sensitive genes (31, 32; Table 3).

We have described the characteristics of a strain which contains both a *cya* mutation and a second-site suppressor mutation in the *crp* gene. NCR30, in the absence of cAMP, expresses many of the operons known to require the cAMP-CRP complex. The CRP isolated from this strain differs from the protein isolated from wild-type *E. coli* in many respects. NCR30 CRP binds cAMP with approximately five times greater affinity than does wild-type CRP (Fig. 1). In its native form the mutant protein is sensitive to digestion by subtilisin, trypsin, and chymotrypsin, a characteristic shared by the wild-type protein only after it has been modified by cAMP (Fig. 2). Unlike wild-type CRP, NCR30 CRP promotes, to a degree, the synthesis of β-galactosidase in vitro in the absence of an effector (Fig. 3, Table 3). Finally, β-galactosidase synthesis in reaction mixtures containing NCR30 CRP was stimulated by the addition of cGMP and cCMP as well as cAMP, a characteristic not shared by reaction mixtures that received the wild-type protein (Table 3). These results provide biochemical evidence that the phenotypic reversion of NCR30 occurred as a direct result of a mutation in *crp*.

The data support a mechanism of CRP-mediated *cya* gene suppression by which the *crp-57* allele encodes a protein (CRP) that exists in a cAMP-CRP complex-like conformation in the absence of cAMP and that, although altered in effector specificity, can function as a positive control element in the absence of effectors.

It is clear from the data presented in Tables 1 and 3 that the *crp-57* mutation promotes only partial expression of the potential of CRP as a positive control element in vivo and in vitro. The full positive control potential of the mutant CRP is expressed only upon binding of an effector and is comparable to that observed with wild-type CRP in the presence of cAMP. This indicates that the conformation of the mutant protein is similar but not identical to the cAMP-CRP complex. The data also show that the mutant CRP is not "locked" into a single conformation but that its conformation can be modified through the binding of effectors. This is supported by the fact that the binding of cAMP to NCR30 CRP results in an apparent decrease in the rate at which subtilisin, trypsin, or chymotrypsin degrades the protein and protects the NCR30 αCRP from further digestion by subtilisin (Fig. 2). These observations are indicative of an effector-induced conformational change in CRP (10, 11).

It has been suggested that CRP isolated from another *cya* suppressor strain requires effector binding to promote the expression of the positive control activity of the protein (26). This conclusion was based on the observation that the mutant protein had increased affinity for cAMP, was apparently activated by cGMP, and

required cAMP to promote the binding of the protein to λ h80 d*lac* DNA. The CRP isolated from NCR30 is similar to the protein described by Takebe et al. (26) in that it binds cAMP with greater affinity than wild-type CRP (Fig. 1) and is activated by other cyclic nucleotides (Tables 2 and 3). The physiological significance of these unique characteristics is obscure. It is difficult to assign physiological significance to the increased affinity of the NCR30 CRP for cAMP when it apparently functions as a positive control element in the absence of cAMP both in vivo and in vitro. Similarly, the apparent K_d for cGMP binding (30 μM) is three to four orders of magnitude greater than the reported steady-state intracellular concentrations of the nucleotide in wild-type *E. coli* (3). Although we have not measured the concentration of cGMP in NCR30, Shibuya et al. (25) and Takebe et al. (26) have shown that the levels of the nucleotide in either *cya* strains or a *cya* suppressor strain are lower than the levels observed in wild-type *E. coli*. This argues that cGMP would not function as an effector of the mutant CRP in vivo. We cannot assess the role that cCMP might play in promoting the synthesis of catabolite-sensitive operons in NCR30 since the nucleotide has not been described in *E. coli*.

It is interesting to note that although the inherent positive control activity of NCR30 CRP is stimulated to comparable levels by cAMP, cGMP, or cCMP, the protein shows increased affinity for only one of the three nucleotides. Anderson et al. (2) have presented evidence that the 2'-hydroxyl and cyclic 3',5'-phosphoryl groups are important determinants in the binding of a nucleotide to wild-type CRP, whereas the N^6-amino group of cAMP plays a role in promoting the structural alteration that activates the protein as a positive control element. Gronenborn et al. (14) have recently implicated an interaction between wild-type CRP and the adenine of cAMP in the conformational selection of *syn*-cAMP upon the binding of the nucleotide to the protein. Neither cGMP nor cCMP has a functional group comparable to the N^6-amino group of cAMP and, unlike cAMP, the nucleotides are bound by both wild-type and mutant CRP with similar affinities. This suggests that the increased affinity of NCR30 CRP for cAMP may simply reflect the additional involvement of an interaction between the N^6-amino group of cAMP and the protein. If this is the case, then the altered cAMP binding characteristic of NCR30 CRP is significant in terms of the mechanism(s) of cAMP binding and need not reflect a physiological significance.

Takebe et al. (26) also placed a good deal of emphasis on the observation that the CRP isolated from a *cya* suppressor strain showed a cAMP-dependent response in binding to λ h80 d*lac* DNA in concluding that the protein required an effector in expressing positive control activity. The DNA binding assay used in these experiments does not detect site-specific binding of CRP to DNA; it does provide a measure of the nonspecific affinity of the protein for DNA templates (20, 23). Nonspecific binding of CRP to DNA is enhanced by cAMP. It is reasonable to assume that a mutation that altered the ability of CRP to activate catabolite-sensitive promoters in the absence of an effector would also enhance the affinity of the protein for DNA in general. The data presented by Takebe et al. (26) show that both the wild-type and mutant CRP retained an equal amount of λ h80 d*lac* DNA on nitrocellulose filters in the absence of cAMP. It is important to note, however, that the protein concentrations used in the assay were not equal; the concentration of mutant CRP was one-half that of the wild-type protein. This implies that at equimolar concentrations and in the absence of cAMP the mutant protein exhibited twice the affinity of the wild-type CRP. We suggest that the results of the DNA binding study reported by Takebe et al. (26) are not inconsistent with the conclusion that the CRP of *cya* suppressor strains can function alone in mediating positive control over the expression of catabolite-sensitive operons.

The mechanism of CRP-mediated stimulation of gene expression is not known; however, two models exist (13). One maintains that direct contact between CRP and RNA polymerase promotes catabolite-sensitive operon expression, whereas the other model invokes a mechanism of telestability. The results of our study do not provide evidence for or against either mechanism; they do, however, conflict with the telestability mechanism proposed by Ebright and Wong (8). These authors have proposed that the adenine moiety of cAMP intercalates the *lac* promoter region and participates in Watson-Crick base pairing with a thymine residue of the DNA. The cAMP-thymine base pairing would lead to local destabilization of the promoter and enhance RNA polymerase binding. This mechanism clearly does not accommodate two characteristics of the NCR30 CRP: that it appears to function as a positive control element in the absence of cAMP and that its positive control activity is enhanced by both cGMP and cCMP as well as by cAMP.

Finally, the results of this study support the conclusion that cAMP is not the sole mediator of catabolite repression effects in *E. coli* (1, 6, 15, 21, 26–29). It is clear that our understanding of the mechanism(s) underlying the phenomenon of catabolite repression is far from complete. It appears that *cya* suppressor strains offer a

unique genetic background in which to study the cAMP-independent mechanism(s) involved in mediating catabolite repression. An in vitro system containing the NCR30 CRP synthesizes β-galactosidase in the absence of cAMP (Fig. 3, Table 3). This system may be applicable as an assay for effectors of *lac* gene expression. Such an assay system will permit a systematic approach to identifying subcellular fractions having either negative or positive effects on catabolite-sensitive operon expression and aid in the isolation of those components responsible for mediating catabolite repression in *cya* suppressor strains.

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LITERATURE CITED

1. **Alexander, J. K.** 1980. Suppression of defects in cyclic adenosine 3',5'-monophosphate metabolism in *Escherichia coli*. J. Bacteriol. **144**:205–209.
2. **Anderson, W. B., R. L. Perlman, and I. Pastan.** 1972. Effect of adenosine 3',5'-monophosphate analogues on the activity of the cyclic adenosine 3',5'-monophosphate receptor in *Escherichia coli*. J. Biol. Chem. **247**:2717–2722.
3. **Boone, T., and G. Wilcox.** 1978. A rapid high-yield purification procedure for the cyclic adenosine 3',5'-monophosphate receptor protein from *Escherichia coli*. Biochim. Biophys. Acta **541**:528–534.
4. **Botsford, J. L., and M. Drexler.** 1978. The cyclic adenosine 3':5'-monophosphate receptor protein and regulation of cyclic adenosine 3':5'-monophosphate synthesis in *Escherichia coli*. Mol. Gen. Genet. **165**:47–56.
5. **Cook, W. R., V. F. Kalb, A. A. Peace, and R. W. Bernlohr.** 1980. Is guanosine 3',5'-monosphophate a cell cycle regulator? J. Bacteriol. **141**:1450–1453.
6. **Dessein, A., M. Schwartz, and A. Ullmann.** 1978. Catabolite repression in *Escherichia coli* mutants lacking cyclic AMP. Mol. Gen. Genet. **162**:83–87.
7. **Dobrogosz, W. J.** 1966. Altered end-product patterns and catabolite repression in *Escherichia coli*. J. Bacteriol. **91**:2263–2269.
8. **Ebright, R. H., and J. R. Wong.** 1981. Mechanism for transcriptional action of cyclic AMP in *Escherichia coli*: entry into DNA to disrupt DNA secondary structure. Proc. Natl. Acad. Sci. U.S.A. **78**:4011–4015.
9. **Eilen, E., and J. S. Krakow.** 1977. Cyclic AMP-mediated intersubunit disulfide crosslinking of the cyclic AMP receptor protein of *Escherichia coli*. J. Mol. Biol. **144**:47–60.
10. **Eilen, E., and J. S. Krakow.** 1977. Effects of cyclic nucleotides on the conformational states of the α-core of the cyclic AMP receptor protein. Biochim. Biophys. Acta **493**:115–121.
11. **Eilen, E., C. Pampeno, and J. S. Krakow.** 1978. Production and properties of the α-core derived from the cyclic adenosine monophosphate receptor protein of *Escherichia coli*. Biochemistry **17**:2469–2473.
12. **Emmer, M., B. deCrombruggh, I. Pastan, and R. Perlman.** 1970. Cyclic AMP receptor protein of *E. coli*: its role in the synthesis of inducible enzymes. Proc. Natl. Acad. Sci. U.S.A. **66**:480–487.
13. **Gilbert, W.** 1976. Starting and stopping sequences for the RNA polymerase, p. 193–205. *In* R. Losick and M. Chamberlin (ed.), RNA polymerase. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Gronenborn, A. M., G. M. Clore, B. Blazy, and A. Baudras.** 1981. Conformational selection of *syn*-cAMP upon binding to the cAMP receptor protein. FEBS Lett. **136**:160–164.
15. **Guidi-Rontani, C., D. Danchin, and A. Ullmann.** 1980. Catabolite repression in *Escherichia coli* mutants lacking cAMP receptor protein. Proc. Natl. Acad. Sci. U.S.A. **77**:5799–5801.
16. **Krakow, J. S.** 1975. Cyclic adenosine monophosphate receptor: effect of cyclic AMP analogues on DNA binding and proteolytic inactivation. Biochim. Biophys. Acta **383**:345–350.
17. **Krakow, J. S., and I. Pastan.** 1973. Cyclic adenosine monophosphate receptor: loss of cAMP-dependent DNA binding activity after proteolysis in the presence of cyclic adenosine monophosphate. Proc. Natl. Acad. Sci. U.S.A. **70**:2529–2533.
18. **Kumar, S. A., N. S. Murthy, and J. S. Krakow.** 1980. Ligand-induced change in the radius of gyration of cAMP receptor protein from *Escherichia coli*. FEBS Lett. **109**:121–124.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Majors, J.** 1975. Specific binding of CAP factor to *lac* promoter DNA. Nature (London) **256**:672–674.
21. **Melton, T., L. L. Snow, C. S. Freitag, and W. J. Dobrogosz.** 1981. Isolation and characterization of cAMP suppressor mutants of *Escherichia coli* K12. Mol. Gen. Genet. **182**:480–489.
22. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 18, 48. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Nissley, P., W. B. Anderson, M. Gallo, I. Pastan, and R. Perlman.** 1972. The binding of cyclic adenosine monophosphate receptor protein to deoxyribonucleic acid. J. Biol. Chem. **247**:4264–4269.
24. **Pastan, I., and S. Adhya.** 1976. Cyclic adenosine 5'-monophosphate in *Escherichia coli*. Bacteriol. Rev. **40**:527–551.
25. **Shibuya, M., T. Takebe, and Y. Kaziro.** 1977. A possible involvement of *cya* gene in the synthesis of guanosine 3':5'-monophosphate in *E. coli*. Cell **12**:521–528.
26. **Takebe, T., M. Shibuya, and Y. Kaziro.** 1978. A new extragenic suppressor of *cya* mutation. J. Biochem. **83**:1615–1623.
27. **Ullmann, A.** 1974. Are cAMP effects related to real physiological phenomena? Biochem. Biophys. Res. Commun. **57**:348–355.
28. **Ullmann, A., F. Tillier, and J. Monod.** 1976. Catabolite modulation factor: a possible mediator of catabolite repression in bacteria. Proc. Natl. Acad. Sci. U.S.A. **73**:3476–3479.
29. **Wanner, B. L., R. Kodaira, and F. C. Neidhardt.** 1978. Regulation of *lac* operon expression: reappraisal of the theory of catabolite repression. J. Bacteriol. **136**:947–954.
30. **White, R. J.** 1968. Control of amino sugar metabolism in *Escherichia coli* and isolation of mutants unable to degrade amino sugars. Biochem. J. **106**:847–858.
31. **Wilcox, G., P. Meuris, R. Bass, and E. Engelsberg.** 1974. Regulation of the L-arabinose operon BAD *in vitro*. J. Biol. Chem. **249**:2946–2952.
32. **Zubay, G.** 1973. *In vitro* synthesis of protein in microbial systems. Annu. Rev. Genet. **7**:267–287.