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Cyclic AMP in Prokaryotes

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INTRODUCTION

When this topic was last reviewed, the role of cyclic AMP (cAMP) in mediating the glucose effect on the induction of catabolic enzymes in *Escherichia coli* and its relatives was familiar (39, 310). The regulatory protein that binds cAMP and mediates transcription activation at several promoters was first termed catabolite gene-activating protein (337). We now know that the cAMP receptor protein (CRP)-cAMP regulatory complex not only is involved in positive regulation of several catabolic functions but also is required for flagellum synthesis, toxin production, and other functions not directly related to catabolism. In addition, the CRP-cAMP regulatory complex serves as a negative regulator of transcription at several promoters. It seems appropriate to refer to the protein involved in this complex as CRP, as originally proposed by Emmer et al. (102). Its ability to bind cAMP is the common element. Its specific role in regulating gene expression varies.

In the enterics the CRP-cAMP regulatory element represents, in some respects, a global regulatory network (139). The cell responds to the availability of glucose by regulating the intracellular concentration of cAMP. This in turn, in concert with the CRP, affects the expression of many catabolic operons. However, the substrate for each operon must also be present. There are now many more reports of cAMP in many nonenteric bacteria. Little is known of the role of the nucleotide in these bacteria. In many cases cAMP appears not to mediate the glucose effect observed for induction of catabolic enzymes. Mutants lacking adenylate cyclase have not yet been isolated in these less familiar bacteria. No cAMP-dependent functions have yet been identified. Proteins capable of binding cAMP have been found in some cases, but no function for these proteins has yet been established.

This review covers what is known about the mechanisms controlling the expression of the gene for adenylate cyclase, control of adenylate cyclase activity, and CRP-cAMP complex function in the enterics since these topics were last reviewed (1, 39, 51, 82, 274, 310). The review discusses the role of cAMP in regulation of diverse functions in addition to the familiar inducible catabolic operons in enteric coliform bacteria. It also includes what is known about cyclic nucleotides in a host of less familiar bacteria. Limited evidence indicates that the nucleotide is not found in all bacteria (30).

This same report presented immunological data indicating the absence of CRP antibody-cross-reacting material in *Pasteurella multocida*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* (30). The results of this study suggest that the CRP-cAMP regulatory complex is not ubiquitous among bacterial species.

CYCLIC NUCLEOTIDES IN ENTERIC COLIFORMS

Regulation of Adenylate Cyclase Activity

The synthesis of cAMP is catalyzed by the enzyme adenylate cyclase, encoded by the *cya* gene, which is located in *E. coli* at map position 85.7 min (17). CRP, the protein that mediates cAMP effects in this bacterium, is encoded by the *crp* gene, which is located at map position 73.5 min (17). Physiological studies provided an early indication that *cya* expression is negatively controlled by the CRP-cAMP complex (42).

Adenylate cyclase activity is transcriptionally regulated. The extent of *cya* regulation in whole cells has been addressed by using Mud *cya lac* fusions in both *E. coli* and in *Salmonella typhimurium* (19, 174, 271). Bankaitis and Bassford (19), using both operon and protein fusions, reported a twofold variation in the expression of *lacZ* in response to carbon source variation. Jovanovitch (174) observed a twofold variation in *cya* promoter activity with respect to the carbon source and a ninefold repression of *cya* promoter activity in cells grown in the presence of high levels of exogenous cAMP. Similar results were obtained by Roy et al., who also found evidence indicating that *cya* is poorly translated in vivo (271). The initiator codon for *cya* is UUG as opposed to the typical AUG. They found that the gene was not translated efficiently even when the UUG codon was replaced by AUG. Reddy et al. (258) conducted a similar series of experiments in which the *cya* initiator UUG codon was replaced by either GUG or AUG. Their results showed that the translation efficiency of *cya* mRNA originating from the λp_L promoter was 1:2:6 in constructs having either the UUG, GUG, or AUG initiator codons, respectively (258). Apparently *cya* expression is limited at the translational level. Furthermore, the work of Reddy et al. (258) showed that in *E. coli*, a sixfold increase in *cya* expression is lethal. Apparently cells require tight control of *cya* expression to maintain viability.

Cloning and DNA sequence determination of *cya* provided the means for obtaining direct and unambiguous support for this conclusion. The *cya* control region was shown to contain three promoters; the strongest (*cyaP2*) contains a operator region having a sequence homologous to the consensus sequence for CRP binding (6, 272). DNase I footprinting studies showed the CRP-cAMP complex to bind the *cya* operator and protect between positions −20 and +11 (5, 9). Quantitative analysis of the *cya* mRNA levels in whole cells showed that the CRP-cAMP complex can cause a four- to fivefold repression of adenylate cyclase (233, 234).

With fusions made in vitro and including only the major *cyaP2* promoter, no repression of adenylate cyclase could be detected (271). However, Kawanyjaum et al. constructed similar fusions but included the two minor promoters identified through S1 nuclease mapping. They found a fivefold variation in the expression of *lacZ* when values were corrected for the copy number of the plasmid present (179).

The *cya* region in *S. typhimurium* has been rigorously mapped (307). As was the case for the *cya* gene from *E. coli*, three promoters were identified and a sequence homologous to the consensus CRP-binding site was located immediately downstream from the major promoter. A strain with a single-copy *cya lacZ* fusion located on the chromosome in addition to the wild-type *cya* allele was constructed and used to study the regulation of *cya* in a wild-type background (105). It was found that the level of *lacZ* expression varied four- to fivefold. This variation was eliminated both in isogenic strains containing a *crp* allele and by mutation of the *cyaP2* promoter CRP-binding site. This definitively proves that the CRP negatively regulates adenylate cyclase expression. The CRP-cAMP complex functions as a repressor of the *cya* gene in both *E. coli* and *S. typhimurium*, binding to a site that overlaps the major *cya* promoter to block productive RNA polymerase interaction.

Adenylate cyclase activity is posttranslationally regulated. The range of variation in *cya* expression is much smaller than the variation in the ability of the cell to synthesize cAMP (42). Recently, it has been shown that mutations in *crp* do not result in increased production of cAMP if mutations in the phospoenolpyruvate-dependent sugar phosphotransferase system (PTS) are also present, mutations particularly in *crr*, which encodes enzyme III for glucose phosphorylation (70, 85, 86). This research has been expanded (205). Using site-directed mutagenesis, this group introduced well-characterized mutations in *ptsI*, *ptsH*, and *crr*. One strain was constructed with a deletion of all three genes. Strains containing deletions in *ptsI* and *crr* made about 3% as much cAMP as the wild type. The strain containing a deletion in only *crr* made 6%. Both the *ptsI* and the *crr* gene products appear to be involved in regulation of adenylate cyclase activity. Further experiments showed that the effect of enzymes I and III is limited to regulation of adenylate cyclase activity and not *cya* expression. Posttranslational control of adenylate cyclase activity via PTS components involving covalent modification of adenylate cyclase has been proposed (257, 274). Both PTS sugars and adenylate cyclase serve as substrates for the PTS and accept phosphate from PEP. In the absence of glucose, adenylate cyclase is fully phosphorylated and has high activity. In the presence of glucose, the sugar rather than adenylate cyclase is preferentially phosphorylated, adenylate cyclase is dephosphorylated and less active, and rates of cAMP synthesis decrease.

cAMP Receptor Protein

One of the better-characterized transcription factors of *E. coli* is the CRP. The primary structure of CRP has been deduced from DNA sequence analysis of the gene (*crp*) encoding CRP (6, 67, 68). The primary structures of CRP from two other enteric bacteria have been determined: *S. typhimurium* CRP differs from the *E. coli* protein at one position (A118S), whereas the *Shigella flexneri* CRP is identical to the *E. coli* protein (68).

CRP has been purified to homogeneity and characterized by a variety of physical and biochemical techniques in vitro, including X-ray analysis of CRP crystals grown in the presence of cAMP. CRP is a 47,238-Da protein made up of two identical subunits, each of which can bind one molecule of cAMP (13, 326). In CRP-cAMP crystals, each subunit folds into two domains (326). The amino-proximal domain contains extensive β-sheet structure and forms the cAMP-binding pocket. The carboxy-proximal domain consists primarily of α-helix structure that forms the DNA-binding surface of the protein. cAMP binding to CRP modifies CRP tertiary structure and induces sequence-specific DNA rec-

ognition. In the absence of cAMP, CRP is resistant to proteases and exhibits sequence-independent affinity for DNA. In the presence of cAMP the protein is rapidly degraded by proteases, exhibits increased affinity for DNA, and demonstrates sequence-specific DNA binding. The results of a variety of biochemical studies, including the results of protease digestion experiments, all support the conclusion that cAMP induces CRP transcriptional control activity through the alteration of CRP tertiary structure (extensive citation is found in references 82, 149, and 326).

Heyduk and Lee (154) have recently provided evidence that at least three conformations exist for CRP: free CRP and two CRP-cAMP conformations, CRP-$(cAMP)_1$, a CRP-cAMP complex dimer having one cAMP-binding site occupied by cAMP, and CRP-$(cAMP)_2$, a CRP-cAMP complex dimer having both cAMP-binding sites occupied by cAMP. Their results, derived from CRP protease digestion assays, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) modification of Cys-178, and tryptophan fluorescence and 8-anilenino-1-naphthalene-sulfonic acid (ANS)-CRP complex fluorescence studies, support the establishment of the CRP-$(cAMP)_1$ conformer between the range of >0 and 200 μM cAMP and the CRP-$(cAMP)_2$ conformer between the range of 200 μM and 15 mM cAMP. The CRP-$(cAMP)_1$ conformer binds *lacP* DNA better (by a factor of 100) and with higher sequence specificity than does the CRP-$(cAMP)_2$ conformer (154, 155). Physiologically relevant cAMP concentrations in bacteria are within the range of 0 to 10 μM (39). This, coupled with the findings that the stoichiometry of CRP to cAMP in both CRP-cAMP-*gal* or CRP-cAMP-*lac* complexes is 1:1 (159, 280), makes it likely that the CRP-$(cAMP)_1$ conformer is the relevant active form of the activator in vivo (304).

There is great interest in determining the events that take place in cAMP-mediated allosteric activation of CRP. Kypr and Mrazek (190) have presented a structural model that predicts that cAMP binding to CRP converts a predominantly α-helical region of the protein to a β-sheet structure that forms the cAMP-binding site observed in CRP-$(cAMP)_2$ crystals. This model is based on a comparison of the CRP-cAMP crystal structure with a predicted CRP secondary structure derived from amino acid sequence analysis. De Grazia et al. (80) have provided evidence, based on Raman spectral analysis, that the binding of two cAMP molecules to CRP results in a secondary-structure change in the protein consistent with the conversion of α-helix to β-sheet. A more recent study indicates, however, that the Raman spectra of unbound CRP shows little difference from that of CRP modified by the binding of a single cAMP (325a). Together, the results of Heyduk and Lee (154, 155) and De Grazia et al. (80) indicate that cAMP modification of CRP tertiary structure includes the formation of a CRP-$(cAMP)_1$ form of the protein that shows little or no measurable change in CRP secondary structure from the unliganded form of the protein. The CRP-$(cAMP)_2$ conformer predominates at higher cAMP concentrations and demonstrates measurable differences in secondary structure from the unliganded form of the protein. To date crystal structure information is available for only one form of CRP, CRP-$(cAMP)_2$. A comprehensive understanding of the allosteric mechanism by which cAMP activates CRP will clearly benefit from the analysis of both the unliganded CRP and CRP-$(cAMP)_1$ crystal structures.

CRP-cAMP complex functions as a transcription control element in a dimer form. To test the idea that changes in the CRP monomer-dimer equilibrium might function as a mechanism that regulates CRP activity, Brown and Crothers (46) used both biotinylated and radiolabeled preparations of CRP to measure the dimerization of CRP subunits. CRP dimers that dissolved in a buffer system at near-physiological pH and ionic strength were found to be relatively stable, having a subunit exchange rate half time of 300 min. The addition of calf thymus DNA led to an acceleration in the rate of subunit exchange, indicating that nonspecific DNA-CRP interactions destabilize, to a large degree, CRP subunit interactions. cAMP, on the other hand, stabilized CRP subunit interactions, slowing the rate of subunit exchange by a factor of approximately 2 at 20 μM cAMP and by a factor of 22 at 200 μM cAMP. cAMP-mediated CRP dimer stabilization was observed in the presence and in the absence of DNA; DNA was found to lower the cAMP concentration required for CRP dimer stabilization. Considering the relative concentration of nonspecific DNA present in cells, it is possible that variations in cAMP concentration between 1 and 10 μM cAMP have significant effects on CRP dimer formation.

Positive-control mutants. The λ *c*I protein bound at the operator site O_{R2} acts as both a repressor of the λp_R promoter and an activator of the promoter λp_{RM}. Mutant forms of *c*I that bind O_{R2} yet fail to activate λp_{RM} have been isolated. A very elegant description of these positive-control mutants and their role in describing a mechanism for *c*I-mediated activation at λp_{RM} has been provided by Ptashne (253); *c*I-mediated activation of λp_{RM} involves *c*I contacting RNA polymerase.

Positive-control mutants of CRP that have the same general characteristics of the λ *c*I positive-control mutants have been described; they bind to the appropriate DNA sequence in CRP-dependent promoters in a cAMP-dependent manner and apparently induce a bend in the DNA, yet either partially or fully fail to activate transcription (25, 164).

cAMP-independent mutants. Mutant strains defective in de novo cAMP synthesis (i.e., adenylate cyclase or *cya* mutants) contain no detectable cAMP and fail to synthesize proteins encoded by CRP-dependent operons (39). Extragenic *cya* suppressor mutations that harbor both the original *cya* lesion and compensatory mutations that overcome the cAMP deficiency have been described (extensive citation is found in reference 149). Genetic mapping localized these second-site mutations (*crp**) to a region of the chromosome that includes *crp*. More recently, DNA sequence analysis of *crp* alleles cloned from several *cya crp** mutant strains confirmed the earlier genetic work and yielded information on both the nature and the sites of specific mutations in *crp** (8, 118, 119, 149).

Biochemical analysis of several allelic forms of CRP* has established the rationale for the mechanism of CRP*-mediated compensation of a cAMP deficiency (34, 148–150, 260). CRP* forms of CRP contain one or more amino acid substitution(s) that confer a conformation to CRP* resembling that of the active, cAMP-modified conformation of wild-type CRP. The evidence for this is derived from protease digestion experiments that show that CRP* forms exhibit protease sensitivity in both the presence and absence of added cAMP. Protease sensitivity is characteristic of the active, CRP-cAMP complex form of wild-type CRP but not of the inactive, native form. Analysis of four CRP* forms in a purified in vitro transcription system that used supercoiled DNA template directly demonstrated that the structural changes in CRP* had functional significance in that all were found to activate *lacP* in the absence of cAMP (149, 150).

Wild-type CRP binds cAMP with an apparent K_m of 1 μM and requires approximately 1 μM cAMP to half-maximally activate the *lacP* in vitro (149). Three forms of CRP*, none of which contains mutations in the cAMP-binding pocket,

require only 20 to 300 nM cAMP to half-maximally stimulate *lacP* in vitro (149, 150). Although indirect, these transcription data suggest that these forms of CRP* have higher affinity for cAMP that does wild-type CRP. Ren et al. have directly measured the binding affinity of one CRP* for cAMP under high-salt conditions and found that indeed this CRP* has about fivefold-greater affinity for cAMP than does wild-type CRP (261). These data are consistent with the conclusion that CRP* forms have, as the result of mutation, a structure similar to the CRP-cAMP complex form of wild-type CRP.

A second class of CRP* mutant has been reported (23, 124). Mutants of this class differ from those described above in that the mutation(s) that confers the cAMP-independent phenotype is in the stem structure of a putative rho-independent terminator located in the 3′-noncoding region of *crp*. The mechanism by which these mutations confer the CRP* phenotype remains unknown. Aiba et al. (7) have reported the results of a study designed to define the role of the *crp* terminator in CRP expression. The *crp* terminator structure was found to be an important factor in stabilizing *crp* mRNA. In contrast to results reported by George and Melton (124), they found no evidence that a specific 4-base deletion located in the *crp* terminator conferred a CRP* phenotype. The reason for this discrepancy in results is not clear.

CRP-dependent promoter activation and repression. CRP-cAMP complex activates transcription at several promoters and represses transcription from others. The mechanisms involved in CRP-mediated transcription activation will be considered below. The mechanisms by which CRP-cAMP complex repress transcription are varied. CRP-cAMP-mediated repression of *cya* was discussed above; here repression occurs through a mechanism that involves CRP-cAMP complex binding to the *cya* operator between positions −20 and +11 overlapping the RNA polymerase-binding site (6). A unique situation is presented by the *gal* operon, which contains two tandem promoters that initiate the synthesis of mRNA species that differ by five bases at the 5′ end (163). The binding of CRP-cAMP complex to the *gal* regulatory region simultaneously represses the activity of the *galP2* promoter by a factor of 2 and activates the *galP1* promoter by a factor of 2 (163). The mechanism involved in CRP-mediated repression of both the *cyaP2* and *galP2* promoters is one in which repressor binding interferes with the functional binding of RNA polymerase to DNA; for *gal*, CRP binding redirects RNA polymerase to a second promoter (163).

CRP-cAMP complex represses the expression of the *crp* gene itself (4, 66). In contrast to the mechanism involved in CRP-mediated repression of *cyaP2* and *galP2*, CRP-mediated repression of the *crp* occurs through an indirect mechanism. CRP-cAMP complex binding to *crpP* activates the synthesis of an antisense RNA that originates from a divergent promoter (242, 243). The antisense RNA specifically inhibits *crp* transcription and is thought to form what amounts to a transcription-terminating hairpin loop structure (242, 243).

A third distinct mechanism for CRP-mediated transcription repression is illustrated by *deoP2* regulation (20, 285). CRP-cAMP complex binds to the *deoP2* promoter in a tandem array that spans positions −26 through −53 and positions −80 through −106, resulting in a 30-fold stimulation of *deoP2* activity (288, 289, 319). The ability of CytR to repress *deoP2* activity shows an absolute requirement for CRP-cAMP complex bound at both sites in *deoP2* (289). Apparently, CytR, unlike other repressor proteins that bind specific DNA sequences in a promoter, recognizes a CRP-cAMP-*deoP2* complex. CRP functions as both an activator of *deoP2* and, under the appropriate conditions, a corepressor.

Two additional systems controlled by the CRP-cAMP complex include members of the phosphate regulon and enzymes important in polyamine biosynthesis. Wanner (325) has shown that products of the *psiE* and *psiO* genes of the phosphate regulon, are expressed to higher levels in *cya* and *crp* strains. Similarly, CRP-cAMP negative control over ornithine and arginine decarboxylase biosynthesis has been reported (333). The specific activity of these enzymes was found to vary by a factor of 2 depending on the carbon source. The specific activity of both enzymes was higher in strains containing a *cya* mutation and decreased when cells were grown with exogenous cAMP; the cAMP effect was eliminated by mutation of *crp*. In a coupled transcription-translation system Wright et al. (334) observed a cAMP- and CRP-dependent repression that reduced enzyme levels to 90% of the control values.

Much of what we know about CRP-mediated transcription regulation has been developed from both genetic and biochemical studies involving the wild-type or mutant derivatives of the lactose operon (*lac*) control region, as well as several other CRP-dependent systems including those involved in the metabolism of arabinose (*ara*), galactose (*gal*), maltose (*mal*), and melibiose (*mel*). Transcription activation is a complex process. Our understanding of the specific details regarding the mechanism by which CRP-cAMP complex activates a given CRP-dependent promoter remains incomplete. This section reviews data obtained from systems that have relatively few components involved in the activation process, namely CRP-cAMP complex, RNA polymerase, and promoter DNA.

CRP-cAMP binds to DNA sequences located upstream from what are generally classified as weak promoters and modifies those promoters in a way that enhances promoter recognition by RNA polymerase. CRP-dependent promoter landmarks include a start site for transcription (+1), the −35 to −10 region that binds RNA polymerase and show a poor fit to the promoter consensus sequence TGGACA-N_{17}-TATAAT (151), and a specific sequence of bases either at or upstream of the −35 region that serves as a binding site for CRP-cAMP complex. A recent surge of experimental evidence implicates the importance of three factors involved in CRP-dependent promoter activation, including the DNA sequence of the CRP-binding site, positioning of this sequence within a promoter, and a role for protein-protein contact.

CRP-binding-site sequence and position. Inspection of 26 known CRP-binding sites has yielded a 22-bp palindromic consensus site of the sequence AAATGTGATCT*AGA TCACATTT (28). Ebright et al. (99) provided direct evidence that CRP-cAMP complex has 450-fold greater affinity for the $CRP_{(consensus)}$ sequence than for $CRP_{(lac)}$, which differs from the consensus sequence at seven positions. Although the consensus sequence is limited to 22 bp, distal DNA sequences play a role in determining the affinity of CRP to a given site in DNA (74, 213). The clear implication of these data is that the affinity of CRP-cAMP complex for different CRP-binding sites is controlled by the DNA sequence. Variations in sequence among CRP-binding sites undoubtedly provide one means by which CRP activated promoter strength is fixed in vivo.

Until very recently an obstacle to the development of a

single mechanism to account for CRP-mediated activation at all CRP-dependent promoters has been the fact that the distance from the +1 site to the center of the CRP-binding site is frequently different among CRP-dependent promoters. Gaston et al. (120) and Ushida and Aiba (314) have recently reported a series of experiments, the results of which have been interpreted to provide a common general mechanism for CRP-dependent promoter activation. Both the *melR* and *lac* promoters were modified to provide a set of sequences having a CRP-binding site centered from 41.5 to 83.5 bp (*melR*) or 51.5 to 96.5 bp (*lac*) upstream from the start site of transcription. The promoter strength for all of the constructs was low in the absence of CRP-cAMP. CRP-cAMP-dependent *melR* promoter and *lac* promoter derivative activity demonstrated periodicity. Constructs whose CRP-binding sites were located at near-integral turns of the helix (i.e., $n \times 10.5$ bp) from the start site of transcription were activated by CRP-cAMP complex. Constructs that introduced half-integral turns of the helix from the start site of transcription exhibited little, if any, CRP-cAMP-mediated stimulation of promoter activity. The conclusion drawn from these studies is that CRP can activate transcription when bound to DNA on the same face of the helix within a range of helical turns from the RNA polymerase-binding site. In addition, the data showed that the extent of CRP-dependent promoter activation decreased with increasing CRP-binding-site distance from the start site of transcription. Both CRP-binding-site sequence and position relative to the start site of transcription also appear to be important determinants of CRP-dependent promoter strength (321).

CRP bends DNA. Without exception, CRP-cAMP binding to specific sites in DNA containing a CRP-dependent promoter mediates a structural change near the promoter. Work published from several laboratories indicates that CRP-cAMP binding to *lacP* results in DNA bending (27, 74, 93, 182, 183, 213, 335, 336). CRP-induced *lacP* DNA bending originates at the center of the CRP-binding site (213, 335). The magnitude of the bend is estimated at 90 to 130° and is dependent upon the sequence of bases in the binding site (74, 93, 182, 183, 213, 335, 336). A specific role for CRP-mediated bending in the process of promoter activation has yet to be established. DNA bending is likely to facilitate protein-protein interactions important in CRP-dependent promoter activation (addressed below). On the other hand, DNA bending could play a more direct role in the promoter activation process. Bracco et al. (43) recently reported the construction of hybrid *gal* promoter sequences in which the −35 region and the *gal* CRP-binding site were replaced either by sequences derived from *lac*, 57 bp containing both $CRP_{(lac)}$ and the $-35_{(lac)}$, or by sequences that either would or would not produce a natural bend in the DNA. The results of in vivo expression assays showed that *lac-gal* hybrid promoters were active in vivo and that their activity was stimulated by CRP-cAMP. Unbent DNA-*gal* hybrids exhibited little promoter activity in the presence or absence of CRP-cAMP, whereas bent DNA-*gal* hybrids had relatively strong promoter activity that, again, was unresponsive to CRP-cAMP. For unknown reasons the in vivo results were not reproduced in vitro in a purified transcription system (43). The lack of correlation between the two systems makes it unclear whether the bend per se or the bend sequence interacting with additional factors mediated *gal* transcription activation in vivo (43).

CRP interacts with RNA polymerase. Three lines of evidence support protein-protein interactions in CRP-mediated promoter activation. The first centers on the isolation of CRP-positive control mutants (25, 164). These mutant forms of CRP exhibit cAMP-dependent binding to DNA yet either partially or fully fail to activate the CRP-dependent promoters. The second comes from results of DNase I footprinting experiments which indicate that the CRP-cAMP complex and RNA polymerase facilitate mutual tight binding to CRP-dependent promoter DNA. In the absence of RNA polymerase, CRP-cAMP failed to protect its binding site in both the pBR-P4, *lacUV5*, and *melR* promoter DNA from DNase I cleavage (254, 300, 327). In the presence of RNA polymerase all three promoters show a protected region that includes both the CRP- and RNA polymerase-binding sites. Similarly, analysis of CRP*, CRP*-cAMP complex, or CRP*-cGMP complex binding to *lac* promoter DNA showed that although all three forms of the CRP* activate *lacP*, only the CRP-cAMP complex would footprint the DNA in the absence of RNA polymerase. Again, RNA polymerase footprinted DNA and all three forms of CRP* protected a region that spanned the binding sites for both proteins (260). The third stems from observed in vitro interactions between CRP and RNA polymerase (33, 248, 296). CRP binds to RNA polymerase holoenzyme in a cAMP-dependent manner at physiological concentrations of CRP (K_d = 1 to 3 μM) and at near-physiological ionic strength. These interactions are modulated by the σ subunit of RNA polymerase. CRP binding to the core polymerase is considerably weaker and shows no effect of cAMP, indicating that sigma factor plays, either directly or indirectly, a role in promoting the interactions between RNA polymerase and CRP-cAMP complex (33, 248, 296). The characteristics of CRP positive-control mutants strongly suggest that these contacts are important in the activation process.

Recently, reports have emerged that suggest a more complex interaction of proteins involved in *lacP* regulation than was previously thought. First, Straney and Crothers (301) provided evidence that the *lac* repressor forms what amounts to a repression loop that serves as a nucleation site for RNA polymerase binding and closed-complex formation at *lacP* on *lacL8UV5* DNA. Lac repressor and RNA polymerase simultaneously bind the *lac* control region. Lac repressor binding to the operator increases the binding constant for RNA polymerase but blocks the isomerization of the polymerase from the closed to the open complex. A similar situation has been described for wild-type *lacP*, for which closed-complex formation is dependent upon the CRP-cAMP complex. Hudson and Fried (160) have shown that CRP-cAMP complex and the Lac repressor can simultaneously bind to *lacP*. The binding is synergistic; i.e., the affinity of each protein for its site in *lac* DNA is greater in the presence of the second. The data are interpreted to provide a mechanism similar to that proposed by Straney and Crothers (301). At wild-type *lacP*, the interactions between CRP, Lac repressor, and RNA polymerase act to sequester polymerase in a closed complex poised to immediately respond to the removal of inducer (160). Preliminary evidence suggests that such a complex is formed in vitro (160). The fact that strains deficient in Lac repressor synthesize β-galactosidase clearly indicates that these interactions are not an absolute requirement for *lacP* function in vivo; this discovery does, however, represent a refinement of our understanding of the complexity of the systems.

DNA superhelical density affects CRP function. Much of the work that relates to CRP-mediated control of promoter activity has been conducted by using deproteinized linear DNA fragments as a template. When applying the models for

lacP control developed in in vitro systems to *lacP* regulation in vivo, it is important to recognize that the bacterial chromosome exists not as a linear fragment in whole cells but as a covalently closed supercoil with associated basic proteins similar to eukaryotic histones (49, 111, 324).

The development of assay systems suited to the study of transcription that originates from a covalently closed plasmid DNA template has stimulated the investigation of DNA superhelical density (SHD) effects on promoter activity in vitro. DNA supercoiling stimulates both CRP-cAMP complex and Lac repressor interactions at *lacP* in vitro and in vivo (38, 328). Similarly, the histonelike protein HU increases both CRP-cAMP complex and Lac repressor interaction (by factors of 20 and 12, respectively) with *lacP* contained on a supercoiled DNA template (109).

The results of a systematic study measuring the effect of DNA superhelical density on CRP-dependent *lacP* activation that utilized a set of plasmids differing only in their mean superhelical density showed that plasmid SHD affects the time required for RNA polymerase-*lacP* open-complex formation. RNA polymerase-$lacP^+$ open-complex half times, in reactions that contained the CRP-cAMP complex, were shown to decrease, by a factor of 35, with increased negative template SHD. Meiklejohn and Gralla (230) proposed that RNA polymerase interaction with *lacP* depends on three contact points including the −10 and −35 regions of the promoter and the CRP-cAMP complex centered at position −61.5. These contacts are correctly aligned if *lacP* is underwound ($\sigma > -0.05$). At $\sigma < -0.05$ the alignment of these contacts becomes suboptimal and *lacP* becomes less active as the template becomes less negatively supercoiled at σ values between −0.05 and −0.02.

CRP-cAMP complex constitutes one of the global regulatory mechanisms involved in the regulation of transcription in bacteria (reviewed in reference 139). Changes in DNA SHD represent a second global transcriptional regulatory mechanism (reviewed in reference 324). Earlier studies showed that CRP-dependent promoter activity was unusually sensitive to DNA gyrase inhibitors or was aberrantly low in strains carrying mutations in the genes encoding topoisomerase (*topA*) and DNA gyrase (*gyrA* and *gyrB*) (324). More recent data indicate that DNA SHD varies in response to environmental changes that elicit glucose-mediated repression of CRP-mediated promoter activity in wild-type *E. coli* and those that elicit the reversal of glucose-mediated repression of CRP-mediated promoter activity in *cya crp** strains (18).

One unusual and as yet unexplained property of some (NCR91, *cya crp-91*) but not all (*cya crp-222*) *cya* suppressor strains is their sensitivity to glucose-mediated repression of CRP-dependent enzyme synthesis (149). The mechanism of catabolite repression in wild-type *E. coli*, carbohydrate transport-mediated inhibition of cAMP synthesis, clearly fails to account for glucose-mediated repression of CRP-dependent enzyme synthesis observed in many *cya crp** strains: strains which lack cAMP and utilize CRP* to promote CRP-dependent enzyme synthesis. Four plausible explanations could account for this observation. The first involves glucose-mediated inducer exclusion wherein glucose transport inhibits lactose transport into cells. Here, *lacP* activity would become limited not by decreasing CRP* activity but rather by increasing Lac repressor activity. This mechanism can be ruled out by the observation that *cya crp* lacI* strains that fail to produce an active *lac* repressor remain sensitive to glucose-mediated repression (148). Second, positive effectors of CRP* may be present in *cya crp** strains and mediate the bulk of CRP* activity in vivo. Culture medium-dependent variations in effector concentration could produce variations in CRP* activity in a manner analogous to that described for cAMP-mediated control over wild-type CRP activity. The physiological significance of the finding that CRP* forms exhibit broader effector specificity than wild-type CRP, particularly their activation by cyclic GMP (cGMP), has been addressed and was considered unlikely on the basis of titration data which showed that the levels of cGMP required to stimulate CRP* activity in vitro are well above physiological concentrations of cGMP (149). A third mechanism that might account for glucose-mediated repression in *cya crp** strains involves variations in CRP* concentration. In vitro *lacP* activity promoted by CRP* forms varies with the concentration of CRP* (149). Mechanisms for varying cellular CRP* concentration include changes in *crp** expression and CRP* stability. CRP-cAMP functions as a repressor of *crp* gene expression in wild-type *E. coli* (4, 242, 243). Different CRP* forms might differentially affect the steady-state concentration of cellular CRP*. This does not, however, provide a mechanism by which an individual CRP* could affect its own synthesis in response to an environmental stimulus. The stability of different forms of CRP* or its mRNA in vivo could vary from strain to strain and vary with the growth condition of any given strain. This mechanism cannot be ruled out at present. A fourth mechanism that could account for environmentally mediated changes in CRP-dependent promoter activity in *cya crp** strains involves changes in CRP-dependent promoter structure. Harman et al. (149) observed that the activity of in vitro *lacP* transcription reactions containing either a catabolite repression-sensitive (91 CRP) or a catabolite repression-resistant (222 CRP) form of CRP* exhibit differential responses to spermidine. Spermidine inhibited 91 CRP-mediated *lac* transcription and had little effect on transcription reactions mediated by 222 CRP. Spermidine inhibition of 91 CRP-mediated *lac* transcription was not observed in reaction mixtures that contained 91 CRP and cAMP. The CRP*-specific response of in vitro transcription reactions to spermidine draws a striking parallel to the *cya crp** strain-specific glucose sensitivity of β-galactosidase synthesis in vivo. This parallel is extended by the observations that both spermidine-mediated inhibition of 91 CRP activity in vitro and glucose-mediated repression of β-galactosidase synthesis in strain NCR91 are relieved by cAMP. On the basis of this observation, it was proposed that spermidine-induced changes in *lacP* structure might affect either 91 CRP promoter recognition or the recognition of the 91 CRP-*lacP* DNA complex by RNA polymerase (149).

One mechanism that could mediate cAMP-independent catabolite repression in *crp** strains assumes that repression-sensitive forms of *crp** are more restricted in their recognition of promoter DNA than either wild-type CRP or catabolite repression insensitive forms of CRP* and that environmentally mediated changes in template SHD would limit their efficacy in vivo. This mechanism fits well with existing data that suggest a relationship between template DNA SHD and CRP and CRP*-mediated gene expression. The data are summarized as follows. Plasmid DNA becomes more relaxed, at least transiently, upon the addition of glucose to wild-type *E. coli* cells growing in medium containing acetate as the carbon source (18). Plasmid relaxation closely parallels the onset of transient catabolite repression of β-galactosidase synthesis under these conditions. Glucose-mediated repression of β-galactosidase synthesis in a *cya crp** strain of *E. coli* is reversed by the addition of cAMP

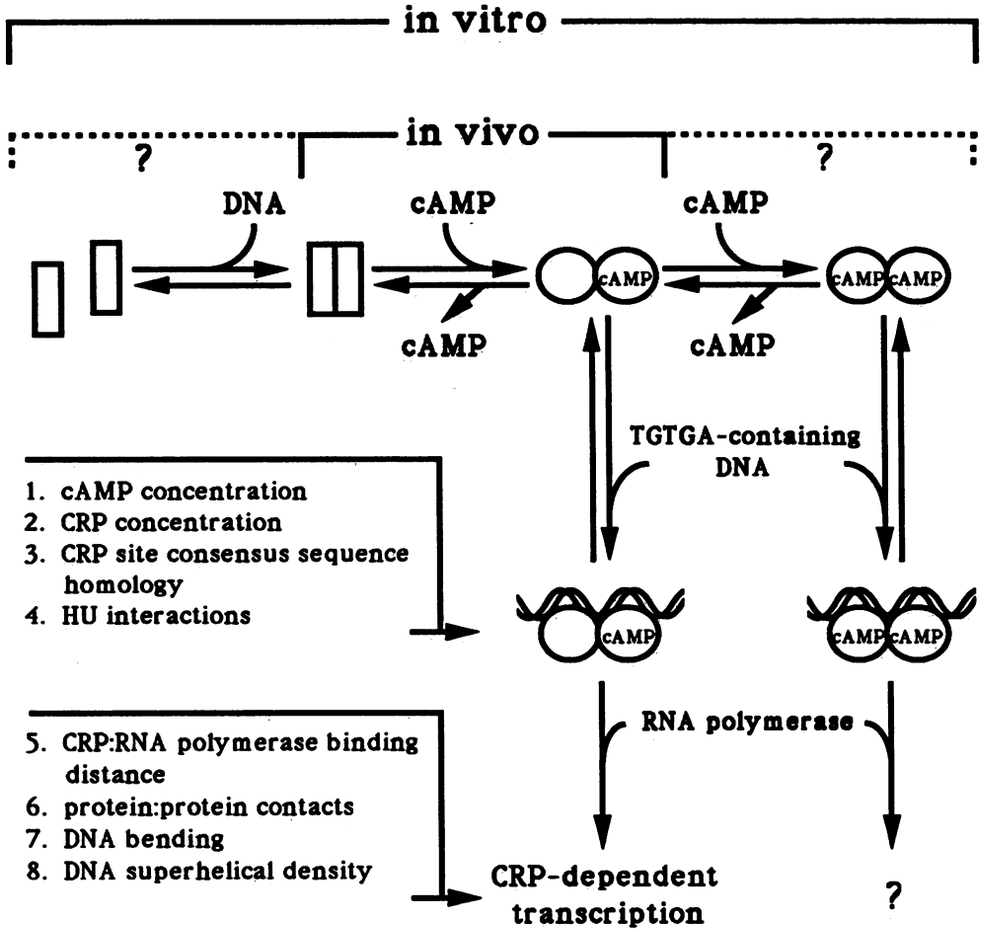

FIG. 1. Summary of CRP-cAMP-mediated binding and activation of CRP-dependent promoters in *E. coli*. "In vitro" designates the properties of the two-component system for which there are biochemical data. "In vivo" represents the properties of the two-component system for which data have been obtained in whole-cell experiments. Rectangles represent either the monomeric or the dimeric form of unliganded CRP. Circles represent the cAMP bound form(s) of CRP whose conformation differs from that of unliganded CRP. DNA refers to nonspecific DNA lacking sequences homologous to the consensus CRP-binding sequence. TGTGA-containing DNA is represented by a double-stranded helical structure and refers to DNA that contains sequences homologous to the consensus CRP-binding sequence. No data exist to indicate that (i) nonspecific DNA sequences cause unliganded CRP to monomerize in vivo, (ii) a stoichiometry of CRP-cAMP complex can be 1:2 in vivo, and (iii) a CRP-cAMP complex having 1:2 stoichiometry can function in activating transcription in vivo or in vitro; these regions are therefore identified by question marks.

to cultures grown under aerobic conditions or by a shift of those cultures from aerobic to anaerobic growth conditions (198). Dorman et al. (19) have provided evidence that plasmid DNA isolated from cells grown under anaerobic conditions is more negatively supercoiled than plasmid DNA isolated from aerobically cultured cells. In fact, 91 CRP-mediated *lacP* activity is more restricted at lower template SHDs than is *lacP* activity mediated by either the wild-type or 222 CRP (198a). The data are consistent with a promoter structure-directed mechanism for limiting 91 CRP activity.

What has been presented here concerning both the function of CRP-cAMP complex and the mechanisms by which a cell can regulate CRP-cAMP complex activity in mediating control of transcription is summarized in Figure 1. Detailed discussions of the characteristics of specific protein-DNA interactions involving both CRP and other DNA-binding proteins, promoter structure-function relationships, and DNA supercoiling effects on promoter activity can be found in references 27, 35, and 157.

Complex Control Systems Involving CRP-cAMP

Induction of the *hut* operon in *Klebsiella aerogenes* presents an interesting example of multiple controls. In this bacterium, the operon is under the control of cAMP and CRP and also the NTR system (239). Induction of the operon responds not only to carbon source limitation but also to the need of the cell for nitrogen. The mechanism for the role of the CRP-cAMP complex is unique. On the basis of in vitro experiments with the cloned genes, it has been proposed there are two promoters for the *hut* genes. CRP-cAMP complex blocks transcription at a "nonproductive" promoter, resulting in induction of the operon. When CRP-cAMP is not available, a transcript, which may not code for a protein, is made from the second nonproductive promoter. This system is further complicated. Although *hut* induction is under NTR control, the effect is indirect. Recent evidence indicates that the *nac* gene provides a function necessary for

hut expression and that *nac* expression is under NTR control (217).

A sequence showing homology to the CRP consensus has been found in the *gln* operon, which encodes glutamine synthetase (259). However, the function has not been established. Exogenous cAMP has been shown to affect the specific activity of several enzymes involved in ammonium assimilation (252). Many of these enzymes are under NTR control. It has not been determined whether there is interaction between NTR and the cAMP-CRP regulatory complex. However, the NTR system responds to the ratio between 2-ketoglutarate (a catabolite) and ammonium. It is inviting to propose a role for cAMP. Two functions involved in amino acid transport, *dhu* and *argT* in *S. typhimurium*, provide an interesting example of carbon and nitrogen control. Both of these genes are derepressed in response to nitrogen starvation and are affected by mutations in *ntrC*. The expression of *argT*, but not *dhu*, is affected by the carbon source; ArgT levels are low in cells grown on glucose. The *argT* promoter sequence includes a sequence homologous to the CRP consensus sequence (297).

Regulation of the enzymes involved in the catabolism of arabinose involves multiple control mechanisms. CRP-cAMP complex mediates direct positive control of both the *araC* gene and the *araBAD araE araFG* regulon (206, 299). The *araC* gene product, complexed with arabinose, also mediates positive control of the *ara* regulon and, in the absence of arabinose, functions as a repressor of the *araBAD* and *araC* genes. A detailed mechanism for CRP-mediated *ara* regulon induction is unclear; one role, described for *araBAD* induction, appears to involve the destabilization of an AraC repression loop formed by AraC binding to *araI* and *araO2* (214).

The expression of both the *tnaA* and *ilvB* genes, whose products are involved in amino acid catabolism, are subject to multiple control involving a CRP-cAMP-dependent expression mechanism as well as translational control mechanisms (113, 121, 135, 215). The promoters for both genes contain a sequence similar to the CRP consensus sequence; both of these regions bind CRP in vitro and, when deleted, eliminate CRP-cAMP stimulation of the respective downstream genes. Translational control over transcription is a common theme in the systems involved in either anabolic or catabolic amino acid pathways. The *tnaA* and *ilvB* genes represent examples of CRP-cAMP involvement in both processes.

The *malT* gene product, complexed with maltotriose, and the CRP-cAMP complex are both required to activate transcription from the divergent *malK* and *malE* promoters. A control region consisting of about 240 bp contains four CRP-binding sites, and at least four *malT* binding sites separate *malK* and *malE*. Raibaud et al. (255) have developed a model involving the participation of CRP-cAMP complex and *malT* in the formation of a nucleoprotein structure that leads to the activation of divergent transcription. The specific role of CRP in this complex is not known: it could serve as a scaffold protein designed to form a specific structure for RNA polymerase or as a protein that makes specific contacts with RNA polymerase.

It has been known for some time that synthesis of the EII^{Glc} and other components of the PTS, dependent on cAMP, is stimulated two- to threefold by the addition of cAMP to *cya* strains. *crp* mutations block this stimulation, indicating that the effect is mediated through the CRP (262). For *ptsH*, *ptsI*, and *crr* this has been expanded with *lacZ* fusions. Again a threefold stimulation was observed in a *cya* strain provided with exogenous cAMP (86). The promoter region has been sequenced, and a typical CRP consensus sequence is present (85). The physiological significance of this two- to threefold stimulation is uncertain. Nevertheless, *cya* and *crp* strains grow readily in minimal medium with glucose as the carbon source. The basal levels of the enzymes must be sufficient even in the absence of cAMP.

Other Cellular Processes Regulated by CRP-cAMP

There are many isolated reports that CRP-cAMP is involved in regulating a host of cellular processes. Contrary to earlier conclusions made concerning CRP-cAMP involvement in controlling the expression of catabolically related functions, many CRP-cAMP-controlled functions, summarized in Table 1, have nothing to do with the induction of alternative catabolic enzymes. A glucose effect, catabolite repression, does not always indicate a role for cAMP, as seen with production of fimbriae (100, 101, 276). 2-Ketobutyrate (75, 77) and an unidentified compound (310) have been implicated in catabolite repression, suggesting that cAMP may not be the only compound involved.

In an attempt to measure the extent to which cAMP influences protein synthesis, *E. coli* strains isogenic except for the *cya* allele were grown in glucose minimal medium containing [^{35}S]methionine and [^{35}S]cysteine and the proteins contained in cell extracts were separated by two-dimensional electrophoresis (41). The amounts of radioactivity in proteins having 0.1% or more of the total incorporated label were compared. Of approximately 250 proteins common to both extracts, 19 contained at least fivefold more label in the cya^+ culture than in the *cya* culture. These 19 proteins made up 4.11% of all the proteins present in the cya^+ extract and accounted for 0.61% of the total protein in the *cya* strain.

Nine proteins contained at least fivefold more label in extracts derived from the *cya* culture than in extracts derived from the cya^+ culture. Only two of these nine proteins were detected in the cya^+ culture. These data indicate that some proteins are not normally expressed in cya^+ cells grown on glucose, suggesting either that there is sufficient CRP-cAMP complex in glucose-grown cells to mediate strong negative control over the synthesis of some proteins or that there is a cAMP-dependent posttranscriptional modification mechanism that affects either mRNA stability, mRNA translation, or the position of these translation products in the gel. The nine proteins that were negatively regulated by cAMP accounted for 1.71% of the total protein in the *cya* culture. Together, these data indicate that some cAMP-dependent functions, both positive and negative, are expressed even in cells grown on minimal glucose medium, in which cAMP levels are low (41).

There is a similar report involving proteins separated by two-dimensional electrophoresis and compared visually in which the authors concluded that approximately as many proteins are negatively controlled by cAMP as are positively controlled (221). Mova et al. (234) have reported that the expression of a major porin is negatively controlled by cAMP.

The evidence that the expression of a protein is influenced by cAMP is not always rigorous simply because the effect of exogenous cAMP on cell physiology is not always certain. Cells regulate the intracellular concentration of cAMP by regulating its synthesis (42, 258, 274), its excretion (227), and its degradation by cAMP phosphodiesterase (40). Cells grown in the presence of large amounts of exogenous cAMP,

TABLE 1. Various functions regulated by cAMP in *E. coli* and *S. typhimurium*[a]

Gene	Function	Criteria[b]	Reference(s)
ani	pH-regulated gene expression	D	112
ansB	L-Asparaginase II	B, D, F, G	170
appR	Acid phosphatase	D	79
cat	Chloramphenicol acetyltransferase	D	200
ccd	Deoxycytidine deaminase	F	320
cea	Colicin E1 induction	D	98, 275, 284, 322
cidA	*N*-Acetylglucosamine catabolism	D	317
cir	Colicin I receptor	D	141
cpdB	2',3'-Cyclic phosphodiesterase	A, F	211, 212
crp	cAMP receptor protein	A, B, C, D, E, F	See text
cup	Carbohydrate uptake	B	219
cya	Adenylate cyclase	A, B, C, D, E, F	See text
dnaA	cAMP, allosteric effector	E	161
exuT	Galactoside metabolism	D	32
fadBC	Fatty acid utilization	B, F, G	61, 89
fic	Filamentation	D	178
flaAB	Flagellum synthesis	D	22
flaD	Flagellum synthesis	D	189
fru	Fructose regulon expression	D	109
fur	Iron uptake regulon	D, F, G	83
glpD	*sn*-Glycerol-3-phosphate dehydrogenase	B, F	60, 278
glgC	Glycogen synthesis	E, G	196, 197, 268, 313
ilvA	Threonine deaminase expression	D, F, G	121, 215
ilvB	Acetohydroxyacid synthase I	D	113, 329
melR	Melibiose activator synthesis	E, F	327
pck	PEP[c] carboxylase expression	A, B, G	135, 134
sdh	Succinate dehydrogenase operon	F	331
speC	Ornithine decarboxylase	B, E	333, 334
spf	Unstable spot 42 RNA	B, F	250
tdc	Threonine dehydratase	B	143
toxAB	Enterotoxin production	A, B, D, F, G	129, 222
tra	Transfer genes in F plasmids	C	187
tsx	Outer membrane protein	C, D, G	45
ubiG	Ubiquinone synthesis	A, D, G	128
uxaCA	Galactoside metabolism	D	32
	Aerotaxis, cAMP not involved	D	283
	CloDf13, cloacin excretion	?	245, 322
	Constitutive enzyme synthesis	C	52
	Heat shock response	C	84
	2-Oxyglutarate dehydrogenase	C	287
	pBR322, P4 promoter	A, B	254
	pH-sensitive growth	D	2, 112
	Phasmid P4, copy number	B	192
	Pilus formation	D, F	136
	Thiosulfate reduction	A, B	62

[a] This table is an updated version of Table 1 in reference 40.
[b] Criteria: A, function sensitive to catabolite repression; B, exogenous cAMP affects function; C, measurement of cyclic nucleotides; D, genetic evidence of *cya* and *crp* mutant strains; E, in vitro biochemical evidence; F, sequence data showing a consensus *crp*-binding site; G, studies with reporter gene fusions.
[c] PEP, phosphoenolpyruvate.

typically 1.0 to 5.0 mM, no longer control the intracellular concentration of cAMP. Exogenous cAMP does indeed compensate for the pleiotropic effects of mutations in *cya*. It is not certain that very high levels of exogenous cAMP have no other effects on cells. There is good evidence that there are 3,000 to 6,000 copies of the CRP in a cell (67). Typically a cell contains only a few hundred molecules of cAMP (39, 40). Cells grown in the presence of high concentrations of exogenous cAMP presumably contain a CRP population largely complexed with cAMP and hence contain several times the concentration of CRP-cAMP complex found in cells cultured in the absence of cAMP. Recent evidence indicates that, depending on the concentration of cAMP, the CRP-cAMP complex can contain either one or two bound cAMP molecules. We do not yet know whether CRP complexed with two molecules of cAMP, a situation likely to occur in cells grown in the presence of high cAMP concentrations, has physiological relevance. In addition, CRP may well have a function independent of its clearly defined role as the mediator of cAMP effects.

Genetic evidence based on mutations in *cya* or *crp* is not always definitive in establishing a direct role for the CRP-cAMP complex in gene expression. A mutation in *cya* or *crp* could affect the expression of a gene that in turn affects the expression of the gene of interest. Two clear examples are known. cAMP affects expression of the genes essential for the catabolism of melibiose in *E. coli* (327). In addition to melibiose, the CRP-cAMP complex is required to express the α-galactosidase and α-galactosidase permease encoded by the *melAB* operon. The effect of the cAMP-CRP complex is not, however, directed to the *melAB* promoter but rather to *melR*, a gene encoding a positive activator of *melAB* (327).

Rigorous proof of this was presented in a series of experiments that showed MelR provided in *trans*, and whose synthesis was coupled to a CRP-cAMP-independent promoter was, in the presence of melibiose, capable of promoting cAMP-independent *melAB* expression in vivo (327). Similarly, the expression of the *malPQ* operon, essential for the catabolism of maltose, is controlled by maltose and the *malT* gene product. Synthesis of MalT is dependent upon the CRP-cAMP complex (255). In addition, the putative role of cAMP in the process of cell division appears to be indirect.

cAMP and cell division. There is good evidence supporting a role for cAMP in regulating the cell cycle in *Saccharomyces cerevisiae* (73, 228). A similar role for cAMP in regulation of cell division in *E. coli* has been sought (156). Strains having *cya* mutations, including nonreverting *cya* deletions, are viable and divide; clearly, there is not an obligate requirement for the nucleotide in the process. Kumar (185) reported many years ago that *cya* mutants have altered morphology. It is perhaps naive to think that mutations in such a critical regulatory mode would not somehow affect cellular division. Two genes involved in cellular division after SOS inducing shock, *sfiA* and *sfiC*, require cAMP to function normally (166). Kumar et al. (186, 187) have presented evidence that expression of the *fts* gene, which codes for a protein localized to the inner membrane and involved in the process of cellular division (249), requires cAMP. In cultures growing with synchronized division, a *cya lacZ* fusion was expressed during cell elongation but not during cell division, suggesting that *cya* promoter activity and cell division are coupled (315, 316, 318). Synchronously dividing cells containing a cAMP-independent *lacUV5 cya* fusion and induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) demonstrated a two- to threefold increase in cAMP levels, ceased division, and grew as filaments during the time when cAMP production was elevated. CRP was required for cAMP to mediate this effect. This approach was also used for strains having *fic-1* and *sulB* mutations; the results of these experiments again showed that filamentous growth was induced by elevated levels of cAMP. Minicell production, again an aberrant cell division, is affected by cAMP (184).

The field has been reviewed well by D'Ari et al. (78). They showed that cells containing *cya* or *crp* mutations have abnormal morphology and are abnormally small. They further showed that *cya* and *crp* strains were resistant to mecillinam, an antibiotic that interferes with septum formation, suggesting that a function involved in septum formation is affected. *fts* expression was found to be elevated in *cya* and *crp* strains; however, through experiments involving a *fts lacZ* fusion, D'Ari et al. showed that *fts* expression itself is not cAMP dependent. They also provided evidence that the expression of *sfi*, involved in the SOS response, was affected by cAMP. This work is particularly appealing because they constructed *cya* and *crp* strains with Tn*5* insertions in clean genetic backgrounds. The results of this study provide no compelling evidence for direct involvement of cAMP in cell division. cAMP affects cell division only indirectly through several as yet unidentified cAMP-dependent functions that are not obligatory.

cAMP and starvation functions. Approximately 30 unique proteins are induced when cells are starved for carbon, nitrogen, or phosphate (226). This appears to be yet another global response of cells to environmental changes. The starvation response has been studied by using two-dimensional electrophoresis (291) and Mud *lacZ* operon fusions (292). Of these 30 proteins, approximately 20 are cAMP dependent and are not induced in strains with mutations in *cya* or *crp* (36). cAMP levels increase when cells are starved for carbon (42); it is appealing to propose that the genes derepressed in response to carbon starvation are regulated in response to cAMP levels. However, the proteins that are dependent on cAMP for expression do not appear to be critical for survival of the bacteria when starved (226, 277). One of these cAMP-dependent genes, *cst* (carbon starvation), has been cloned, and its expression has been studied in vitro (36). cAMP stimulated expression in vitro just as in vivo. The gene appears to be dependent on σ^{70}. The function encoded by the gene has not been identified; *cst* strains maintain the ability to metabolize acetate and lactate, metabolites that accumulate as glucose is exhausted.

The situation with these starvation genes is complicated. The gene products of many are induced in response to heat shock, anaerobiosis, and osmotic shock (169). This suggests that many starvation response gene products belong to a larger class of proteins that are expressed whenever cells are subjected to stressful growth conditions.

cAMP and motility. It has been proposed that the process of chemotaxis toward sugars transported by the PTS involves cAMP and cGMP (31). More recently, this has been questioned (144). Two lines of evidence indicate that neither cAMP nor cGMP is involved in the process. Tribhuwan et al. (309) and Vogler and Lengeler (323) showed with carefully constructed *cya* strains that formation of flagella, a *cya*-dependent function, and the requisite PTS enzymes II were induced in cells grown in the presence of exogenous cAMP. The cAMP could be removed, and cells were then capable of normal chemotaxis. These studies showed that the synthesis of flagella and chemoreceptors is cAMP dependent but that chemotaxis per se is not. Once these components of the chemotactic response were present, the nucleotide was no longer required. Neither group was able to reproduce experiments implicating cGMP in the chemotactic response (31). Vogler and Lengeler (323) pointed out that the cGMP level found in *E. coli* is equivalent to <1 to 3 molecules per cell and could well be an artifact.

This offers a good example of how a model can be very appealing but is not supported by subsequent work. A priori it was anticipated that cAMP would be involved in chemotaxis to PTS sugars. These sugars, unlike other chemotactic attractants, are independent of *che* mutations and the receptor methylating systems (202). PTS transport, chemotaxis, and cAMP metabolism are clearly interrelated. Mutations in some PTS components, particularly *ptsI* and *crr*, affect cAMP production (274), and it is thought that adenylate cyclase is activated by phosphorylation carried out by the PTS in the absence of glucose (274). However, more intensive work has shown no direct involvement of cAMP or cGMP in chemotaxis (31).

cAMP and anaerobiosis. Anaerobic growth of *E. coli* is reduced in *cya* strains (247). When cells grow anaerobically, more cAMP is made than when cells grow aerobically (311). Under these conditions, levels are lowest when cells grow with glucose, indicating that synthesis of the nucleotide, in cells growing anaerobically, responds to the carbon source (311).

More than 50 proteins are induced when *E. coli* grows anaerobically (256). Some are involved in anaerobic respiration and are required for the utilization of alternative terminal electron acceptors such as nitrate or fumarate. Others are specific for fermentative metabolism. Some of these functions are sensitive to a glucose effect.

At least three different mechanisms control production of

enzymes involved in anaerobiosis: *oxyC*, *fnr*, and the two-component *narL-narX* regulatory system (37, 117, 173, 176, 188, 199, 298). It appears that, superimposed on this, is a role for cAMP in regulating some but not all of these functions.

FNR regulates some functions induced in response to anaerobiosis (282). The protein is very similar to the CRP in both primary amino acid sequence and predicted secondary structure (24, 94, 152, 240). The site-directed mutation of three amino acids permits FNR to activate transcription of the *lac* operon. The cloned gene, when introduced into a *crp* strain, complemented the *crp* mutation sufficiently well to permit cells to grow with lactose, mannose, galactose, and arabinose but not with maltose, melibiose, xylose, or mannitol. The mutated FNR could still activate transcription of *frd*, a gene induced only when cells grow anaerobically (293, 294), indicating that the mutant protein retained FNR activity.

Studies of regulation of the three hydrogenases found in *S. typhimurium* have shown that OxyC is involved in regulating fermentation functions induced in response to anaerobiosis. The role of FNR is confined to regulating functions involved in anaerobic respiration. Mutations in *cya* and *crp* reduce, but do not eliminate, expression of the fermentative hydrogenase. The same mutations have no effect on the FNR-dependent hydrogenase involved in anaerobic respiration (168). Apparently some functions, albeit not alcohol dehydrogenase (256), involved in fermentative metabolism are cAMP dependent.

E. coli can utilize trimethylamine-*N*-oxide or dimethyl sulfoxide as terminal electron acceptors. The functions, encoded by *dmsABC*, are repressed by oxygen and nitrate but not by fumarate. Mutations in *fnr*, as expected, decrease *dmsABC* expression, indicating that FNR acts as a positive regulatory element. *dmsABC* expression responds to the carbon source and is lowest when cells are grown with glucose; however, this is not relieved by exogenous cAMP, indicating that CRP-cAMP-mediated catabolite repression is not involved (64). This points out yet again that observing a "glucose effect" is not necessarily indicative of a role for cAMP in the regulation of a function.

There is at least one report of an enzyme dependent on the *fnr* gene product for expression (i.e., L-asparaginase II) that is also CRP-cAMP complex dependent (171). Mutations in *crp* and *cya* as well as *fnr* affect expression (58, 273). The gene has been sequenced, and there is a typical FNR sequence and a unique region that shows homology to the consensus CRP-binding sequence (171).

The FNR protein has been purified and characterized. It is a dimer of 28,000 Da. It was proposed that the protein senses and responds to oxidation-reduction potential; it is part of a two-component regulatory system (240). It does not bind to cAMP (311). Initially, there was some confusion from studies of cells growing with glycerol and fumarate. It was suggested that FNR binds cAMP (312). Later it was shown that cAMP is required for optimal levels of glycerol-3-phosphate dehydrogenase and that FNR is required for induction of fumarate reductase, the electron acceptor (311).

Alcohol dehydrogenase is one of the proteins involved in fermentative metabolism that is induced in response to anaerobiosis. This induction is prevented in anaerobically grown cells exposed to glucose. There is a report of studies on this enzyme in which a *lacZ* fusion was used (256). The response of alcohol dehydrogenase to glucose is independent of cAMP. Exogenous cAMP did not relieve the glucose effect, and alcohol dehydrogenase activities in wild-type cells and in cells having mutations in *cya* and *crp* are similar.

In *S. typhimurium*, the level of pyruvate formate-lyase, encoded by *pfl*, increases in response to anaerobiosis and pyruvate. In strains containing *pfl lacZ* fusions, mutations in *cya* or *crp* decrease the expression two- to threefold. It is uncertain whether there is a direct involvement of CRP and cAMP or whether the mutations increased the intracellular level of pyruvate and the increased pyruvate resulted in *pfl* induction (330). Mutations in *oxyC* also reduced expression of the gene; in an *oxyC cya* strain, *pfl* expression was decreased sixfold.

E. coli produces two catalases, HPI and HPII. Both are induced as the cells enter the stationary phase of growth or when cells are shifted from aerobic to anaerobic conditions. Glucose inhibits this induction, and exogenous cAMP fails to reverse the glucose effect. Mutations in *crp* do dramatically relieve the glucose effect as cells enter stationary growth (231). This suggests that the CRP has effects that are independent of cAMP. The observed regulatory pattern is complex. The HPII catalase, encoded by the *katE* gene, is regulated by the *katF* gene product, a sigma factor-like protein (235). It is not clear how cAMP and CRP fit into the situation, but the results suggest that CRP may have functions independent of cAMP.

D'Ari et al. (78) found a *cya* mutant that was unable to grow anaerobically with glucose, but they also found that a *crp* mutant in the same genetic background grew quite well, supporting the observation by Unden and Guest (312) that *cya* but not *crp* was critical for anaerobic expression of fumarate reductase. A *crp** mutation permitted anaerobic growth, indicating that an active CRP can substitute for the missing function in the *cya* strain (78). This suggests that there may be a regulatory protein for anaerobic functions other than the CRP that utilizes cAMP.

CYCLIC NUCLEOTIDES IN NONENTERIC BACTERIA

cAMP and Disease

Toxins that stimulate adenylate cyclase in mammals. The cholera toxin and the heat-labile enterotoxin from *E. coli* stimulate adenylate cyclase in cells lining the large intestine. Their activity results in the activation of host adenylate cyclase through AMP-ribosylation of the α-subunit of G_S. The increase in the intracellular level of cAMP is responsible for the loss of fluid and electrolytes typical of the diseases caused by these bacteria (224, 225).

Adenylate cyclase in pathogenesis. In *S. typhimurium*, mutations in *cya* and *crp* make cells avirulent (72). Strains with deletions in both genes were constructed by Tn*10* mutagenesis and curing the strains of the transposon. The mutants grew more slowly than the wild type and were completely avirulent for cells in culture and for infant mice. This indicates that cAMP-dependent functions are required for virulence in these bacteria. The mutant strains have proven to be useful for preparation of vaccines against a variety of bacteria including *Streptococcus sobrinus* (71).

At least two bacteria, *Bordetella pertussis*, the bacterium that causes whooping cough, and *Bacillus anthracis*, the bacterium that causes anthrax, make a toxic adenylate cyclase. Both bacteria excrete the enzyme, which enters the host cell and raises the intracellular concentration of cAMP. The adenylate cyclases excreted by *B. pertussis* and *B. anthracis* are calmodulin dependent. Calmodulin is supplied by infected host cells. The elevated cAMP levels compromise the host response to the pathogen and contribute to the virulence of the bacteria (225, 227).

(i) ***B. pertussis.*** The situation in *B. pertussis* was reviewed briefly (147). The adenylate cyclase activity of the bacterium is stimulated more than 1,000-fold by calmodulin in vitro. The enzyme is difficult to purify (28, 115). Usually it is extracted from intact cells with 4 M urea. There are several reports suggesting that it is made as a 200-kDa precursor that is cleaved posttranslationally to a 43-kDa fragment (267). This fragment has both adenylate cyclase activity and distinct activity as a toxin (153, 191, 266). Mutants lacking adenylate cyclase activity are avirulent; virulence is restored when the cloned *cya* gene is introduced (130, 224). Two closely related species, *B. parapertussis* and *B. bronchiseptica*, cause a mild disease similar to whooping cough. Both species have the *cya* gene, but neither has a functional promoter for the gene (14, 142). Synthesis of adenylate cyclase is affected by Mg^{2+} (47, 48, 124).

The mechanism used to translocate the adenylate cyclase into the host cell has been investigated (114). The bacterium makes a protein that appears to facilitate entry of the adenylate cyclase (106, 122, 137, 138). This protein enables purified adenylate cyclase to enter host cells (90). Adenylate cyclase is rapidly degraded by an ATP-dependent proteinase once it enters the host cell (130). Nevertheless, so much enzyme enters the cells and it is so active that the intracellular concentration of cAMP increases over 20-fold. In lymphocytes, this increased level of cAMP compromises the immune response, enabling the bacterium and opportunistic pathogens to become established.

Adenylate cyclase from *B. pertussis* has been cloned in *E. coli*. However, the activity was not expressed in *E. coli* until another gene coding for calmodulin from mouse brain was also introduced (76). The adenylate cyclase gene from *B. pertussis* was modified to include a less effective promoter because the production of cAMP at very high concentrations was toxic for *E. coli* (218).

The adenylate cyclase gene from *B. pertussis* has been sequenced. There is no hydrophobic N-terminal signal sequence typical of leader sequences. The cloned gene would complement a *cya* strain of *E. coli* but only if a cloned calmodulin gene was also present (76, 132). The cloned gene product, of 1,706 amino acids (18.4 kDa), was smaller than predicted. Only the amino-terminal 450 residues (4.8 kDa) are required for calmodulin-dependent adenylate cyclase activity; the remaining 1,300 amino acids are homologous to the *hylA* gene of *E. coli*, coding for a hemolysin. This indicates that the protein functions not only as an adenylate cyclase but also as a hemolysin (133).

It is not known whether the extracellular cAMP produced by *B. pertussis* has any effect on the physiology of the bacterium or whether the bacterium has a second adenylate cyclase activity to regulate its own functions. It has been shown that when cells are grown in $MgSO_4$, but not $MgCl_2$, adenylate cyclase activity is much lower and many antigenic proteins are made at decreased levels. The bacterium contains a protein that binds cAMP, but there are no proteins that cross-react immunologically with antibodies prepared against *E. coli* CRP. The authors concluded that the decrease in adenylate cyclase activity parallels the decrease in these other proteins and was due to the $MgSO_4$ supplement.

Recently it has been demonstrated that the adenylate cyclase with the associated hemolysin requires posttranslational activation (21, 48). A mutant unable to carry out the activation was isolated, and characterization of this mutant showed that the posttranslational processing was required for the expression of both adenylate cyclase activity and hemolysin activity either in vitro or in cells (16).

(ii) ***B. anthracis.*** *B. anthracis* makes three well-defined toxins, PA (85 kDa), LF (83 kDa), and EF (89 kDa). Individually, none of these proteins is toxic. In combination, they interact to produce two different pathogenic responses. PA and LF, when injected into test animals, cause death. PA and EF, when injected into test animals, cause edema. PA is thought to be a receptor-binding component needed for entry of the other toxins. EF is an adenylate cyclase (203, 204). The enzyme causes the intracellular concentration of cAMP to increase as much as 200-fold in CHO cells (204). Unlike the enzyme from *B. pertussis*, adenylate cyclase from *B. anthracis* enters the cell by endocytosis (138). Inhibitors of receptor-mediated endocytosis, cytochalasin D for example, inhibit entry of adenylate cyclase from *B. anthracis* but not *B. pertussis* (139).

All three toxins are encoded by a plasmid in *B. anthracis*. The gene encoding the EF toxin was first cloned by using an oligonucleotide probe based on the N-terminal sequence of amino acids (264). The gene was also cloned into *E. coli* by complementation of a *cya* strain containing cloned calmodulin (232). This gene has been sequenced (263, 264, 308). It is included in an open reading frame corresponding to a protein with 800 amino acids, including a 33-amino-acid signal sequence (86 kDa). The sequence includes a region homologous to regions in mammalian proteins that interact with calmodulin and an ATP-binding site. These regions and a third region are similar to regions in the enzyme from *B. pertussis*. This similarity has been exploited to carry out directed mutagenesis of the genes from *B. pertussis* to better define the calmodulin- and ATP-binding sites (131). EF toxin of *B. anthracis* cross-reacts with polyclonal antibodies prepared against the adenylate cyclase from *B. pertussis*. These antibodies also cross-react with adenylate cyclase isolated from mouse brain.

It has been proposed that the adenylate cyclase from *B. pertussis* and *B. anthracis* have a common origin (103). *B. anthracis* DNA has a G+C content of 29%, whereas *B. pertussis* DNA has a G+C content of 65%. *B. anthracis* is gram positive and is normally found in the soil. *B. pertussis* is gram negative and is normally found associated with mammals. This suggests that the enzyme may have been acquired from the host.

cAMP in phototrophic bacteria. In *Rhodospirillum rubrum*, a purple photosynthetic bacteria, a pyruvate-phototrophy diauxie is observed (290). Exogenous cAMP reduces the duration of this diauxie. There are reports of an adenylate cyclase activity and a cyclic phosphodiesterase activity in this bacterium (107). The intracellular levels of cAMP have been measured in *Rhodopseudomonas capsulatus* (146).

Rhodocyclus gelatinosus, another purple photosynthetic bacteria, is capable of oxidizing CO to CO_2 and H_2O as well as phototrophic growth. A CO-phototrophy diauxie is observed when CO is replaced by H_2 in cells growing in the light. When cells were grown with CO, the intracellular concentration of cAMP was measured at 30 to 40 pmol mg of $protein^{-1}$. When cells were grown phototrophically, the intracellular concentration of cAMP was measured at 9 pmol mg of $protein^{-1}$. cAMP appears to be excreted as CO is exchanged. Exogenous cAMP reduced the amount of pigments involved in photosynthesis, suggesting a role for the nucleotide in regulation of expression of genes involved in the photosynthetic process (236).

A ninefold change in intracellular cAMP levels has been observed in the cyanobacterium *Anabaena cylindrica* when cells grown in the dark were exposed to light (241). The cAMP level increased very rapidly when cells grown in the

light were shifted to the dark. There is also a report indicating that exogenous cAMP interferes with the formation of the photosynthetic apparatus in *A. variabilis* (286). Adenylate cyclase from *Anabaena* spp. has been purified and characterized. This bacterium produces a protein that binds Ca^{2+} and is similar to mammalian calmodulin.

cAMP in *Streptomyces* species. cAMP has been implicated in morphogenesis in several dimorphic bacteria. It was anticipated there might be a role for the nucleotide in sporulation. In addition, these bacteria produce antibiotics as secondary metabolites after the exhaustion of the principal carbon source. It was anticipated that cAMP might play a role in regulating antibiotic production. It is now possible to carry out rigorous genetic investigations with this group of prokaryotes. It should be possible to isolate *cya* mutants to investigate possible roles for the nucleotide in sporulation and antibiotic production.

In an earlier review of this topic (39), there were five preliminary reports of cAMP in various *Streptomyces* species; again, there are only preliminary reports. The antibiotic puromycin is produced by *S. alboniger*. No change in either ATP levels or cAMP levels were observed in cultures as the glucose became exhausted and the cells initiated antibiotic production. This suggests that cAMP has no role in the production of this antibiotic in this bacterium (56, 57).

There is one report in which the authors measured cAMP levels in aerial hyphae. Cells were grown on agar medium covered with dialysis membranes. The cell hyphae above the membrane were harvested, and cAMP levels were measured. The authors found that when cells were grown in a very rich undefined medium containing glucose, cAMP levels were much higher, 0.5 to 2.0 pmol mg (dry weight)$^{-1}$, than when cells grew in the same rich medium containing dextrin (302).

S. fradiae produces the antibiotic tylosin. A mutant strain that produces threefold-greater amounts of the antibiotic and also produces excess cAMP has been isolated. Chloroquin, an antimalarial drug, increased the production of both the antibiotic and cAMP (63).

S. griseus produces streptomycin. The production of this antibiotic is initiated only when growth of the bacteria is limited by the availability of phosphate. It was shown that cAMP levels increased as the culture aged and that this increase occurred independently of phosphate availability. The authors measured cAMP concentrations as high as 1,600 pmol mg of protein^{-1}, 2 orders of magnitude greater than is typically found in eubacteria. cAMP accumulated in the extracellular medium in modest amounts, which decreased as the culture aged. This suggests that the bacterium produces a cyclic phosphodiesterase that acts on extracellular cAMP (306).

S. hygroscopicus produces the antibiotic turimycin. A brief report indicates that cAMP production varies as much as 10-fold with the carbon source available to the cells (127). Production of turimycin varies with the carbon source about threefold. These authors found that adding carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to destroy the proton gradient caused the intracellular concentration of cAMP to increase dramatically. They concluded that cAMP production is linked to the proton motive force.

S. venezuelae produces chloramphenicol. Cells exhibit a glucose-lactose diauxie when grown with limiting concentrations of the two carbon sources. No change in the intracellular levels of cAMP was observed (57).

The streptomycete that has been studied in the most detail with respect to cAMP metabolism does not produce antibiotics but is nevertheless significant to the field of applied microbiology. *Thermomonospora curvata* is a thermophilic streptomycete that produces cellulase. Cellulase activity is induced by cellobiose. In a mutant shown to produce higher than normal amounts of cellulase, excess cAMP was also produced, suggesting that cAMP was involved in regulation of cellulase production (110, 332). 2-Deoxyglucose, a sugar transported into the bacterium but not metabolized further, caused the intracellular concentration of cAMP to increase. In cells made permeable by toluene, exogenous cAMP stimulated the production of cellulase. Inhibitors of cyclic phosphodiesterase, theophylline and caffeine, stimulated the effect of exogenous cAMP, suggesting the presence of a cyclic phosphodiesterase activity that is physiologically significant (332). When cells were grown in chemostats with limiting cellulose, it was possible to show a correlation between cAMP levels and cellulase production. These authors presented evidence for four different cyclic phosphodiesterase activities. They proposed that the intracellular concentration of the nucleotide is determined by degradation (125, 126).

cAMP in *Rhizobium* species. The gene encoding the adenylate cyclase of *Bradyrhizobium japonicum* has been cloned. Clones were obtained by selecting for chimeric plasmids that complemented *cya* mutations in *E. coli* (145). The adenylate cyclase and at least two cyclic phosphodiesterases have been partially characterized from this bacterium (53). *B. japonicum* has a hydrogenase that permits chemoautotrophic growth on hydrogen. Hydrogen uptake is inhibited by the presence of malate in the growth medium. This inhibition is reversed by the addition of exogenous cAMP (229). To the best of our knowledge, the involvement of cAMP in the regulation of hydrogen uptake has not been pursued.

Rhizobium fredii, a fast-growing *Rhizobium* species that grows symbiotically with soybeans, has been found to have three distinct cyclic phosphodiesterase activities (208, 210). A protein capable of binding cAMP has also been isolated from this bacterium (216).

A gene encoding adenylate cyclase has been cloned from *R. meliloti*, again selecting for complementation of *cya* mutations in *E. coli* (181). The cloned gene has been characterized (193) and sequenced (50). The sequence is very different from the *cya* genes of either *E. coli* (6) or *B. pertussis* (132). Some similarity with the sequence of the enzyme from eukaryotes was noted; it was proposed that the enzyme was acquired for eukaryotes. When the gene encoding glutamine synthetase II of rhizobia was cloned and sequenced, it was proposed that this enzyme, again very similar to plant glutamine synthetase and very unlike glutamine synthetase I of rhizobia, was acquired from plants. A more critical analysis of the data does not support this conclusion (281). No requirement for calmodulin has been shown for the enzyme from *R. meliloti*. Eukaryotic adenylate cyclases all seem to require calmodulin.

The situation is further complicated in *R. meliloti*. The cloned *cya* gene was mutated by using site-directed mutagenesis and gene replacement. No difference in phenotype could be observed in the mutant strain with a transposon insertion in *cya*. Furthermore, the strain with the mutant gene continued to make appreciable amounts of cAMP (244). A gene library prepared from the mutant strain was found to contain a second gene that complemented a *cya* strain of *E. coli*, indicating that the bacterium has at least two distinct *cya* genes (240a).

The function of cAMP in *R. meliloti* is uncertain. The

TABLE 2. Reports of cyclic nucleotides in miscellaneous bacteria

Microorganism	Property	Criteria[a]	Reference(s)
Aeromonas spp.	"Suicide" growth on glucose	A, B	237
Archaebacteria (3)	Measurable cAMP	C	201
Arthrobacter oxidans	Glucose repression of 6-hydroxynicotine oxidase	A	44
Bacillus circulans	β-Xylanase expression	B	104
Bacillus subtilis	cGMP and sporulation	B	220
	Measurable cAMP in O_2-limited cells	C	218
Chlamydia trachomatis	Development	B	177
Erwinia chrysanthemi	Pectate lyase expression	A, B	65, 180
Klebsiella aerogenes	*hut* operon induction	B, E	239
Klebsiella pneumoniae	TCA[b] cycle enzymes	A, B	175
	Nitrogen metabolism	A, B	94
Legionella pneumophila	Cell growth	B	140
Mycobacterium smegmatis	Fatty acid synthesis	B	3
Mycoplasma pneumoniae	Glass attachment	B	108
Pseudomonas fluorescens	Antibiotic production	A, B	167
Vibrio cholerae		A, B	54, 55
Vibrio fischeri	Bioluminescence	B, C, D	95–97
Vibrio parahaemolyticus	Protease production	D	305
Marine microorganisms	cAMP uptake	C	11, 12

[a] Criteria: A, glucose-mediated repression of enzyme synthesis; B, cAMP-mediated stimulatory effects; C, cyclic nucleotide measurement; D, genetic evidence, apparent *cya* and *crp* mutants; E, effects of in vitro gene expression; F, DNA analysis, regions homologous to CRP consensus sequence.
[b] TCA, tricarboxylic acid.

bacterium can fix CO_2 by using RuDP carboxylase when growing with formate. Succinate represses RuDP carboxylase (229). Exogenous cAMP has no effect.

There are reports of cGMP in *B. japonicum* (59, 172, 207, 209). The concentration was found to be in the order of nanomoles per milligram of protein, fewer than 10 molecules per cell. The concentration of cAMP was found to vary with aeration. Exogenous cGMP, at 1 mM, inhibits nitrogen fixation ex planta in the bacterium; the nucleotide was also found to inhibit growth.

Rhizobia are a diverse group of bacteria. In *R. meliloti*, polyol dehydrogenases, mannitol dehydrogenase for example, are inducible several hundred-fold. This induction is inhibited 70% by glucose, an example of catabolite repression analogous to what is observed in *E. coli*. In contrast, the same activity in *R. leguminosarum* is not affected by the presence of glucose (88, 223).

Cyclic di-GMP, is found in *Agrobacterium tumefaciens*, a bacterium related to the fast-growing rhizobia (e.g., *R. meliloti*). *A. tumefaciens* produces cellulose, and this synthesis is stimulated by exogenous cyclic di-GMP. An enzyme that degrades cyclic di-GMP was inhibited by excess Ca^{2+}. The bacterium accumulates excess cellulose when growing with high levels of Ca^{2+}, presumably as a result of the Ca^{2+} inhibition of the enzyme (10). This nucleotide has been found in another bacterium that also produces cellulose, *Acetobacter xylinum* (270).

Brief reports of cAMP in other bacteria. Cyclic nucleotides have been detected in a variety of bacteria. However, with only a few exceptions, the role of cAMP in cellular processes has not been studied extensively. In none of these bacteria has a well-defined function for cAMP been elucidated. For the most part, we know simply that exogenous cAMP influences the expression of a function, that cAMP can be detected in cells, that the cells have a detectable adenylate cyclase activity, and that the cells have a detectable cyclic phosphodiesterase activity. No mutants devoid of adenylate cyclase activity have been isolated.

The least rigorous evidence for a role for cAMP in regulation is a glucose effect. In the enteric coliforms, catabolite repression is a complex process involving not only cAMP and the CRP but also inducer exclusion (251, 274). In *Arthrobacter oxidans* induction of 6-hydroxynicotine oxidase is sensitive to a glucose effect but exogenous cAMP does not reverse this effect (44). Similarly, in *Alcaligenes latus* exogenous cAMP does not reverse glucose repression of H_2 oxidation (92).

The effect of exogenous cAMP is not always unequivocal proof of a role for the nucleotide. It usually takes very high concentration of the nucleotide, 3 orders of magnitude greater than are found in any cells, to have any effect. The effect could be a nonspecific effect of the nucleotide.

Biochemical evidence for adenylate cyclase argues that the cell does make cAMP but says nothing about the function of the nucleotide. The presence of cyclic phosphodiesterase could indicate that the function serves to regulate the concentration of cAMP in the cell as it does in vertebrates; alternatively, it could serve to simply break down exogenous cAMP, as appears to be the case in *S. typhimurium* (40).

The most rigorous evidence for a role of cAMP is the isolation of adenylate cyclase mutants. With the exception of *M. meliloti* (229) and *Vibrio fischeri* (95), adenylate cyclase mutants have not been described among bacteria other than the familiar enteric coliforms and some pathogenic bacilli.

It should be noted that cAMP has been found in *B. subtilis* (194). A report definitively shows that cAMP is made in appreciable quantities when cells are grown with limiting oxygen (218). However, it does not appear to be involved in catabolite repression, at least of aconitase (*citB*) expression (269). There is another report showing that cAMP is not found in *Clostridium perfringens* (279). These authors used well-established techniques to isolate and purify the nucleotides in a well-controlled series of experiments.

There is an interesting report of a *Xanthomonas campestris* protein involved in the phytopathogenicity of the bacterium. The gene was cloned from a gene library for proteins that would complement a *cya crp* double mutant of *E. coli*. The product will complement some catabolic operons in *E. coli*. It is similar to *crp** gene products, mutations that make the CRP independent of cAMP for its activating function (8, 34, 118,

119, 148–150, 260). The cloned gene from the bacterium has 45% identity with CRP. No cAMP could be found in the cells, and no effect of exogenous cAMP was observed (81).

Table 2 lists some bacteria in which cyclic nucleotides have been found.

CALMODULIN IN PROKARYOTES

Calmodulin is a small protein that binds Ca^{2+} and mediates many Ca^{2+}-dependent functions, often in conjunction with cAMP, in eukaryotic cells. An adenylate cyclase has been partially purified from *Anabaena* spp., a filamentous cyanobacterium that differentiates to form a nitrogen-fixing heterocyst. The enzyme has a molecular mass of 183 kDa and is activated by Ca^{2+} complexed to calmodulin from bovine brain and by a calmodulinlike protein produced by the bacterium. This protein activated adenylate cyclase from bovine brain, indicating functional similarity between the two proteins (29).

Calmodulinlike proteins have been found in *E. coli* (165). This protein was isolated in the course of isolating a Ca^{2+}-dependent cAMP phosphodiesterase from cells grown in nutrient broth. The protein is stable and activates bovine brain cAMP phosphodiesterase, ATPase from human erythrocytes, and myosin light chain-like authentic calmodulin. However, it has been pointed out that the cells were grown in nutrient broth and the protein could have been derived from the culture medium (116). A calmodulinlike protein has been found in *B. subtilis* (116, 146a, 158).

The S protein from *Myxococcus xanthus*, a protein produced in large amounts during cell differentiation, has been sequenced and shows some similarity to the sequence of the mammalian calmodulin gene product (87, 162). However, it has been pointed out that the sequence is not consistent with the helix-loop-helix motif of authentic calmodulin (303). The protein does bind Ca^{2+}. Its ability to activate calmodulin-dependent enzymes was not tested.

A protein similar to calmodulin has been isolated from *Streptomyces erythraeus* the streptomycete that produces erythromycin (195). The protein is acidic, has a low molecular weight, and displays a Ca^{2+}-dependent conformational change. It did not substitute for authentic calmodulin to activate bovine cAMP phosphodiesterase. The gene has been sequenced, and the sequence is consistent with the helix-loop-helix motif of calmodulin (303).

CONCLUSIONS

cAMP is essentially ubiquitous. It is found in animal cells (265), fungi (246), and plants (15, 238). Its status in plants is uncertain; in tissues like tobacco callus that should be reproducible, the levels are highly variable. In a recent study no detectable (<0.5 pmol g of tissue^{-1}) cAMP could be found in lettuce, sunflowers, rice, pine seeds, or tobacco (295). The authors conclude that all cAMP found in plants is artifactual.

In vertebrates, synthesis of cAMP is stimulated by peptide hormones interacting with protein receptors in the cell membrane. In higher cells, cAMP is degraded by cyclic nucleotide phosphodiesterases and intracellular levels can vary rapidly. In the enteric coliforms the intracellular concentration of cAMP is rigorously controlled. Adenylate cyclase activity is controlled through a mechanism that senses environmental conditions. This signal is mediated by transport proteins located in the cell membrane. The nucleotide is degraded by cAMP phosphodiesterase; it is also readily excreted.

In higher organisms, the nucleotide acts by activating an allosteric protein kinase that, in turn, activates enzymes. The most familiar role for cAMP in vertebrates is in the regulation of glycogen and triglyceride metabolism. The nucleotide regulates cellular activities that are unrelated to energy metabolism. cAMP made in response to adrenocorticotropin stimulates the production of steroid hormones including cortisone and aldosterone; in response to follicle-stimulating hormone it stimulates the production of estradiol and progesterone. cAMP in concert with Ca^{2+} and calmodulin regulates the contractility of smooth muscle.

cAMP, at least in the enteric coliforms, functions not as an activator of a protein kinase (69), but rather as an activator of CRP, an allosteric DNA-regulatory protein that modulates the transcription of several genes. With the exception of the finding that cAMP functions as an allosteric effector of DnaA (161), all cAMP effects in bacteria appear to be mediated through its binding to CRP. On the surface, cAMP functions differently in bacteria and vertebrates. However, vertebrates, unlike bacteria, rarely regulate housekeeping functions at the genetic level. Rather, these functions are regulated through posttranslational mechanisms. In the context of the way in which cells regulate essential metabolic activities, cAMP plays a comparable role in bacteria and in vertebrates and does so through a mechanism appropriate to the cell type.

cAMP, at least in the enteric coliforms, functions most dramatically to regulate the expression of inducible catabolic operons. In normal situations, some cAMP is always present; even cells grown in the presence of glucose produce detectable levels of the nucleotide (39). Most inducible catabolic operons are expressed to some degree when cells are cultured in medium containing glucose. The nucleotide modulates the expression of alternative catabolic enzymes in response to the environmental conditions.

Functions that are not related directly to energy metabolism and whose production is influenced by cAMP are generally not absolutely dependent on cAMP for expression. Even in *cya* and *crp* mutants, which either contain no cAMP or fail to produce an active CRP, there is substantial basal activity of these functions. It may be that when cells grow in a real-world situation, these functions, heat-stable enterotoxin production for example, offer a selective advantage to the cell and that this mode of regulation is appropriate.

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