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Cyclic Adenosine 5'-Monophosphate in *Escherichia coli*

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INTRODUCTION

One of the major aims of molecular biology is to understand how gene activity is controlled. Most of the effort in this area has been centered around *Escherichia coli* and related organisms. Some of the earliest detailed studies on regulation of gene activity in *E. coli* were performed by Jacob, Monod, and co-workers, who investigated the expression of the genes for lactose (*lac*) utilization. On the basis of genetic and physiological studies, Jacob and Monod (45) proposed a model of gene expression in which the *lac* genes were prevented from being expressed by a specific repressor molecule. This repressor was postulated to act by binding at a regulatory site on the *lac* genes. Inducers such as lactose acted by removing the repressor from deoxyribonucleic acid (DNA) and allowing ribonucleic acid (RNA) polymerase to synthesize *lac* messenger RNA (mRNA) which was next translated into proteins. Within a few years after Jacob and Monod had proposed their model, Gilbert and collaborators succeeded in purifying the *lac* repressor and showing that it was a protein with a high affinity for *lac* DNA (34). However, they were unable to show in a cell-free system employing purified *lac* DNA and RNA polymerase that the *lac* repressor would repress transcription, since *lac* RNA was not made when RNA polymerase was incubated with *lac* DNA. Thus other regulatory factors were probably required for *lac* operon transcription.

It has been known for many years that the ability of cells to ferment lactose was inhibited when glucose was also present in the growth medium (64). Glucose did this by inhibiting the synthesis of the lactose-degrading enzymes. This ability of glucose to inhibit the synthesis of a variety of enzymes was called "the glucose effect."

E. Sutherland and co-workers, who had already discovered the existence of cyclic adenosine 5'-monophosphate (AMP) in animal cells, were interested in the possible existence of the cyclic nucleotide in other organisms. They found that cyclic AMP was present in *E. coli* and that its level was affected by the substrate on which the organism was grown (60). Cells growing on glucose had particularly low levels, and these low levels rose dramatically when glucose was exhausted only to fall again upon glucose readdition.

The observation that glucose lowered cyclic AMP levels, coupled with the findings that glucose repressed the synthesis of the lactose-degrading enzyme β-galactosidase and a variety of other inducible catabolic enzymes, suggested that glucose might exert its repressive action

by lowering cyclic AMP levels. To test this idea, Perlman and Pastan (81) added cyclic AMP to cultures of *E. coli* and found that the cyclic nucleotide would overcome the repressive effects of glucose on the synthesis of β-galactosidase and tryptophanase; subsequently, cyclic AMP was found to control the synthesis of other enzymes (see reference 75). In addition to controlling the synthesis of a variety of inducible and catabolic enzymes, cyclic AMP has been found to regulate the synthesis of a morphologically distinct entity, the flagella of *E. coli* (121), and also to regulate the life cycle of some bacterial viruses (42).

To elucidate how cyclic AMP controlled gene expression it was necessary to duplicate its action in a cell-free system. This was first achieved by Chambers and Zubay (18), who showed that β-galactosidase synthesis was increased about 20-fold when a crude cell-free system containing *lac* DNA was supplemented with cyclic AMP. Later, Parks et al. were able to demonstrate the synthesis of galactokinase in cell-free extracts; galactokinase synthesis was also highly dependent on the presence of cyclic AMP (73).

Two classes of mutants have been isolated in which the synthesis of enzymes under cyclic AMP control is greatly diminished. One class has a defective adenylate cyclase (*cya*), and such cells make β-galactosidase and other glucose-repressible enzymes when cyclic AMP is added to the culture medium (82). A second class of mutants (*crp*) is missing a protein that binds cyclic AMP (30, 124). Mutant cells lacking the cyclic AMP receptor protein (CRP) will not make β-galactosidase or related enzymes even when the medium is supplemented with cyclic AMP, and cell-free extracts of such mutant cells will only make β-galactosidase or galactokinase when the extracts are supplemented with both cyclic AMP and CRP.

Shortly after cyclic AMP was shown to stimulate β-galactosidase synthesis in cell-free extracts, de Crombrugghe et al. (26) showed that cyclic AMP also stimulated *lac* mRNA synthesis in such extracts. Finally, Anderson et al. (5) were able to obtain homogeneous CRP. Using this purified CRP, *lac* gene expression was obtained using only the following purified components: *lac* DNA, cyclic AMP, CRP, RNA polymerase, and nucleoside triphosphates (24). Further, as predicted by Jacob and Monod (45), *lac* repressor was directly shown to act by preventing initiation of *lac* RNA synthesis (19).

In this review we mainly consider three general areas: (i) cellular reactions under cyclic AMP control; (ii) how cyclic AMP controls gene expression; and (iii) how the intracellular levels of cyclic AMP are controlled.

ROLE OF CYCLIC AMP IN REGULATION OF GENE EXPRESSION

A Positive Element in Gene Expression

Monod (64) discovered that *E. coli* grew in a two-step pattern when glucose and lactose or one of a number of other sugars were supplied in the growth medium. He found that cellular growth ceased when glucose was exhausted and resumed after a lag period which corresponded to the time required for the induction of the enzymes needed for utilization of lactose; the synthesis of the enzymes needed to metabolize lactose was repressed to low levels as long as glucose was present. The phenomenon of specific inhibition of induction of other enzymes by glucose became known as the "glucose effect." The glucose effect is not limited to the enzymes of sugar catabolism; glucose represses the synthesis of other degradative enzymes and some *E. coli* structural proteins.

Table 1 shows a list of proteins in *E. coli* known to be subject to glucose repression. The list is by no means complete; more exhaustive lists and references have been published previously (75, 93). Two points stand out—one is that almost all the proteins are inducible; the other is that their presence is not essential for cell survival and growth in glucose-supplemented medium. Even the proteins involved in cell chemotaxis and flagellar synthesis are subject to such a generalized control. In all these cases, addition of cyclic AMP reverses the glucose repression. The effect is specific; adenosine 5′-triphosphate (ATP), adenosine diphosphate (ADP), 5′-AMP, and 3′-AMP are ineffective (81). These studies emphasize the central role that cyclic AMP occupies in the regulation of gene expression.

A number of hypotheses have been proposed to explain the glucose effect (see reference 58). Magasanik favored the hypothesis that glucose, being catabolized faster than other sugars, produced high levels of catabolic intermediates, one of which caused repression of the synthesis of inducible enzymes. He therefore called the phenomenon "catabolite repression." The phenomenon has been studied extensively with β-galactosidase, one of the *lac* enzymes. When glucose is added to cultures of *E. coli* growing on glycerol and containing isopropylthio-β-galactoside, a gratuitous inducer of the *lac* enzymes, glucose produces a severe repression of β-galactosidase synthesis for about 0.5 h. This is known as "transient" repression. Then β-

galactosidase synthesis resumes, but at a low rate. This reduced rate of synthesis is called permanent repression. A typical experiment is shown in Fig. 1. Although glucose has been shown to inhibit the transport of inducers of *lac* enzymes (20), a potential source of glucose repression, both transient and permanent repression have been shown to be the result of a more direct effect of glucose at the level of enzyme synthesis. After the discovery by Makman and Sutherland (60) that addition of glucose drastically lowered the concentration of cyclic AMP, Perlman and Pastan suggested that the molecule was needed to promote the synthesis of inducible enzymes and that the repressive effect of glucose was due to a diminished concentration of cyclic AMP in the cells (81). Indeed, it was found that cyclic AMP addition not only stimulated the synthesis of β-galactosidase in a nonglucose medium but also overcame both transient and permanent repression by glucose, as shown in Fig. 1. The profound initial drop in cyclic AMP level upon the addition of glucose to cells growing in glycerol explains the severe transient repression. The subsequent recovery of cyclic AMP levels to an intermediate value accounts for the less profound permanent repression (75).

Before we discuss further how cyclic AMP controls enzyme synthesis, we would like to clarify a few points about glucose repression. (i) Glucose repression of β-galactosidase synthesis is independent of specific repression by the *lac* repressor, because in *lac* repressor-defective mutants β-galactosidase can be repressed by glucose (56). (ii) To lower the intracellular concentration of cyclic AMP and produce both transient and permanent repression, glucose does not need to be extensively metabolized. Cells defective in glucose utilization (lacking hexose phosphate-isomerase) are not resistant to glucose repression (55). Analogues of glucose that are phosphorylated but not further metabolized, 2-deoxyglucose or α-methyl glucoside, can mimic the effect of glucose (20, 111); α-methyl glucoside has been shown to lower intracellular cyclic AMP levels (75).

TABLE 1. *Typical genes under cyclic AMP control*

lac operon	Tryptophanase
gal operon	D-Serine deaminase
ara operon	Chemotactic genes
mal operons	Flagellar genes
Mannitol utilization	
Sorbitol utilization	β-glucuronidase
gly regulon	Chloramphenicol acetyltransferase (*cam*)

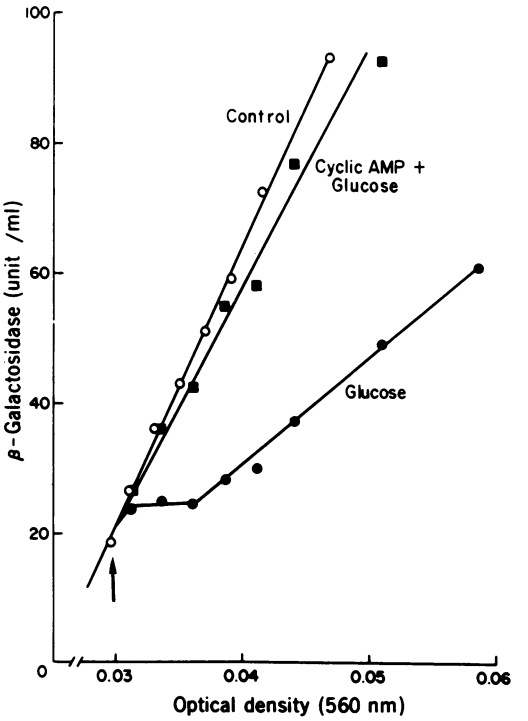

FIG. 1. *Effect of glucose and cyclic AMP on the synthesis of β-galactosidase. Cells growing in glycerol minimal medium were induced to make β-galactosidase by the addition of IPTG. Glucose (●) or glucose plus cyclic AMP (■) were added at the arrow (from reference 75).*

The finding that exogenous cyclic AMP would relieve the "glucose effect" suggested but did not prove that cyclic AMP had an important physiological role. A physiological role for cyclic AMP was confirmed by isolation and analysis of mutants of *E. coli* that were defective in adenylate cyclase, the enzyme that catalyzes the conversion of ATP into cyclic AMP (44, 110). Perlman and Pastan isolated adenylate cyclase-defective mutants (referred to as *cya* in this article) of *E. coli* based on the failure of these mutants to utilize both lactose and galactose on an indicator plate (82). The mutants were also unable to utilize sorbitol, maltose, arabinose, mannitol, or glycerol; utilization of each of these requires the induction of enzymes known to be subject to glucose repression (Table 1). They also did not make flagellar proteins. The capacity to synthesize these proteins in the *cya* mutants was restored after addition of cyclic AMP. Since the addition of cyclic AMP to the wild-type cells did not stimulate the synthesis of total RNA, protein, or anabolic and amphibolic enzymes, including

glycolytic enzymes, it was not surprising that the *cya* mutants could still ferment glucose and that the mutation was nonlethal. Many *cya* mutants isolated subsequently behave similarly. Generally, even in a rich medium *cya* mutants grow slowly in the absence of cyclic AMP and their growth is stimulated by cyclic AMP (26a). This raises the possibility that cyclic AMP is an essential component of the cell, and that the *cya* mutants are only partially defective. Recently, Brickman et al. (13) have isolated a large number of *cya* mutants. Most of these mutants recombine with each other and therefore carry single-site mutations. However, since one of these mutants does not recombine with three other point mutants, it appears to be a multisite or deletion mutant. Since a deletion in a gene usually eliminates the biochemical function, it is likely that cyclic AMP is not essential for cell survival and growth. The *cya* mutations map between the *rho* and *metE* loci on the *E. coli* chromosome (121; A. Das, personal communication).

CRP. Among the mutants isolated as defective in the induction of many sugar-catabolizing enzymes was a class that retained high adenylate cyclase activity. These have been subsequently found to overproduce cyclic AMP (87). This second group maps close to the cluster of genes coding for some ribosomal proteins, *str, spc, fus* (79). These mutants have been shown to be defective in a protein to which cyclic AMP specifically binds and which is called CRP (30). CRP, also known as catabolite activator protein (124) and catabolite gene activator (94), has been purified to homogeneity (5). *crp* mutants have the same phenotype as *cya* mutants, but do not respond to the addition of external cyclic AMP, which restores *cya* mutants to a wild-type phenotype. A crp+/crp diploid strain has the wild-type phenotype, suggesting that CRP has a positive regulatory role (84). In its role as a positive control element in gene activation, cyclic AMP always needs CRP. Cells with mutations in the *crp* region are readily obtained. Extracts of one mutant, strain 5333, have been found to have lost their capacity to bind cyclic AMP. Using an antibody to CRP, Anderson and Pastan (3) were able to detect only a 50% reduction of CRP cross-reacting protein. Thus, this mutant may have an altered nucleotide-binding site in CRP.

Sanders and McGeoch (97) have isolated a mutant of CRP in which the nucleotide-binding site appears to be altered. The alteration results in a protein that recognizes both cyclic AMP and cyclic guanosine 3',5'-monophosphate (cyclic GMP), and permits either nucleotide to promote β-galactosidase synthesis. Although CRP from wild-type cells binds both cyclic GMP and cyclic AMP, only the latter can promote enzyme synthesis.

M. Gallo, M. Gottesman, and I. Pastan (unpublished observations) have isolated a temperature-sensitive CRP mutant. The cells are able to ferment lactose at 30°C but are defective at 39°C. When cells maintained at 30°C are shifted to 39°C, there is a transient rise in β-galactosidase synthesis, followed by a decline with a half-life of approximately 10 min. The delay presumably reflects the half-life of functional CRP in the cell. This mutant strain should be useful in trying to establish whether or not all actions of cyclic AMP in *E. coli* are mediated by CRP.

The biochemical properties of wild-type CRP are discussed in the section, Cyclic AMP Receptor.

Direct stimulation of gene expression. How does cyclic AMP promote gene expression? Does it increase the synthesis of various mRNA's in the cell, does it increase the translation of mRNA into proteins, or does it do both? Measurements of the synthesis of inducible enzymes and the synthesis of corresponding mRNA show that cyclic AMP regulates the synthesis of mRNA for inducible operons. Nakada and Magasanik (65) estimated the concentration of *lac* mRNA by the capacity of the cells to synthesize β-galactosidase after removal of the inducer. They showed that glucose decreased the synthesis of this mRNA. Perlman and Pastan (83) showed that cyclic AMP addition restored the amount of *lac* mRNA made in glucose-grown cultures. *lac* mRNA concentrations, as measured by direct hybridization to *lac* DNA under various conditions, revealed a correlation between the rate of β-galactosidase synthesis and the rate of *lac* mRNA synthesis (112). Some of the results are shown in Table 2. By the use of a competition hybridization assay, Varmus et al. (113) showed that, in induced cells, the con-

TABLE 2. *Correlation of lac mRNA levels, lac mRNA synthesis, and β-galactosidase synthesis*[a]

Additions	*lac* mRNA levels	*lac* mRNA synthesis	β-galactosidase synthesis
None	4	7	1
IPTG	100	100	100
IPTG, glucose	10	9	14
IPTG, glucose, cyclic AMP	80	79	85

[a] *E. coli* 3.000 was grown in medium containing 0.5% glycerol, and was then incubated with 5×10^{-4} M IPTG; with IPTG and 0.25 M glucose; and with IPTG, glucose, and 3 mM cyclic AMP (taken from references 112 and 113).

centration of *lac* mRNA in *E. coli* was controlled by cyclic AMP. Both transient and permanent repression by glucose decreased *lac* mRNA production, and this decrease was reversed by cyclic AMP. Neither the adenylate cyclase mutant nor the CRP mutant synthesized *lac* mRNA, but the former regained the ability to do so if supplied with exogenous cyclic AMP. Cooper and Magasanik (21) have also found that both transient and permanent repression by glucose is due to specific inhibition of the rate of *lac* mRNA synthesis relative to the rate of formation of bulk cellular RNA.

Using the antibiotics rifampin, which specifically inhibits the initiation of transcription, and actinomycin D, which inhibits polymerization of RNA, Jacquet and Kepes (46) measured the time required for cyclic AMP to produce an effect on β-galactosidase synthesis that was resistant to inhibition by one of the two drugs. They suggested that cyclic AMP stimulates an early step similar to the rifampin-sensitive step of transcription.

The demonstration that cyclic AMP directly acts at the level of gene activity has been aided by the development of a cell-free system (S30) that catalyzes DNA-dependent synthesis of various inducible enzyme (18). In such systems, *lac, gal, cam,* and *ara* DNA templates promote the synthesis of *lac, gal, cam,* and *ara* enzymes, respectively (18, 25, 73, 123), and cyclic AMP stimulates the synthesis of all of these enzymes (Table 1). The action of cyclic AMP requires the presence of CRP, because the nucleotide fails to promote in vitro synthesis of the enzymes if the S30 is made from a *crp* mutant. Sample data from the *gal* system are shown in Table 3.

There have been reports that cyclic AMP stimulates *lac* gene expression by stimulating translation of *lac* mRNA (1). The evidence for this is indirect. Such a role of cyclic AMP seems to be ruled out by (i) the demonstration that isolated *gal* or *lac* mRNA, synthesized in the presence of cyclic AMP and CRP, can be translated efficiently by a S30 protein-synthesizing system without the cyclic nucleotide (92, 102); and (ii) the observation that a mutant *lac* operon (UV5) does not need cyclic AMP and CRP to promote β-galactosidase synthesis (104). The *lac* RNA from UV5 is normal in structure and does not require cyclic AMP for its translation.

TABLE 3. *Dependence of galactokinase synthesis on CRP and cyclic AMP*[a]

DNA	Cyclic AMP	CRP	Synthesized galactokinase (mU/ml)
λ	+	+	0
λ*galP*$^+$	−	−	40
λ*galP*$^+$	−	+	70
λ*galP*$^+$	+	−	80
λ*galP*$^+$	+	+	2320

[a] Protein synthetic mixtures contained S-30 prepared from a *crp* mutant strain (73).

Possible Role as a Negative Element

In the previous section, we discussed the experiments that indicated that cyclic AMP acts in *E. coli* as a positive regulatory element for turning on gene expression. Is that the only way cyclic AMP controls gene expression? Experiments of Prusiner et al. (89) indicate that the synthesis of some enzymes in *E. coli* may be under negative control by cyclic AMP. The five enzymes they studied are involved in the interconversions of glutamate and glutamine; they are glutamate dehydrogenase, glutamine synthetase, glutamate synthetase, glutaminase A, and glutaminase B. The levels of the first two enzymes are elevated in cells grown in glucose, whereas the levels of glutamate synthetase and glutaminase A are lowered by cyclic AMP. The level of glutaminase B remains unaffected. Both effects of cyclic AMP, stimulation and depression of enzyme synthesis, have been confirmed by the use of mutants defective in adenylate cyclase and CRP. A *cya-* or *crp*-defective mutant has decreased levels of glutamate dehydrogenase and glutamine synthetase and increased levels of glutamate synthetase and glutaminase A. Another enzyme system that is activated in CRP mutants is the adenylate cyclase system (see section, Adenylate Cyclase). CRP mutants have greatly increased levels of cyclic AMP (87, 116) due to enhanced adenylate cyclase activity. Perhaps cyclic AMP in combination with CRP represses the synthesis of a component of the adenylate cyclase complex. Since CRP in conjunction with cyclic AMP is known to work at the gene level, it is likely that both the positive and negative effects of cyclic AMP and CRP occur at this level. The negative control by cyclic AMP is new and worth further study, but this action of the nucleotide may be indirect. Cyclic AMP might be needed for the synthesis of a product which in turn represses the synthesis of glutamate synthase, glutaminase A, and a component of the adenylate cyclase system. A definite answer to this type of question can be obtained by studying the synthesis of these enzymes in cell-free extracts.

Another indication of a putative negative control of gene activity by cyclic AMP has come from the study of the *gal* operon of *E. coli*. Among five so-called promoter-defective *gal-*

negative mutants of this operon, two, P_{211} and P_{83}, become *gal* positive in either a *cya* or *crp* mutant background (S. Adhya, in preparation). In these double mutants, somewhat increased levels of *gal* enzymes are made constitutively compared with the low levels made by promoter-defective mutants alone. The addition of cyclic AMP restores the *gal*-negative phenotype in the *cya* mutant cells, but not in the *crp* mutant cells. Curiously, mutation in another gene, *lon*, the product of which is unknown, also partially suppresses the same promoter mutations. However, mutations in *cya*, *crp*, or *lon* do not suppress other *gal* promoter mutants. The relationships between cyclic AMP, CRP, *lon* gene product, and the suppressible *gal* promoter mutations raise the possibility that there exists an additional cyclic AMP-mediated control of *gal* operon expression in which cyclic AMP has a negative action. *lon* mutants have a pleiotropic phenotype and overproduce the enzymes of capsular polysaccharide synthesis (61).

Does Cyclic AMP Have Other Actions in *E. coli*?

In *E. coli*, all actions of cyclic AMP that have been examined in detail are at the level of gene activity. In animal cells, cyclic AMP has been found to have a wide variety of effects and many of these do not involve regulation of gene activity. Instead, cyclic AMP directly controls enzyme activity by promoting the phosphorylation of preformed proteins (95). An early report that *E. coli* contained a cyclic AMP-dependent protein kinase (52) suggested that the nucleotide might have similar actions in *E. coli*. However, subsequent studies in our laboratory and others have failed to confirm the presence of this enzyme in *E. coli* (see below). In addition, cyclic AMP can stimulate *lac* and *gal* transcription when ATP is replaced by the analogue adenylyl imidodiphosphate (App-NH-p) (77a). The latter is not a substrate for protein kinase but will support RNA synthesis. This result makes it clear that protein phosphorylation is not required for cyclic AMP and CRP to promote transcription.

Protein kinase is usually assayed by measuring the transfer of the gamma phosphate of [γ-^{32}P]ATP to histone. *E. coli* contains an enzyme, polyphosphate kinase, that converts the gamma phosphate of ATP into long-chain polyphosphate which, like histone phosphate, is insoluble in trichloroacetic acid. Further, the activity of polyphosphate kinase is strongly stimulated by histone (52a). It seems likely that the activity originally ascribed to protein kinase (52, 87a) was really due to polyphosphate kinase (52a; D. Powers and A. Ginsburg, personal communication).

In an attempt to analyze how cyclic AMP activates gene expression in animal cells, Tompkins and co-workers isolated mutant lymphoma cells refractory to cyclic AMP action (reviewed in reference 39). In wild-type lymphoma cells cyclic AMP induced the synthesis of the enzyme cyclic AMP phosphodiesterase, whereas in mutant cells the enzyme could not be induced. When extracts of the mutant cells were examined, they were found to be deficient in cyclic AMP-dependent protein kinase activity. Thus, even enzyme induction in animal cells appears to require cyclic AMP-catalyzed phosphorylation. It seems likely that the activity of chromatin-associated proteins is controlled by phosphorylation.

OPERON ACTIVATION BY CYCLIC AMP

Of all the biological roles ascribed to cyclic AMP, gene activation in *E. coli* has been studied in most detail. The existence of a cyclic AMP-dependent generalized positive control system for the activation of a number of genes, in addition to the existence of specific control systems for each of these, enables the organism to repress the expression of unnecessary genetic information. The key to elucidating the mechanism of cyclic AMP action has been due to a successful fusion of biochemical and genetic techniques. The detailed information now available raises interesting possibilities about the molecular mechanism of control of gene expression.

In 1961, Jacob and Monod proposed the "operon" model of gene expression (45). An operon is a segment of DNA consisting of two or more contiguous genes subject to joint control through one region, the promoter-operator (*p-o*) complex, located at one end of the unit. The promoter site determines where and how transcription is initiated, and the operator locus is the site of repressor action (45, 99). An operon is expressed as one unit of transcription into a polycistronic message, which is then translated into cistron-specific proteins.

The *gal* and *lac* operons of *E. coli* are prototypes for operons activated by cyclic AMP. Cyclic AMP activates both operons by the same mechanism. Cyclic AMP acts at the level of initiation of transcription. This is based on the following evidence.

(i) Cyclic AMP deficiency, caused by growing cells in glucose-containing medium, causes severe reduction of *lac* operon expression. This effect is overcome by addition of cyclic AMP

(81). However, in a cell containing a point mutation in the *lac* promoter (mutation L8) in which the residual expression of the operon is still subject to the glucose effect, a 10-fold higher concentration of cyclic AMP is needed to overcome the repression (80). Further, in a cell with a deletion mutation of the *lac* promoter (L1), the 2% residual level of *lac* expression is unaffected by lowering cyclic AMP levels by the addition of glucose (80, 105). These findings indicate that the *lac* promoter is the site at which cyclic AMP acts. Since the promoter locus determines where transcription of an operon begins, the cyclic nucleotide must act at the level of transcription.

(ii) Induction of cyclic AMP-dependent operons is inhibited by acridine orange, a substance known to intercalate between the bases of the DNA helix. This inhibition is overcome by cyclic AMP. However, a *lac* promoter mutant, in which the basal level of expression is independent of the cyclic nucleotide, is resistant to inhibition by acridine orange. In this mutant cell, the induction of other cyclic AMP-dependent operons is still sensitive to the intercalating drug (98).

(iii) More definitive evidence about the role of cyclic AMP has come from in vitro studies. These have shown that cyclic AMP and CRP act at the level of transcription of an operon and they are required at the initiation step for making *gal* and *lac* operon-specific transcripts (24, 70). A purified system for the transcription of *gal* and *lac* operons has been developed. The required components for the transcription of the two operons are identical. They are *gal* or *lac* DNA; RNA polymerase holoenzyme (core enzyme + sigma factor); cyclic AMP; CRP; and uridine 5'-triphosphate, cytidine 5'-triphosphate, guanosine 5'-triphosphate, and ATP.

One of the nucleoside triphosphates is radioactive to monitor RNA synthesis by measuring the amount of radioactivity incorporated into the latter. It should be noted that any other components that might play a further role in regulation of *gal* or *lac* transcription would be removed during purification of RNA polymerase and CRP. In vitro *lac* transcription is known to be further stimulated by factor H (22) or TFppGpp in the presence of ppGpp (2).

The *lac* and *gal* DNA templates used in the purified system are DNA of λ*lac* or λ*gal* transducing phages. Obviously these DNA preparations additionally contain phage genes with phage-specific promoters. The *lac*- or *gal*-specific RNA made from these templates in vitro is measured by selective hybridization with separated stands of λ*lac* or λ*gal* DNA. Usually phage-specific RNA, also made in the system, is removed in a prehybridization step with phage λ DNA that does not contain *lac* or *gal* genes. The specificity of the system can be determined by using DNA as template carrying various types of promoter mutations, and the results can be compared with the in vivo properties of the mutants. An example of the in vitro *gal* transcription system is shown in Table 4.

When a *gal* DNA template is incubated with RNA polymerase holoenzyme, λ genes are transcribed but *gal* genes are not. The addition of cyclic AMP alone does not promote *gal* transcription. However, when cyclic AMP is present along with homogeneous CRP, transcription of the *gal* operon is activated (70). Similar results are obtained when the DNA template is the *lac* operon present as transducing phage DNA (24). These results prove conclusively that cyclic AMP acts at the level of transcription. That the stimulation of *gal* mRNA synthesis by cyclic AMP and CRP is from the *gal* promoter, and not from some neighboring phage promoter, was demonstrated with a *gal* DNA template carrying promoter-defective mutations (66). With those templates there was 25-fold less *gal* RNA made than with a wild-type template.

In vitro studies have shown that in both the *lac* and *gal* systems, cyclic AMP and CRP acting together are required for RNA polymerase to form an open complex (also known as preinitiation or rapidly initiating complex) with DNA at the *lac* or *gal* promoter. This complex is formed in the absence of nucleoside triphos-

TABLE 4. *Stimulation of gal transcription by cyclic AMP and CRP*[a]

Template	Additions	Total [³H]RNA cpm (×10³)	*gal*-specific [³H]RNA cpm (×10³)
galP⁺	None	114	4
galP⁺	CRP, cyclic AMP	363	93
galP⁻	CRP, cyclic AMP	217	3

[a] The reaction mixture for the synthesis of RNA contained 23 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.9); 15 mM $MgCl_2$; 100 mM KCl; 0.1 mM sodium ethylenediaminetetraacetic acid; 0.1 mM dithiothreitol; 0.15 mM ATP, UTP, and GTP; 0.075 mM [³H]CTP (specific activity, 11.7 Ci/mmol); 45 μg of λ*gal* DNA per ml and 23 μg of RNA polymerase per ml. When added, CRP concentration was 8 μg/ml and cyclic AMP concentration was 0.17 mM (taken from reference 70 and P. Nissley, unpublished data). The *galP*⁻ mutation used was P_{211}.

phates, and *gal* or *lac* mRNA is made after addition of nucleoside triphosphates together with rifampin. The latter rapidly inactivates free RNA polymerase (reviewed in reference 17). When the addition of cyclic AMP and CRP is delayed and these are then added with rifampin, no *gal* or *lac* RNA is made. These results strongly imply that the role of cyclic AMP and CRP is to effect the formation of the RNA polymerase-promoter open complex, i.e., the action of cyclic AMP is at the promoter level.

What is the role of cyclic AMP and CRP in the formation of the complex? CRP, in the presence or absence of cyclic AMP, does not bind to RNA polymerase (69). Rather, the cyclic AMP-CRP complex appears to recognize and bind to *gal* and *lac* DNA and "promote" or "stabilize" RNA polymerase binding. Although CRP, in the presence of cyclic AMP, binds to every type of DNA tested (69, 94), higher-affinity binding to the *lac* promoter site has been demonstrated (59). CRP in the presence of cyclic AMP binds to a DNA fragment (generated by a restriction nuclease) carrying the *lac* promoter-operator region. The specific binding is only observed at a low CRP-to-DNA ratio. Under similar conditions, CRP does not bind to DNA carrying the L8 promoter mutation. The L8 mutation site seems to have lost CRP recognition (see discussion below). A cyclic AMP-dependent promoter locus must then contain, besides an RNA polymerase recognition element, additional information for recognition of the cyclic AMP-CRP complex. The cyclic AMP-dependent operons may not have identical base sequences in their cyclic AMP-CRP recognition site, because the maximal rate of expression varies from one operon to another, and because different concentrations of cyclic AMP are needed for optimal expressions of different operons, both in vivo and in vitro (2a, 54, 86a).

Genetic analysis of the *lac* promoter. In an extensive genetic analysis of the promoter region of the *lac* operon, the various promoter mutations have been found to fall into three distinct classes (6, 43).

Class I mutations result in reduced β-galactosidase synthesis and map in the operator-distal part of the promoter region (site I).

Class II mutations also result in reduced levels of β-galactosidase synthesis but cluster in the operator-proximal part (site II·III).

Class III mutations cause increased synthesis of β-galactosidase in a *crp* or *cya* background or in a strain carrying a class I promoter mutation. Their map position overlaps with the group II promoter-defective mutations (Fig. 2).

Group I mutants with an expression of 2 to 6% of wild type still made β-galactosidase at a 2% level in a *crp·cya* background. This is identical to the amount of expression of a wild-type promoter in a *crp cya* strain, suggesting an interaction between cyclic AMP-CRP and the operator-distal region of the promoter. Cells

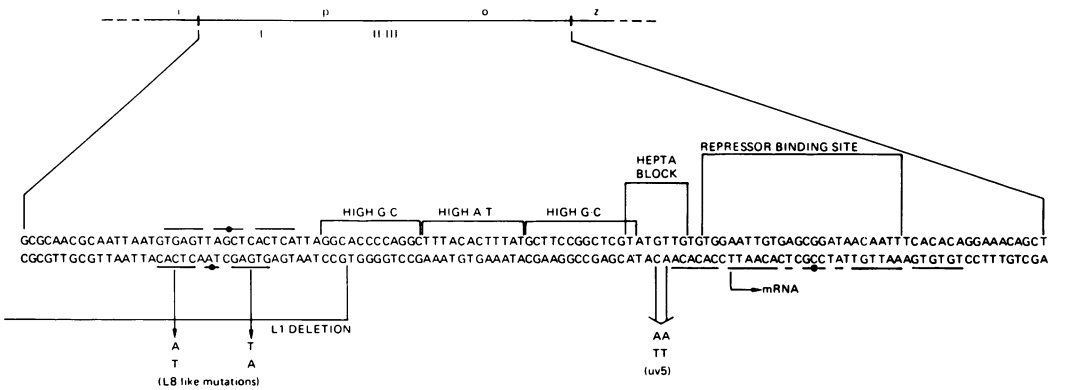

FIG. 2. *Genetic map and nucleotide sequence of the promoter-operator region of the lac operon (27, 35, 43). The promoter locus, p, located between the lac repressor gene, i, and the operator, o, is subdivided into sites I and II·III. Site I is the cyclic AMP and CRP recognition element and carries two overlapping partial twofold symmetry elements indicated by horizontal lines. Point mutations, like L8, which affect cyclic AMP and CRP recognition, always show change of either of two G-C nucleotide pairs located in symmetrical positions in one of the symmetry elements into an A-T pair (W. Reznikoff, personal communication). This suggests that the cyclic AMP·CRP complex "sees" this DNA symmetry. Promoter site II·III contains the A-T-rich block, spanned by two G-C-rich blocks, as well as the heptanucleotide block, the possible implications of which are discussed in the text. The mutation, UV5, to cyclic AMP and CRP-independent promoter affects the hepta block. The operator locus, the repressor-binding site, also contains a partial twofold symmetry, indicated by horizontal broken lines. The horizontal arrow indicates the start of lac transcription.*

with mutations in this region, L8 and L1, were originally found to be resistant to glucose repression in vivo (80, 105), and DNA with the L8 mutation has lost CRP binding in vitro (59). The L1 mutation is a deletion that encompasses the mutational sites of all others in this group.

The group II mutants, in contrast, have lower levels of *lac* operon expression in a *crp·cya* strain than in a wild-type strain. This result suggests that class II mutations are in the RNA polymerase interaction site. Thus, there are two essential sites in the promoter region: the operator-distal part is the site of recognition by cyclic AMP and CRP, and the operator-proximal locus is the RNA polymerase interaction site. The notion of two separate interaction sites in the promoter drew support from the behavior of class III mutations. A similar subdivision of the *lac* promoter region was suggested from earlier in vitro *lac* transcription studies (19).

If site II·III is the binding site of RNA polymerase, then it seems likely that in the absence of cyclic AMP, CRP, or the CRP recognition site (site I), RNA polymerase can bind to its normal recognition site and initiate transcription at a low rate, perhaps 2% of normal. If the RNA polymerase recognition site is altered by a mutation of type III, the enzyme can then transcribe the *lac* operon effectively even in the absence of an interaction of cyclic AMP and CRP with site I. In this model, the role of cyclic AMP and CRP is to enhance the low level of initiation of transcription by RNA polymerase from site II·III. This enhancement is mediated by a direct recognition of site I by cyclic AMP and CRP. Therefore, they are positive effector molecules for operon expression. A number of molecular models about the steps of in vitro initiation of transcription from cyclic AMP-independent promoters have been discussed. In one model, Chamberlin (17) has proposed that RNA polymerase collides with and binds to many non-specific sites on DNA. This interaction between the reactive groups of DNA and RNA polymerase is weak and in rapid equilibrium. The next step is the selective binding of RNA polymerase to the promoter locus. The resulting complex is referred to as an inert complex.

The step that signals the initiation of transcription is the conversion of the inert complex to an open complex form, also known as a preinitiation complex. This complex is stable. Its formation is facilitated by elevated temperatures and low ionic strength and is believed to occur without dissociation of the RNA polymerase from the inert complex (reviewed in reference 17).

The following evidence supports the idea that melting or a change in the conformation of the promoter region is involved in the formation of an open complex at the *gal* promoter. Dimethyl sulfoxide, glycerol, and ethylene glycol, all of which lower the T_m of DNA, stimulate *gal* transcription in the absence of cyclic AMP and CRP when added to a purified transcription system containing RNA polymerase holoenzyme. The stimulatory effect is proportional to the concentration of glycerol up to 20% (66). Glycerol stimulates *gal* transcription by promoting the formation of rifampin-resistant open complex. The formation is strongly dependent on temperature. At lower temperatures, complex formation requires increased glycerol concentrations.

Glycerol seems to act at the same site as cyclic AMP and CRP because (i) transcription factor sigma, which is required for the stable binding of RNA polymerase to a promoter, is also required for the glycerol effect; and (ii) at saturating concentrations of cyclic AMP and CRP where the *gal* promoter is maximally activated, the addition of glycerol has little further effect on *gal* transcription. Moreover, glycerol stimulates *gal* transcription from *gal* templates containing two different promoter mutations. The mutant DNAs are not transcribed in the presence of cyclic AMP and CRP. Glycerol can also replace the requirement of cyclic AMP and CRP for *lac* transcription in vitro (22).

Another condition that promotes the formation of open complexes at the *lac* and *gal* promoters is the absence of Mg^{2+} (67), but this only occurs when the ratio of RNA polymerase to DNA is high. The formation of an open complex at the *lac* or *gal* promoter in the absence of Mg^{2+}, as with glycerol, is faster at higher temperatures. The temperature dependence profile of open complex formation, whether occurring in the presence of glycerol or cyclic AMP and CRP, or in the absence of Mg^{2+}, resembles a DNA melting curve. The presence of cyclic AMP and CRP in the absence of Mg^{2+} lowers the transition temperature required for complex formation without affecting the maximal number of complexes formed.

The formation of open complexes is favored by conditions that are known to disrupt the ordered structure around a double helix, e.g., presence of dimethyl sulfoxide or glycerol, higher temperature, or absence of Mg^{2+}. These observations strongly support the notion that localized melting or alteration of the secondary structure of the promoter is a prerequisite for the formation of an open complex at the *lac* and *gal* promoters. The role of cyclic AMP and CRP in the formation of the complex is to melt or change promoter conformation, so that the in-

ert RNA polymerase DNA complex is converted into a rapidly initiating open complex.

The nucleotide sequence of the entire promoter-operator region of the *lac* operon has been determined (35, 27). The promoter and operator segments of this sequence have been inferred by analyzing the sequence changes in this region induced by various types of promoter and operator mutations. This region contains about 122 nucleotide pairs which include (i) the recognition and binding site of *lac* repressor, the operator; (ii) the recognition and binding site for RNA polymerase, site II·III; and (iii) the recognition and binding site for the cyclic AMP-CRP complex, site I. The entire sequence is reproduced in Fig. 2.

(i) Operator. The region protected by the *lac* repressor from nuclease digestion is indicated in Fig. 2 as the operator site. This region, contains all the O^c mutations sequenced. Interestingly enough, the operator is distinguished by a region of partial twofold rotational symmetry, which could possibly form a looped Gierer structure with potential for protein recognition (33). Such a structure is, however, not present when repressor binds to operator (114). Whether the structure exists in the absence of repressor is not known.

(ii) Promoter–site II·III. The segment to which RNA polymerase binds and initiates transcription is approximately 46 nucleotide pairs long and contains the nucleotide changes produced by two class II and one class III promoter mutations genetically inferred to be alterations at the site of RNA polymerase action, as discussed above. Significant features of this region are: (a) It contains a block of high adenosine-thymine (A-T) content, spanned by two guanine-cytosine (G-C)-rich blocks. The class II promoter mutations map in these G-C-rich blocks, suggesting a role of this block in interaction with RNA polymerase. (b) The region is not protected by RNA polymerase from nuclease digestion. The protected region overlaps with the transcriptional initiation site, as defined by the 5′ end of *lac* mRNA. This RNA polymerase protected region seems to be the site of tight binding (presumably the site of the open complex) needed for initiation of transcription. Since the RNA polymerase entry site is not coincident wth the tight-binding initiation site, RNA polymerase must drift through the region located in between.

(iii) Promoter–site I. The region of cyclic AMP and CRP action is defined by the L1 deletion mutation, and is located upstream from the RNA polymerase region. This segment carries two overlapping partial twofold symmetry elements indicated by horizontal lines. Point mutations, like L8, which affect cyclic AMP and CRP recognition, always show change of either two G-C nucleotide pairs located in symmetrical positions in one of the symmetry elements into an A-T pair (W. Reznikoff, personal communication). This suggests that the cyclic AMP-CRP complex "sees" this DNA symmetry.

Knowing the nucleotide sequence of the *lac* operator-promoter region, Dickson et al. (27) have proposed the following model for initiation of transcription. The A-T-rich block, because of its inherent low transition temperature, is where RNA polymerase enters the DNA. But this block is bounded on both sides by G-C-rich blocks, which would tend to raise the transition temperature of the A-T-rich block, thus inhibiting RNA polymerase entry and formation of an open complex. G-C sequences are known to have a strong stabilizing effect on A-T sequences (16). Dickson et al. (27) suggest that the binding of cyclic AMP and CRP to site I destabilizes the adjacent G-C-rich regions.

Some of the promoters that utilize *E. coli* RNA polymerase have been sequenced. A similar heptanucleotide block is present in all of these (88). This block is located a few nucleotides before the nucleotide where transcription is initiated and is within the region protected by RNA polymerase from nuclease digestion. The prototype sequence of the heptanucleotide block is ATAPyTAC (88). Schaller et al. (100) and Pribnow (88) have implicated this sequence in the formation of a tight binary complex with RNA polymerase. The same block in *lac* has a sequence ATACAAC, with an A replaced by T. A class III promoter mutation, UV5, was isolated as a second site revertant of a class I mutant, L8. The UV5 mutation partially increases *lac* transcription, and the synthesis of β-galactosidase in the absence of either cyclic AMP and CRP or site I (32, 43). Interestingly, UV5 shows two nucleotide changes in the hepta-block resulting in the prototype sequence ATATTAC (quoted in reference 88). How the UV5 mutation makes *lac* transcription independent of cyclic AMP and CRP will be discussed below. Nakanishi et al. (66, 67) have presented evidence that cyclic AMP and CRP most likely act by destabilizing the DNA helix. This property of cyclic AMP and CRP could promote transcription initiation by increasing the formation of open complexes at the A-T-rich entry site. Cyclic AMP and CRP presumably recognize and interact with site I and then alter the structure of DNA, destabilizing the G-C clusters surrounding the A-T block. This model is supported by data obtained in studies on the *gal* operon. *Gal* operon expression is less de-

pendent on cyclic AMP than *lac* operon expression (62). The *gal* promoter region has an A-T-rich block, but the block is not flanked by regions of high G-C content (R. Musso, R. DiFarro, M. Rosenberg, B. de Crombrugghe, J. Sklar, and S. Weissman, in preparation). Thus, the *gal* promoter may be more readily melted than the *lac* promoter. β-glucuronidase is an enzyme whose dependence upon cyclic AMP is much more stringent than β-galactosidase (71; G. Novel, reviewed in reference 108). An extremely leaky mutation in *crp* has been identified that makes the cell defective for β-glucuronidase but not for β-galactosidase. The defect can be corrected by the addition of a high concentration of cyclic AMP. These findings could be explained if relatively higher concentrations of cyclic AMP-CRP complex are needed to activate the β-glucuronidase promoter than the *lac* promoter. We suppose this behavior may be due to an extremely high G-C content in the β-glucuronidase promoter region that prevents melting of the A-T cluster.

If the role of cyclic AMP and CRP is to destabilize the double helix around the A-T block, then how is this need alleviated by changes in the sequence of a heptanucleotide block located near the initiation region some 32 nucleotides away from site I?

Cyclic AMP and CRP enhance the conversion of inert (see Fig. 3) to open complexes by interacting with site I. We propose that the hepta-block, the site at which open complexes are found, influences the equilibrium between inert and open complexes. With the wild-type *lac* promoter, the equilibrium lies toward the inert complexes. The base changes in the hepta-block, caused by the UV5 mutation, shift the equilibrium toward the open complexes by increasing the affinity of RNA polymerase for this site. Suppose 2% of the inert complexes normally gets converted to open complexes in the absence of cyclic AMP and CRP. The number of open complexes can be enhanced either by increasing the number of inert complexes by cyclic AMP and CRP or by shifting the equilibrium toward the open complexes.

CONTROL OF CYCLIC AMP LEVELS

A considerable effort has gone into determining the concentration of cyclic AMP in *E. coli* to determine if the intracellular concentration correlates with the rate of synthesis of β-galactosidase and other enzymes subject to cyclic AMP control. Before discussing the results of these studies, it is necessary to review briefly the methods used in measuring cyclic AMP and in collecting cells for analysis of the nucleotide.

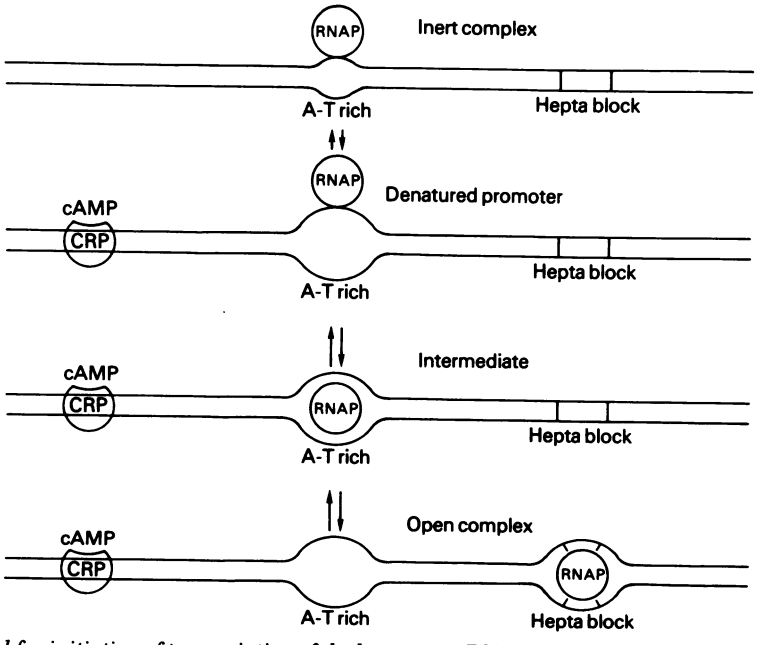

FIG. 3. *Model for initiation of transcription of the lac operon. RNA polymerase forms an initiation complex at the A-T-rich block. Cyclic AMP-CRP complex interacts at a different site (covered by L1 deletion; see Fig. 2) to facilitate melting of the A-T-rich block, spanned by two G-C-rich blocks, thus helping the coversion of the inert complex of the polymerase into an open complex with DNA at the region containing the heptanucleotide block. There is an equilibrium between the two complexes. The equilibrium is determined by the affinity of the polymerase toward the nucleotide sequence of the heptanucleotide block.*

The levels of cyclic AMP in *E. coli* and other cells are approximately 10^{-6} M, much lower than those of ATP and other small molecules whose levels are frequently measured. In addition, cyclic AMP is similar in many of its chemical and physical properties to other adenine-containing nucleotides. Therefore, highly sensitive and specific assays had to be developed to measure the cyclic nucleotide. There are two types of assays currently in use. Both are competitive binding assays and take advantage of the fact that certain proteins bind cyclic AMP with high affinity and considerable specificity. One such protein is a cyclic AMP-dependent protein kinase that can be isolated from beef muscle or other animal tissues (36). The other is an antibody that can be readily prepared against cyclic AMP after the nucleotide is coupled to a suitable protein by a succinyl bridge (107). The sensitivity of the antibody assay method can be further increased by converting the unknown cyclic AMP samples to a succinyl or acetyl derivative (40).

The first assays of cyclic AMP were by Makman and Sutherland (60) who collected their cells by centrifugation. Collecting cells by this method imposes a severe physiological stress for a considerable period of time during which cyclic AMP levels could change. Thus it seemed desirable to collect the cells quickly. To accomplish this, filtration through nitrocellulose filters was subsequently employed. A second complication arises because cells release cyclic AMP into the growth medium. If the cells are grown in a liquid medium and collected on filters, the medium retained on the filter can contain more cyclic AMP than the cells, and the medium must be rapidly washed away after the cells are collected. If a large number of cells are concentrated on a filter, the magnitude of contamination by the medium may be negligible. In any event, the amount of cyclic AMP present in the medium should be determined and if the value is large, a suitable correction should be made. As we shall see, various investigators have handled this problem in different ways.

In our own laboratory we began by collecting cells on nitrocellulose filters. Low flow rates were improved by placing a glass-fiber filter under the nitrocellulose filter to prevent adherence of the nitrocellulose filter to the glass support. We determined cyclic AMP levels on 10-ml samples of cells at the mid-log phase of growth and found the level to be about 5 μM in *E. coli* cells (strain 1100) growing exponentially on glycerol (75). Five minutes after the addition of glucose, the level fell to 0.5 μM, and after pyruvate or α-methyl glucoside, the concentration fell to 1.5 μM (75; see also Table 5). Contamination by medium was not a serious problem, because a sufficient number of cells was collected to reduce medium contamination to a low value.

Subsequently, Buettner et al. (15) reported cyclic AMP levels to be very high in strain AB257: 50 μM in cells growing on succinate, 43 μM on glycerol, and 12 μM on glucose. Although these levels are high, their relative order was consistent with the proposal that cells growing on glucose have a decreased rate of β-galactosidase synthesis because of low cyclic AMP levels. However, Buettner et al. (15) failed to observe a sufficient drop in cyclic AMP levels shortly after glucose was added to cells growing on glycerol to account for a complete arrest of β-galactosidase synthesis. Therefore they concluded "that transient repression could not be explained on the basis of a drastic lowering of the cellular concentration of cyclic AMP." It now appears that Buettner et al. (15) failed to recognize that the cyclic AMP content of the medium trapped on the filters greatly exceeded the cyclic AMP of the cells. Their failure to observe a drop in the cyclic AMP content of glycerol-grown cells upon the addition of glucose can probably be accounted for by the fact that the large amount of cyclic AMP in the glycerol culture fluid obscured the change in intracellular levels.

More recent studies have carefully assessed the contribution of extracellular cyclic AMP. Wayne and Rosen (116) collected cells on membrane (Millipore Corp.) filters and washed the cells with the appropriate prewarmed growth medium to remove cyclic AMP-containing medium before extraction of the nucleotide. In strain 23804, transient repression correlated with a drop in cyclic AMP from 14 μM before glucose to 2 μM shortly after its addition. In strain 1100, a less dramatic fall was also observed: 5.4 to 4.0 μM. During prolonged growth on glucose, the levels of cyclic AMP in strain 23804 were 9 μM, whereas during growth on glycerol the levels were 19 μM. These findings support the notion that cyclic AMP is responsible for the glucose effect. However, Wayne and Rosen failed to observe a sigificant difference in cyclic AMP levels in strain 1100 when growth on glycerol and glucose were compared. Since cyclic AMP levels in strain 1100 varied over a wide range (for example, 2.8 to 9.8 μM when grown on glucose), unforseen technical problems with the assay may have arisen. More recently Epstein et al. (31) have investigated cyclic AMP levels in a number of different strains including X9250, 1100 used by Perlman and Pastan (75) and Wayne and Rosen (116), and AB 257 used by Buettner et al. (15). They

TABLE 5. *Cyclic AMP concentrations in E. coli* (μM)

Source	*E. coli* 1100			*E. coli* AB257			*E. coli* X8251			*E. coli* 23804		
	Glu[a]	Glyc	Tran	Glu	Glyc	Tran	Glu	Gly	Tran	Glu	Glyc	Tran
Pastan et al. (75)	1.5	5.0	0.5									
Buettner et al. (15)				12	43	~10						
Wayne and Rosen (116)	2.9-9.8	1.4-6.2	4.0							4-19	13-24	2
Epstein et al. (31)	0.4	1.5	0.2	0.5	2.7	0.2	0.6	1.7	0.4			

[a] Glu, Cells grown on glucose; Glyc; cells grown on glycerol; Tran, transient repression.

collected their cells in a manner similar to Wayne and Rosen but purified the cyclic AMP more extensively before assay. Apparently this purification reduces the blank values and decreases variation. When strain X9250 was grown on 13 different carbon sources, there was a very good correlation between cyclic AMP levels and β-galactosidase synthesis on 12 of these substrates; these included glucose, glycerol, and succinate (Fig. 4). Only in xylose-grown cells was the correlation poor. Epstein et al. (31) also observed that strains 1100 and AB257 had lower levels of cyclic AMP when grown on glucose than when grown on glycerol. Some of the data from the various studies described are summarized in Table 5. All in all, the view that transient and permanent repression are mediated via lowered levels of cyclic AMP seems to be well supported.

To understand how the levels of cyclic AMP are controlled, studies have been performed on the activities of the enzymes adenylate cyclase and cyclic AMP phosphodiesterase and also on the mechanism by which cyclic AMP is released from the cell into the medium.

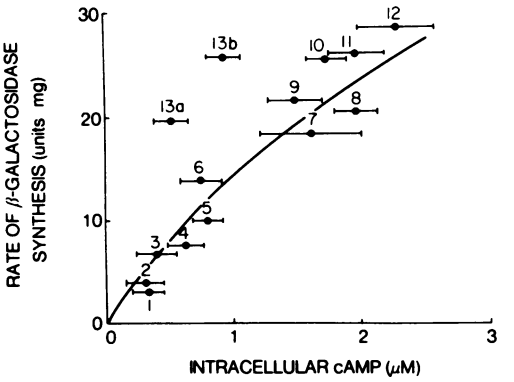

FIG. 4. *Relationship of the rate of β-galactosidase synthesis to intracellular concentration of cyclic AMP in strain X9250. Numbers refer to the carbon source as follows: 1, glucose-6-phosphate; 2, glucose + Casamino Acids; 3, glucose + gluconate; 4, glucose; 5, mannitol; 6, gluconate; 7, fructose; 8, lactate; 9, succinate; 10, glycerol; 11, Casamino Acids; 12, glucosamine; 13a and 13b, xylose (two separate cultures). The curve is based on a linear fit to a double reciprocal plot of the data excluding that for xylose; it does not differ significantly from a straight line through the origin. One unit of β-galactosidase produces 1 μmol of products per min at 37°C. Figure from reference 31.*

Adenylate Cyclase

In all organisms so far studied, cyclic AMP is synthesized from ATP by adenylate cyclase as follows:

$$ATP \rightarrow cAMP + PP_i$$

The conversion of ATP to cyclic AMP is readily demonstrated in extracts of *E. coli*, but the formation of pyrophosphate has not been established.

The presence of adenylate cyclase in *E. coli* was reported from a number of laboratories in 1969 (10, 44, 82, 110). In cell extracts prepared either by sonic disruption or in a French press, a large proportion of the adenylate cyclase activity was found associated with the particulate fraction. The enzyme is readily extracted in a soluble form without the use of detergents and has been purified about 100-fold (109). Using this partially purified material some characteristics of the enzyme have been investigated (109, 110). The protein appears to have a molecular weight of about 110,000. It requires Mg^{2+} for enzymatic activity, with maximal activity expressed at 8 mM Mg^{2+} or higher. The K_m for ATP is 4.5×10^{-4} M. GTP was observed to be a competitive inhibitor of adenylate cyclase with a K_i of 4.2×10^{-4} M. This value is close to the K_m for ATP. Since the partially purified preparation of adenylate cyclase was able to form cyclic GMP from GTP, it was suggested that the same enzyme might be able to form cyclic AMP and cyclic GMP. However, more recent studies suggest that adenylate cyclase and guanylate cyclase are distinct entities. Macchia et al. (57) have purified guanylate cyclase from extracts of *E. coli*. This enzyme does not catalyze the synthesis of cyclic AMP from ATP. Further guanylate cyclase activity is detected in increased amounts in a mutant of *E. coli* deficient in adenylate cyclase (57).

Since glucose and other sugars lower cyclic AMP levels in vivo, a variety of carbohydrates have been tested to see if they inhibit adenylate

cyclase activity in cell-free extracts (44, 86, 110). Both the particulate enzyme and partially purified soluble enzyme were utilized. Most of the substances tested including glucose were inactive, although oxaloacetate at concentrations in excess of 1 mM was inhibitory (44).

The presence of adenylate cyclase in the particulate fraction of the cell suggests that the adenylate cyclase of *E. coli* may be associated with the bacterial cell membrane. It is of interest that in animal cells adenylate cyclase is characteristically located in the plasma membrane (reviewed in reference 76). The animal cell enzyme appears to be a lipoprotein and can only be solubilized with detergents. There is no evidence that the *E. coli* enzyme is a lipoprotein.

Cyclic AMP Phosphodiesterase

The first demonstration of cyclic AMP phosphodiesterase in *E. coli* was by Brana and Chytil (11, 12). One peculiarity they observed was that the enzyme was only fully active in the presence of very high concentrations (0.2 M) of $MgSO_4$. These high concentrations suggested that the $MgSO_4$ might contain trace quantities of another metal that was the true activator. Using partially purified phosphodiesterase, Perlman and Pastan (84) found that quite low concentrations of ferrous ion activated the enzyme and that this activation was inhibited by *o*-phenanthroline and α,α-dipyridyl, reagents that chelate Fe^{2+}. Monard et al. (63) reported that glucose-6-phosphate and other intermediates of carbohydrate metabolism stimulated phosphodiesterase activity when the enzyme was measured in crude extracts of *E. coli*. To explain this stimulation, Perlman and Pastan (84) suggested that the ferrous ion was being kept in a reduced state in cell-free extracts by the oxidation of these carbohydrates. Subsequently, Nielsen et al. (68) investigated the properties of the phosphodiesterase in more detail and confirmed the activation by Fe^{2+}. They also found that the enzyme had an apparent molecular weight of 28,000, a sharp pH optimum of around pH 6.8 to 7.0, and a K_m for cyclic AMP of about 0.5 mM. In contrast to the phosphodiesterases of animals cells, the phosphodiesterase of *E. coli* is not inhibited by methylxanthines (68). However, dipicolinic acid does decrease enzyme activity, apparently by chelating ferrous ion (72).

The presence of phosphodiesterase is not essential for glucose to lower cyclic AMP levels and produce glucose repression. For example, Crooke's strain, employed by Makman and Sutherland (60) in their initial demonstration that glucose lowered cyclic AMP levels, does not contain detectable phosphodiesterase activity (68). In addition, Rickenberg and co-workers (63) have isolated a mutant of *E. coli* K-12 partially resistant to catabolite repression. This mutant has diminished phosphodiesterase activity but still contains more activity than Crooke's strain, in which no phosphodiesterase activity can be detected. It is possible that the presence of phosphodiesterase assists glucose and other sugars to lower cyclic AMP levels.

Cyclic AMP Release

One of the striking findings by Makman and Sutherland (60) was that shortly after the addition of glucose to cells containing a high cyclic AMP concentration, the nucleotide fell to low levels and the fall was accompanied by accumulation of the cyclic nucleotide in the medium. They suggested that glucose acted by preventing cyclic AMP synthesis and promoting its secretion into the medium. Later Peterkofsky and Gazdar (86) measured cyclic AMP accumulated in the media of cells grown to stationary phase in glucose, glycerol, and succinate and found that glucose-grown cells accumulated less nucleotide than glycerol-grown cells, which in turn accumulated less than succinate-grown cells. Their data indicated that in growing cells glucose did not stimulate the release of cyclic AMP but only inhibited its synthesis. The experiments of Makman and Sutherland were done principally on glucose-starved, washed, nongrowing cells. Later studies by Rosen and co-workers (115, 116) showed that the rate of cyclic AMP release was constant during growth and confirmed that cells growing in glucose secreted cyclic AMP at a lower rate than cells in glycerol. Epstein et al. (31) have measured cyclic AMP secretion in growing cells with differing cyclic AMP levels. They found that the secretion rate was a linear function of the intracellular concentration. All these results together indicate that in growing cells the intracellular levels of the nucleotide are regulated by its rate of synthesis and not by its release into the medium.

Rate of Cyclic AMP Synthesis

The inability of various investigators to demonstrate an inhibitory effect of glucose or its metabolites on adenylate cyclase activity in broken-cell preparations indicated that another approach was necessary to understand how glucose controls cyclic AMP levels. Peterkofsky and Gazdar (86) measured the rate of cyclic AMP synthesis in intact cells by measuring the rate at which [^{3}H]adenosine was incorporated into ATP and subsequently into cyclic AMP. To obtain a true assessment of the rate of cyclic

AMP synthesis they determined the specific radioactivity of cellular ATP and the amount of radioactivity found in cyclic AMP. The principal findings from these studies are as follows. (i) Cells that have entered the stationary phase of growth due to glucose depletion synthesize cyclic AMP at a much higher rate than in the logarithmic phase of growth. (ii) The addition of glucose to stationary-phase cells rapidly depresses the rate of cyclic AMP synthesis. Subsequently, Wayne et al. (115) showed that addition of glucose also arrested cyclic AMP synthesis in cells during the logarithmic phase of growth. (iii) Extensive metabolism of glucose is not required for inhibition of cyclic AMP synthesis because a non-metabolizable analogue of glucose, α-methylglucoside, is as effective as glucose in inhibiting cyclic AMP synthesis. (α-Methylglucoside is transported into *E. coli* by the phosphotransferase system as α-methylglucoside-6-P and is not metabolized further.) (iv) Glucose was able to inhibit cyclic AMP synthesis in a strain of *E. coli* lacking cyclic AMP phosphodiesterase (Crooke's strain). This last finding served to support the authors' contention that they were indeed measuring changes in the rate of cyclic AMP synthesis and not of degradation.

How does glucose or α-methylglucoside act to inhibit cyclic AMP synthesis? The transport of glucose, α-methylglucoside, and many other sugars into *E. coli* is catalyzed by the phosphotransferase system, which can be illustrated as follows (51):

$$\text{Phosphoenolpyruvate} + \text{enzyme I} \rightarrow \text{enzyme I-P} + \text{pyruvate}$$

$$\text{Enzyme I-P} + \text{HPr} \rightarrow \text{P-HPr} + \text{enzyme I}$$

$$\text{P-HPr} + \text{enzyme IIA} \rightarrow \text{enzyme IIA-P} + \text{HPr}$$

$$\text{Enzyme IIA-P} + \text{sugar} \xrightarrow{\text{enzyme IIB}} \text{sugar-P} + \text{enzyme IIA}$$

Each enzyme II component is specific for a different sugar. To study a possible relationship between glucose phosphorylation and the ability of glucose to lower cyclic AMP, Pastan and Perlman (77) investigated the ability of glucose to repress β-galactosidase synthesis in mutants of the phosphotransferase system. They found that two enzyme I mutant strains (1101 and MO-X19) and one HPr mutant strain (1103) were more sensitive to transient repression by either glucose or α-methyl glucoside than the *pts*$^+$ parent strains. In contrast, a mutant defective in enzyme IIB for glucose was not affected by α-methyl glucoside. The experiments with the enzyme I and HPr mutants suggested that transient repression by glucose did not require phosphorylation of the sugar. The enzyme II mutant data suggested that α-methyl glucoside might interact with an enzyme II or another component of transport to control cyclic AMP levels. The interpretation of these studies, however, is complicated by the fact that the enzyme I-deficient strain (1101) retained about 1 to 5% of the normal level of enzyme I activity.

Peterkofsky and Gazdar (85) have studied the same mutant strains. They measured cyclic AMP levels in intact cells and adenylate cyclase activity in toluenized cells. They found that only the enzyme I mutant (1101) had low cyclic AMP levels and low adenylate cyclase activity when grown in nutrient broth. The addition of glucose to the wild type and the HPr mutant (1103) lowered cyclic AMP levels and adenylate cyclase activity to those of the enzyme I mutant. The low adenylate cyclase activity of the enzyme I mutant cells was stimulated to wild-type levels when phosphoenolpyruvate was added. Since the mutant studied still had small amounts of enzyme I present, and since phosphoenolpyruvate would be expected to increase the phosphorylation of enzyme I, they suggest that the phosphorylated form of enzyme I is required for activation of adenylate cyclase and that adenylate cyclase is regulated directly or indirectly by a phosphorylation-dephosphorylation mechanism (85).

Saier and Feucht (96) have also concluded that phosphorylation controls the rate of cyclic AMP synthesis from studies of mutants of *S. typhimurium*. They have studied enzyme I and HPr mutants and also another mutant, *crr* (carbohydrate repression resistant), defective in the formation of enzyme IIA for glucose. In a slightly leaky (1%) enzyme I mutant such as strain 1101 (see above), cyclic AMP synthesis was hypersensitive to repression by α-methylglucoside. But in a tight enzyme I or *crr* mutant or in a strain deleted for the HPr, enzyme I, or *crr* genes, cyclic AMP synthesis was markedly decreased even without glucose. To account for the low cyclic AMP levels in these mutant strains, they suggested that enzyme I and HPr function to phosphorylate a central regulatory protein (PRr) and that RPr or phosphorylated RPr allosterically regulates the activity of adenylate cyclase. RPr is presumed to be the product of the *crr* gene, and its absence in *crr* mutants could acount for their low cyclic AMP levels.

The experiments of Saier and Feucht (96) and Peterkofsky and Gazdar (85) have been done using intact cells or toluene-treated cells. Clearly, demonstration of the precise mechanism by which adenylate cyclase activity is

controlled will require detailed studies with purified proteins and perhaps reconstituted membranes. It would seem that such studies may soon be feasible.

Lactose is a sugar that is not transported by the phosphotransferase system; therefore, it was surprising to find that HPr or enzyme I mutant strains were unable to ferment lactose when tested on lactose indicator plates (77). The addition of 1 mM cyclic AMP to the indicator plates enabled these strains to ferment lactose; subsequently, Berman et al. (8) found that an enzyme I mutant strain would ferment glycerol only if cyclic AMP was present.

Dahl et al. (23) examined the effect of introducing an enzyme I mutation into strains that were producing tryptophanase constitutively. They found that tryptophanase synthesis was depressed upon the introduction of an enzyme I mutation and could be restored by the addition of tryptophan and cyclic AMP together but not by cyclic AMP alone. Unfortunately, the nature of the mutation causing constitutive tryptophanase synthesis in their strain is unknown. Dahl et al. (23) attempted to measure cyclic AMP levels in an enzyme I mutant (9111VA) and its parent (911IV). They found that the cyclic AMP level was lower in the mutant (0.9 pmol/mg of protein) than in the parental strain (2.6 pmol/mg of protein). However, in a temperature-sensitive enzyme I mutant, the cyclic AMP levels were unchanged when the cells were grown at temperatures either permissive or restrictive for enzyme I activity. Thus, in the mutant, cyclic AMP levels did not correlate with the ability to synthesize tryptophanase. Since the cyclic AMP levels were measured in cells collected by centrifugation after growth to late-log phase in LB broth, the levels measured may not reflect the levels of cyclic AMP in growing cells utilized for the cyclic AMP addition experiments. Certainly more studies are needed on the levels of cyclic AMP in such mutant strains.

The data summarized above seem to indicate that cyclic AMP levels are low in enzyme I and HPr mutants growing in enriched medium and explain why the addition of cyclic AMP to the growth medium increases β-galactosidase synthesis in enzyme I and HPr mutants.

CYCLIC AMP RECEPTOR PROTEIN

The existence of mutant cells unable to make glucose-repressible enzymes even when cyclic AMP was added to the culture medium suggested that cyclic AMP required some other intracellular molecule to activate gene expression. Zubay and co-workers (124) showed that extracts of such mutant cells (*crp*) could not make β-galactosidase even when cyclic AMP was present. When these extracts supplemented with an extract of normal cells, the defect was corrected. They were able to identify the factor as a protein and to partially purify it.

Emmer and co-workers (30) decided to search in extracts of wild-type *E. coli* for a protein that could bind cyclic AMP. Since the binding of ^{3}H-labeled cyclic AMP to protein is easily measured, they were able to obtain a highly purified CRP. We have discussed in the section, Operon Activation by Cyclic AMP, the stimulation of β-galactosidase synthesis by this protein in extracts of *crp* cells and also its use in a highly purified transcription system containing λ*lac* or λ*gal* DNA, RNA polymerase holoenzyme, and nucleoside triphosphates (24, 70). In such a system very little *lac* or *gal* RNA is made unless cyclic AMP and CRP are added. Then up to 10 to 20% of the total RNA made from λ*lac* or λ*gal* template is *lac* or *gal* RNA.

Physical properties of CRP. A detailed method for preparing homogeneous CRP has recently been published (74). With this method, 50 mg of the protein can be readily prepared from 1 kg of *E. coli*. The protein has a molecular weight of about 45,000 and consists of two identical subunits (5). Other physical properties are described in Table 6.

CRP is a basic protein with a pI of 9.12 (5). This high value probably accounts for the high affinity of the protein for phosphocellulose and DNA. In fact, DNA-cellulose column chromatography has been used to purify CRP (J. Karkow, personal communication). The high isoelectric point of CRP is due to the high content of glutamine and asparagine; the content of lysine and arginine in CRP is not unusually high (5).

TABLE 6. *Summary of some physical properties of CRP from E. coli (5)*

Parameter	Calculated value
M_w, of native protein[a]	44,600
M_w, of reduced-alkylated subunits in 6 M guanidine hydrochloride[a]	22,300
Sedimentation coefficient, $s_{20,w}$, of native protein[b]	3.53
Partial specific volume, $\bar{v}$[c]	0.752 ml/g
Diffusion coefficient, $D_{20,w}$, of native protein[d]	7.7×10^{-7} cm^2 sec^{-1}
Frictional coefficient, f/f_0 of native protein[e]	1.17
Isoelectric point, pI[f]	9.12
Per cent α-helical structure[g]	31%

[a] Determined by sedimentation equilibrium studies.
[b] Determined by sedimentation velocity studies.
[c] Calculated from the amino acid composition.
[d] Calculated from combined sedimentation and molecular weight data.
[e] Calculated from combined sedimentation and molecular weight data, but uncorrected for hydration.
[f] Determined by isoelectric focusing.
[g] Determined by circular dichroic measurements.

Analysis of the amino acid sequence of CRP has been undertaken by D. Schlesinger, H. Niall, I. Pastan, and their co-workers. The *N*-terminal amino acid sequence is NH_2-val-leu-gly-lys-pro-gln-thr-asp-pro-thr-. Each subunit of the protein has five methionine residues and thus should yield six cyanogen bromide peptides. Five peptides have been purified by gel filtration and partially sequenced. In addition, eight maleated tryptic peptides of CRP have been isolated. The structure of four of these has been completed and that of others have been partially determined. All these sequences together account for about 140 of the 200 amino acid residues of CRP and supply overlapping sequences for about 100 residues (D. Schlesinger, personal communication).

Cyclic AMP binding. The initial purification of CRP was based on its ability to bind ^{3}H-labeled cyclic AMP. By this assay, in which the cyclic AMP-CRP complex is precipitated from solution by ammonium sulfate, the complex has a K_a of 1×10^6 liters/mol (30). By equilibrium dialysis at low ionic strength, the K_a is 1.1×10^5 liters/mol (5, 124). Apparently, ammonium sulfate increases the association constant. The K_a has not been measured in the presence of DNA. This should be done because the formation of a ternary complex cyclic AMP-CRP-DNA would be expected to increase the association constant.

A variety of cyclic AMP analogues (Fig. 5) has been tested for their ability to compete with cyclic AMP for binding to CRP. Of these, only cyclic tubericidin monophosphate (cyclic TuMP) was as potent as cyclic AMP (4). Other analogues that inhibited cyclic AMP binding was 8-thio-cyclic AMP > 8-Br-cyclic AMP > N^6-monobutyryl cyclic AMP > cyclic GMP (4).

The presence of CRP in crude extracts of *E. coli* can usually be detected by its capacity to bind ^{3}H-labeled cyclic AMP. Anderson and Pastan (3) have observed that in some freshly prepared extracts of *crp*$^+$ strains of *E. coli*, cyclic AMP binding cannot be detected, but binding will return if the extracts are stored for a few days at 4°C. These experiments suggest that CRP activity may be modulated by factors other than cyclic AMP.

DNA binding. CRP will bind to various types of DNA, including some synthetic polynucleotides. If high concentrations of CRP or DNA are employed, CRP will bind to DNA even in the absence of cyclic AMP. However, if the concentrations of CRP and DNA are reduced, the binding can be shown to be dependent on the presence of cyclic AMP (69, 94). Cyclic GMP will not support the binding of CRP to DNA and in addition inhibits cyclic AMP-dependent binding. A report that cyclic GMP prevent non-cyclic AMP-dependent binding of CRP to DNA (94) has not been confirmed (B. de Crombrugghe, personal communication).

Using the synthetic DNA analogue [^{3}H]-poly(dI-dC)·poly(dI-dC), Krakow (49) has studied the effect of a variety of cyclic nucleotide analogues on the binding of CRP to the polynucleotide. Only cyclic AMP and cyclic TuMP support binding. It is of interest that the synthetic analogue cyclic TuMP is somewhat more active than cyclic AMP. Cyclic GMP, inosine 3′,5′-cyclic monophosphate (cyclic IMP), 8-Br-cyclic AMP, and other cyclic AMP analogues failed to supported binding. Many of the analogues which would not stimulate binding of CRP to d[I-C]n instead competed with cyclic AMP. These nucleotides included cyclic GMP, cyclic IMP, and 8-Br-cyclic AMP.

Anderson et al. (4) employed many of the same analogues in studies on *gal* transcription using a purified transcription system containing CRP, λ*gal* DNA, and nucleoside triphosphates. Only cyclic AMP and cyclic TuMP, the molecules that promoted binding of CRP to d[I-C]n, also promoted *gal* transcription. The analogues that inhibited cyclic AMP-dependent *gal* transcription were the same ones that inhibited DNA binding: cyclic GMP, 8-Br-cyclic AMP, and cyclic IMP. Thus, those nucleotides that promote binding of CRP to DNA promote transcription and those that inhibit binding inhibit transcription (Table 7).

Promoter-specific DNA binding. Once it was established that the cyclic AMP-CRP complex acted to promote the formation of an open complex of RNA polymerase and DNA at *lac* and *gal* promoters, it seemed likely that cyclic AMP-CRP did this by binding to the promoter region. However, attempts to show directly that the complex bound with a higher affinity to DNA containing a normal *lac* or *gal* promoter than to DNA lacking these promoters were unsuccessful (69, 94). In these unsuccessful attempts, intact λ*gal* or λ*lac* DNA was employed. Recently Majors (59) has done binding studies with fragments of *lac* DNA, about 200 base pairs in length, containing the *lac* promoter region. With a fragment containing a normal promoter, he has shown specific cyclic AMP-dependent binding of CRP. A fragment of similar size containing a point mutation (L8), which decreases the effect of cyclic AMP and CRP in vivo, has markedly diminished binding (59). Another fragment containing a small deletion of the promoter region (L1) also has diminished binding. The inability of previous workers to demonstrate *lac* promoter-specific binding of the cyclic AMP-CRP complex to intact

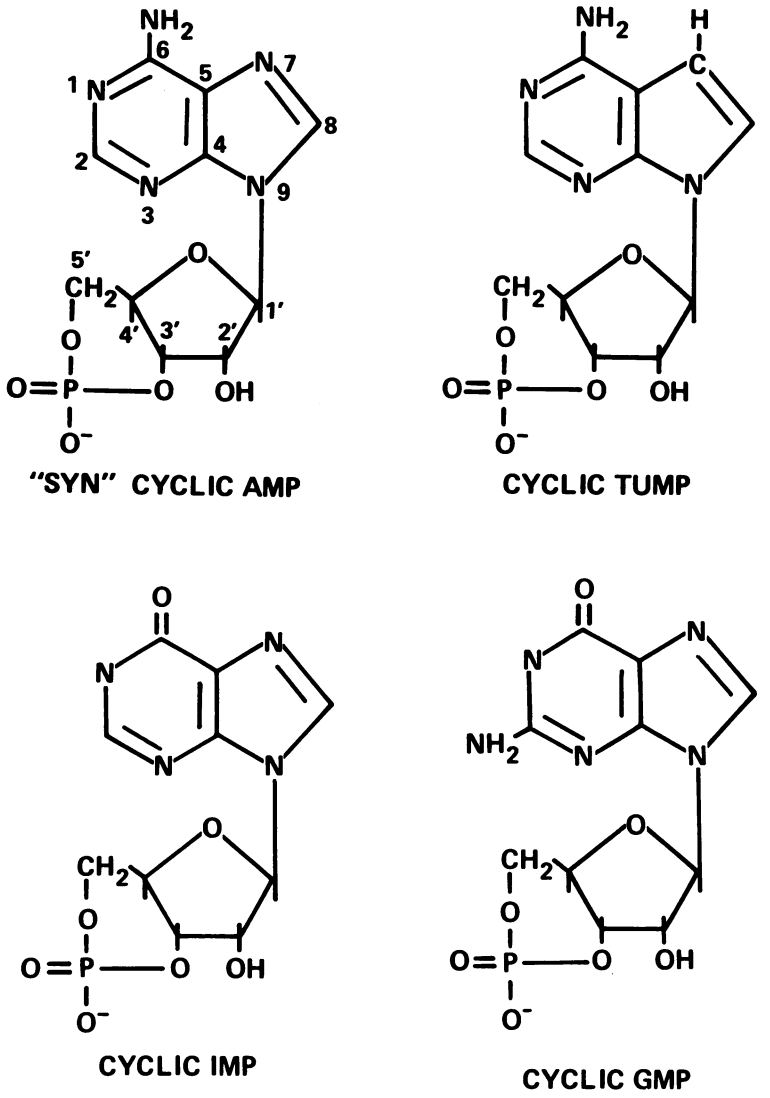

FIG. 5. *Structures of cyclic nucleotides.*

λ*lac* DNA was probably because the binding to a single high-affinity site was obscured by binding to multiple low-affinity sites. Whether binding to these other sites has any physiological significance is not clear.

Lin and Riggs (53) have found that CRP can be covalently linked to DNA. This is accomplished by preparing DNA containing 5-bromodeoxyuridine. Proteins bound to this modified DNA become covalently linked to the DNA upon exposure to ultraviolet light. This approach may be useful in determining the identity of the amino acids of CRP that interact with specific bases at the promoter locus.

Effects of cyclic AMP on CRP structure. The binding of cyclic AMP to CRP brings about a change in the structure of CRP that is associated with its enhanced affinity for DNA. Krakow and Pastan (50) studied the ability of a variety of proteolytic enzymes to degrade CRP in the presence and absence of cyclic AMP. When cyclic AMP is bound to CRP, chymotrypsin, trypsin, and subtilisin all rapidly cleave CRP. A fragment, αCRP, is formed by proteolysis. The fragment-like native CRP will bind to DNA at pH 6 in the absence of cyclic AMP. But at pH 8 where binding of CRP to DNA is dependent on cyclic AMP, no binding of αCRP occurs. Apparently the αCRP fragment retains the DNA-binding site but has lost the cyclic AMP site.

Under the conditions of proteolysis em-

ployed, CRP was resistant to digestion when cyclic AMP was absent. Of the many analogues of cyclic AMP tested, only cyclic TuMP was able to substitute for cyclic AMP (Table 7). Cyclic GMP, cyclic IMP, and 8-Br-cyclic AMP, analogues that did not promote DNA binding or *gal* transcription, also did not convert CRP to the protease-susceptible structure (50). These results with proteases indicate that CRP undergoes a marked change in structure in the presence of cyclic AMP.

Other experiments indicating CRP undergoes a conformational change when exposed to cyclic AMP have been presented by Wu and coworkers (119, 120). They used a fluorescent probe, *N*-(iodoacetylaminoethyl)-1-naphthylamine-5-sulfonate (AENS) to label CRP. When cyclic AMP was added, they observed an increase in fluoresence intensity and a blue shift of the emission maximum of the AENS-CRP complex. This shift indicates a conformational change in the protein. In addition, Wu and Wu (119) used temperature-jump studies to show a change in CRP structure when cyclic AMP was present.

CYCLIC AMP AND BACTERIOPHAGE λ

Weissbach and Jacob (117) studied the "glucose effect" on the infective growth of phage λ in *E. coli*. Phage λ has two modes of life cycle after infection of the host. The λ chromosome either multiplies, resulting in a λ phage burst and lysis of the host (lytic response), or stably integrates into the host chromosome, wherein the lytic functions of the phage chromosome are repressed by the λ repressor protein (lysogenic response). In the latter mode, the host survives. When cells are grown in glucose-containing medium, only 20 to 30% of the cells become infected at a phage multiplicity of 3. In glycerol-containing medium, more than 90% of the cells are infected. In both media the size of the phage burst per infected cell is normal. The λ adsorption proteins on the bacterial cell surface are products of the *lamB* genes, part of the *malB* operon (41, 103). The expression of the *malB* operon is under control of cyclic AMP and CRP. When the concentration of cyclic AMP in the cells is low, synthesis of the lambda adsorption proteins is decreased, and the adsorption of phage λ is diminished (78, 122). *E. coli* mutants defective in either the *cya* gene or the *crp* gene are resistant to λ infection because of diminished expression of the *lamB* genes. Control of λ adsorption by cyclic AMP reflects control of bacterial gene expression by the cyclic nucleotide. However, this is not the only manner by which cyclic AMP controls the life cycle of phage λ. Two other interactions between cyclic AMP and λ have been reported.

TABLE 7. *Effect of cyclic nucleotide analogues on CRP function*

Nucleotide	Binds to CRP	Stimulates CRP binding to DNA	Stimulates transcription	Produces conformational change in CRP
cAMP	+[a]	+	+	+
cTuMP	+	+	+	+
cGMP	+	−	−	−
cIMP	+	−	−	−
8-Br-cAMP	+	−	−	−

[a] +, Nucleotide is effective; −, nucleotide is not effective. The table is a summary of discussions in the text.

The second relationship between λ and cyclic AMP has been studied by Wu et al. (118) and D. Court (in preparation). The rate of host β-galactosidase synthesis is reduced when a phage λ infects a cell. This effect has been ascribed to a λ-coded function and is overcome by deletion of a λ gene or by the addition of cyclic AMP. The λ function probably inhibits β-galactosidase synthesis by lowering the intracellular cyclic AMP concentration.

Using a hybrid phage of λ that carries the tail genes and the absorption specificity of phage 80 (the adsorption of phage 80 does not depend upon cyclic AMP and CRP), a third relationship between cyclic AMP and phage λ growth was observed. When the hybrid phage is exposed to wild-type *E. coli* cells, a normal infection ensues, in which some of the phage enter the lytic pathway and others enter the lysogenic pathway. However, when the hybrid phage infects a *cya* or *crp* mutant of *E. coli*, the phage lysogenize poorly (38), and most phage enter the lytic cycle. This observation with λ in *E. coli* was similar to previous findings (42, 90) that phages P22, MG40, and MG178 preferentially showed a lytic response in *cya* or *crp*-like mutants of *Salmonella typhimurium*. The addition of cyclic AMP to the *cya* mutants enchances lysogeny. The failure to form stable λ lysogens in a *cya* or *crp* host is probably not due to accumulation of inducer and thereby inactivation of phage repressor in the cells, because noninducible λ mutants also fail to give stable lysogens in a *cya* or *crp* host (S. Adhya, unpublished data). A possible hypothesis for the inability of the *cya* and *crp* mutants to establish stable λ lysogens is that λ fails to synthesize its repressor in these hosts. After infection of the wild-type host by the phage, λ repressor, the product of gene cI, is synthesized under the control of the promoter *pre*, known as the establishment promoter for repressor synthesis (91) (Fig. 6). The products

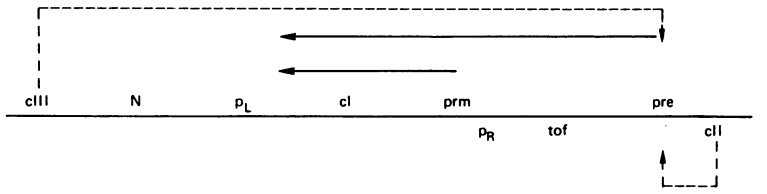

FIG. 6. *Genetic map of phage λ showing the region that controls λ repressor synthesis. cI is the gene coding for repressor, which can be synthesized from either of its two promoters, pre or prm (shown by solid arrows). Two other promoters, pL and pR, which can be turned off by the repressor, are the initiation sites of transcription of genes N and cIII, and tof and cII, respectively. The products of cII and cIII are known to stimulate repressor synthesis by acting at the pre region (shown by arrow-headed broken lines).*

of two other λ genes, *cII* and *cIII*, are also required for normal repressor synthesis from *pre* and integration into the host chromosome (28, 48, 91; D. Court, in preparation). The requirement for the *cII* product is almost absolute. A *cII* mutant, like a *cI* mutant, synthesizes very little λ repressor and lysogenizes extremely poorly. However, in the case of a *cIII* mutant, low levels of repressor are made and lysogenization frequently is higher than in *cI* or *cII* mutants.

Once a phage chromosome is committed to lysogeny, a steady-state level of repressor synthesis is continued under the control of another promoter, *prm*, called the maintenance promoter of repressor (see reference 91). *cII* and *cIII* products are not needed to activate the *prm* promoter.

A mutant of λ (*tof*, also known as *cro*), has been isolated, which on infection of wild-type hosts synthesizes excess repressor (29). A λ *tof* mutant does not show much lytic growth, unless it carries an additional defect in repressor synthesis, for example, a mutation in *cI*, *cII*, or *cIII* genes. Thus a lytic response by a λ *tof* mutant might indicate a reduction of repressor synthesis. Since a *tof* mutant does show lytic growth in a *cya* or *crp* host, this would suggest a requirement for cyclic AMP in λ repressor synthesis (38).

Is repressor synthesis from the establishment promoter, *pre*, controlled by cyclic AMP? The frequency of lysogeny of a *cIII* mutant is negligibly low in a *cya·crp* host (<0.005%), compared with either a *cIII* mutant in the wild-type host (about 4%), or a wild-type phage in a *cya·crp* host (4%) (7, 48). Belfort and Wulff (7) have suggested that some amount of repressor synthesis from *pre* needs *cII* product plus either *cIII* product or the cyclic AMP-CRP system. Jordan et al. (47) have shown that lowering the cyclic AMP level in the cell at the time of infection does not produce any substantial effect on repressor synthesis from the *pre* promoter after infection. This observation does not contradict the idea of Belfort and Wulff, because one would expect that the phage *cIII* product made after infection would at least partially alleviate the requirement of the cyclic AMP system. A real test of the model is to compare the amount of repressor synthesis after infection by a *cIII* mutant in a wild-type host or by a wild-type phage in a *cya* host with that by a *cIII* mutant in a *cya* host. If *cIII* product and the cyclic AMP system are compensatory to each other, then in the latter case the repressor level would be very low compared with the former two.

An explanation for the role of the *cIII* gene product and cyclic AMP and CRP in the establishment of lysogeny has been suggested by Belfort and Wulff (7). A host mutation, *hfl*, causes very high frequency of lysogenization (close to 80%) after infection with λ. In the presence of *hfl* mutation, neither the phage *cIII* gene product nor host cyclic AMP is required for efficient lysogenization. Under such conditions the frequency of lysogenization is more than 70%. The *hfl* mutation is recessive to the wild-type allele. From these results, the authors have suggested that the wild-type *hfl* product is an inhibitor of repressor synthesis from *pre;* the *cIII* product or cyclic AMP and CRP promote repressor synthesis by inactivating the product of the wild-type *hfl* gene.

Mutants of λ, called *tul*, have been isolated which plate turbid on *cya* and *crp* hosts (38; M. Gottesman and S. Adhya, unpublished data). The mutational alterations map in the region of the λ chromosome that determines lysogeny, i.e., the *cI-cII* region (Fig. 6). Genetic and biochemical analysis of the mutants might help to understand the precise role of cyclic AMP and CRP in the process of lysogenization of λ. Whether the role of cyclic AMP in lysogeny is to affect phage or host functions, its fundamental action is probably similar to that described for its activation of *gal* and *lac* transcription (see section, Operon Activation by Cyclic AMP).

CYCLIC GMP

The only other cyclic nucleotide known to be widely distributed in nature is cyclic GMP.

Generally the concentration of cyclic GMP in cells is less than that of cyclic AMP. Cyclic GMP was first detected in *E. coli* by Bernlohr and associates (9). They reported that the levels of cyclic GMP tended to change in the opposite direction from cyclic AMP, suggesting that the two nucleotides might function antagonistically. More recently, Gonzalez and Peterkofsky (37) have performed a more extensive examination of the levels of cyclic GMP. Under a wide variety of conditions of growth, they observed the two nucleotides to change in the same direction, in opposite directions, or one to change without the other changing. On the basis of this study, it is difficult to formulate a role for cyclic GMP in *E. coli*.

Enzymes capable of forming and degrading cyclic GMP have been detected in extracts of *E. coli*. Guanylate cyclase catalyzes the synthesis of cyclic GMP from GTP. Guanylate cyclase has been purified about 1,000-fold by Macchia et al. (57). It has a molecular weight of approximately 40,000 and requires Mn^{2+} ion for optimal activity. The enzyme will not form cyclic AMP from ATP and thus is unrelated to adenylate cyclase.

The only known effect of cyclic GMP is to antagonize the actions of cyclic AMP on transcription. This effect, which occurs at relatively high concentrations of cyclic GMP, has been demonstrated in cell-free extracts. Since the levels of cyclic GMP in intact cells are much lower than those of cyclic AMP, it appears unlikely that this is a major role that cyclic GMP serves. To date, the role of cyclic GMP in *E. coli* remains obscure.

CONCLUSION

The amount of information now available about the role of cyclic AMP in *E. coli* is considerable, but there are a few important questions yet to be answered. At one end of the spectrum is how glucose inhibits adenylate cyclase activity. At the other end is how the cyclic AMP-CRP complex alters the structure of DNA at specific sites to allow RNA polymerase to initiate transcription. Another interesting question is why cyclic AMP-deficient *E. coli* grow poorly even in highly enriched media. Perhaps there is some essential step in cell growth that is modulated by cyclic AMP. The relationship between cyclic AMP and phage λ also merit further investigation. Finally, the role of cyclic GMP is totally obscure.

In this review we have traced how the realization that low cyclic AMP levels were responsible for the repressive "glucose effect" led to the development of a cell-free system in which enzymes could be made. This in turn led to the first experiment in which homogeneous proteins were used to carry out controlled transcription of a bacterial gene in a test tube. In this experiment three pure proteins, RNA polymerase, cyclic AMP receptor, and *lac* repressor and two small regulatory molecules, cyclic AMP and the inducer isopropylthiogalactoside, were employed. The studies with *lac* and later *gal* repressors indicated that one way gene activity was regulated was by negative control. The experiments with cyclic AMP and CRP showed that gene activity was also regulated in a positive manner.

Without the discovery of and subsequent investigations on cyclic AMP, it seems likely that the mechanisms by which bacterial gene activity is regulated would still be obscure, and no model would be available for how hormones and other regulatory molecules may act in animal cells.

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