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# NEGATIVE TRANSCRIPTIONAL CONTROL OF THE ORNITHINE DECARBOXYLASE GENE (*SPEC*) BY CYCLIC AMP IN *ESCHERICHIA COLI*

by

© Jonathan Mark Campion Wright, B.Sc.

A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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## ABSTRACT

The putrescine biosynthetic enzyme, ornithine decarboxylase (E.C. 4.1.1.17), is negatively regulated by cAMP in Escherichia coli. The specific activity of ornithine decarboxylase was determined in crude extracts prepared from wild-type strains of E. coli and from strains carrying a mutation in the adenylate cyclase (E.C. 4.6.1.1) structural gene (cya). These strains were grown with various carbon sources in the presence and absence of cAMP. In wild-type strains ornithine decarboxylase activity was was less after growth on glycerol than after growth on glucose. When cya strains were grown on glucose or glycerol, ornithine decarboxylase activity was the same. Addition of 1 mM cAMP to a glucose-based medium repressed ornithine decarboxylase activity by approximately 50% in both wild-type and cya strains. Futhermore, cAMP exerts its negative control through the cAMP receptor protein (CRP), because a strain carrying a lesion in the crp structural gene failed to exhibit repressed ornithine decarboxylase activity in response to elevated cAMP. These results suggested that negative regulation of ornithine decarboxylase is exerted at the level of transcription. Negative control was shown not to be mediated by a cAMP-induced repressor because synthesis of ornithine decarboxylase in E. coli minicells, and in a cell-free protein synthesizing system, was repressed by cAMP. Repression of ornithine decarboxylase synthesis by cAMP in

*vitro* required functional CRP as evidenced by the lack of repression in a CRP deficient reaction system. The tetracycline resistance of *E. coli* wild-type and *cya* strains harbouring a tetracycline resistance gene (*tet*) under control of the *speC* promoter was repressed by cAMP. Cyclic AMP had no effect on the tetracycline resistance of *crp* strains bearing the *speC:tet* gene fusion, nor on wild-type strains bearing a normal *tet* gene. These results indicate that cAMP and its receptor protein interact with the *speC* promoter region to facilitate negative transcriptional control of the *speC* gene. Negative control of *speC* transcription by cAMP was confirmed by analysis of steady-state levels of ornithine decarboxylase mRNA in *cya* and *crp* strains of *E. coli*. Ornithine decarboxylase mRNA levels were repressed approximately 80% when *cya* strains were grown in the presence of 2 mM cAMP. No repression of ornithine decarboxylase mRNA levels was seen in a *crp* strain cultured in the presence of cAMP.

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## PUBLICATIONS

Much of the work presented in this thesis has been published or submitted for publication. These papers are:

- Wright, J.M., and S.M. Boyle. (1982). Negative control of ornithine decarboxylase and arginine decarboxylase by adenosine-3',5'-cyclic monophosphate in Escherichia coli. Mol. Gen. Genet. 186:482-487.

- Wright, J.M., and S.M. Boyle. (1984). Intergeneric homology of the speC gene encoding the biosynthetic ornithine decarboxylase of Escherichia coli. J. Bacteriol. In press.

- Boyle, S.M., G.M. Markham, E.W. Hafner, J.M. Wright, C.W.Tabor, and H. Tabor. (1984). Expression of the cloned genes encoding the putrescine biosynthetic enzymes (speA, speB, speC) and methionine adenosyltransferase (metK) of Escherichia coli. Gene. In press.

- Wright, J.M., C. Satishchandran and S.M. Boyle. (1984) Transcription of the speC (ornithine decarboxylase) gene is repressed by cyclic AMP and its receptor protein in Escherichia coli. EMBO Journal. Submitted.

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## LIST OF ABBREVIATIONS

- A=adenine; ADP=adenosine 5'-diphosphate; 3'-AMP=adenosine 3'-monophosphate; 5'-AMP=adenosine 5'-monophosphate; ATP=adenosine 5'-triphosphate;

- C=cytosine; cAMP=cyclic adenosine-3',5'-monophosphate; CRP=cAMP receptor protein;

- DNase=deoxyribonuclease; dATP=2'-deoxyadenosine 5'-triphosphate; DNA=deoxyribonucleic acid; dGTP=2'-deoxyguanosine 5'-triphosphate;

- EDTA=ethylenediaminetetraacetate;

- g=gram; G=guanine;

- h=hour;

- IPTG=isopropyl-β-thio-D-galactoside;

- Kb=kilobase pairs;

- l=litre;

- M=molar; min=minute; MOPS=3-(N-morpholino)-propanesulfonic acid; mRNA=messenger ribonucleic acid;

- N=nucleotide;

- poly(A)=polyadenylic acid; ppGpp=guanosine 5'-diphosphate 3'-diphosphate;

- RNase=ribonuclease; RNA=ribonucleic acid; rRNA=ribosomal ribonucleic acid;

- T=thymine; tRNA=transfer ribonucleic acid; Tris=tris(hydroxymethyl)aminomethane;

- U=uracil; UV=ultraviolet.

# Chapter 1

# INTRODUCTION-A REVIEW

## 1.1 Scope of reveiw

Polyamines have been viewed successively "as foul smelling indicators of male reproductive function, then as promoters and regulators of cell growth and more recently as potentially useful markers of human health and disease" (Daves, 1978). Polyamines have been called "a growth industry" (Cohen, 1983) and "molecules in search of a role". (Hopkins and Manchester, 1982). Their history goes back some 300 years with the discovery by Anton van Leeuwenhoek (1678) of the crystallization of spermine phosphate from human semen. Its structure was characterized by Rosenheim over 50 years ago (Rosenheim, 1924). This slow development of the field has changed dramatically during the last 25 years, and numerous articles, reviews and books on the medicine and biology of polyamines have been published.

We now know the biosynthetic pathways for these polycations in procaryotic and eucaryotic cells, and many studies indicate that their biosynthetic enzymes are highly regulated. It seems that the polyamines are physiologically important because they are absolute requirements for the

growth of several microorganisms, and normal growth and differentiation are dependent on these compounds. Moreover, they are ubiquitous in living cells.

The major question still to be answered in the field is, "what are their biological functions"? The polyamines appear to have many roles in cell metabolism. As polycations they bind tightly to nucleic acids and have diverse effects on RNA and DNA biosynthesis and metabolism. They are associated with ribosomes and tRNA, and influence the synthesis of certain factors required for protein synthesis. The polyamines play a role in membrane integrity and probably modulate the activity of membrane-bound enzymes.

This review is designed to introduce the reader to that area of the field of polyamines and gene regulation which may be relevant to the work reported here. Therefore, it is not my intention to comprehensively review the vast literature on the polyamines, nor will I attempt to answer the question of their physiological role. Instead I have limited myself to discussion of selective topics that outline our current understanding of the polyamines in procaryotic physiology, with particular emphasis on the regulation of their biosynthetic enzymes in Escherichia coli. Similarly, a restricted number of specific systems have been chosen to illustrate the role of cyclic AMP in procaryotic gene regulation.

## 1.2 Biosynthesis of simple polyamines

The structural relationship of spermidine and spermine to putrescine immediately suggests the possible origin and metabolism of these bases (Table 1). Putrescine (1,4-diaminobutane) and cadaverine (1,4-diaminopentane) are simple diamines; spermidine and spermine are aminopropyl derivatives of putrescine. Putrescine and spermidine are found in millimolar concentrations in E. coli (Bachrach, 1973; Cohen, 1971). In general, the putrescine content of procaryotic cells is several fold higher than that of spermidine, whereas eucaryotic cells have high levels of spermidine and little putrescine. Spermine has only been found in eucaryotic cells. Several acetylated and oxidized derivatives of the polyamines are inhibitory to many functions in bacteria and mammalian cells, e.g. oxidized spermine inhibits protein and nucleic acid synthesis in cells in tissue culture; inhibits DNA, RNA and protein synthesis in E. coli; and inactivates many RNA and DNA viruses (Bachrach, 1973; Cohen, 1971). The oxidation of polyamines may, therefore, have a physiological role in the inhibition of cellular processes. Also, oxidized polyamines may supply the cell with carbon and nitrogen sources; some bacteria are capable of using polyamines as the sole carbon and nitrogen source. The enzymatic degradation of the polyamines may regulate, in part, the cellular levels of the polyamines, and hence cell physiology. Despite their potential importance these polyamine derivatives will not be discussed here.

Table 1. Structure of simple polyamines

| STRUCTURE | NAME |
|---|---|
| $H_2N-(CH_2)_3-NH_2$ | 1,3-DIAMINOPROPANE |
| $H_2N-(CH_2)_4-NH_2$ | 1,4-DIAMINOBUTANE (PUTRESCINE) |
| $H_2N-(CH_2)_5-NH_2$ | 1,5-DIAMINOPENTANE (CADAVERINE) |
| $H_2N-(CH_2)_3-NH-(CH_2)_4-NH_2$ | SPERMIDINE |
| $H_2N-(CH_2)_3-NH-(CH_2)_4-$ $NH-(CH_2)_3-NH_2$ | SPERMINE |

The pathways for the biosynthesis of putrescine and spermidine are similar in microorganisms and in animal cells, but have certain striking differences (Morris, 1978a; Tabor and Tabor, 1976, 1984) (Figure 1).

In E. coli there are two biosynthetic pathways for putrescine. The first pathway is the decarboxylation of ornithine, an intermediate in the arginine biosynthetic pathway (Morris and Pardee, 1965; 1966). This reaction is catalyzed by ornithine decarboxylase. The second pathway is the decarboxylation of arginine and subsequent hydrolysis of agmatine to form putrescine and urea (Morris and Koffron, 1969; Morris et al., 1970). These reactions are catalyzed by arginine decarboxylase and agmatine ureohydrolase, respectively. In some bacteria and plants agmatine is converted to N-carbamylputrescine which is hydrolyzed by an iminohydrolase to putrescine (reviewed in Cohen, 1971; Tabor and Tabor, 1984). The putrescine biosynthetic enzymes of E. coli are found in cells growing in minimal medium at low substrate concentrations and the enzymes are considered to be produced constitutively (Morris and Koffron, 1969). In certain strains of E. coli, biodegradative forms of ornithine decarboxylase and arginine decarboxylase are induced by low pH and high substrate concentrations (Gale, 1948; Morris and Fillingame, 1974). No biodegradative form of agmatine ureohydrolase has been detected so far (Satishchandran and Boyle, 1984). The biosynthetic and biodegradative ornithine and arginine decarboxylases have

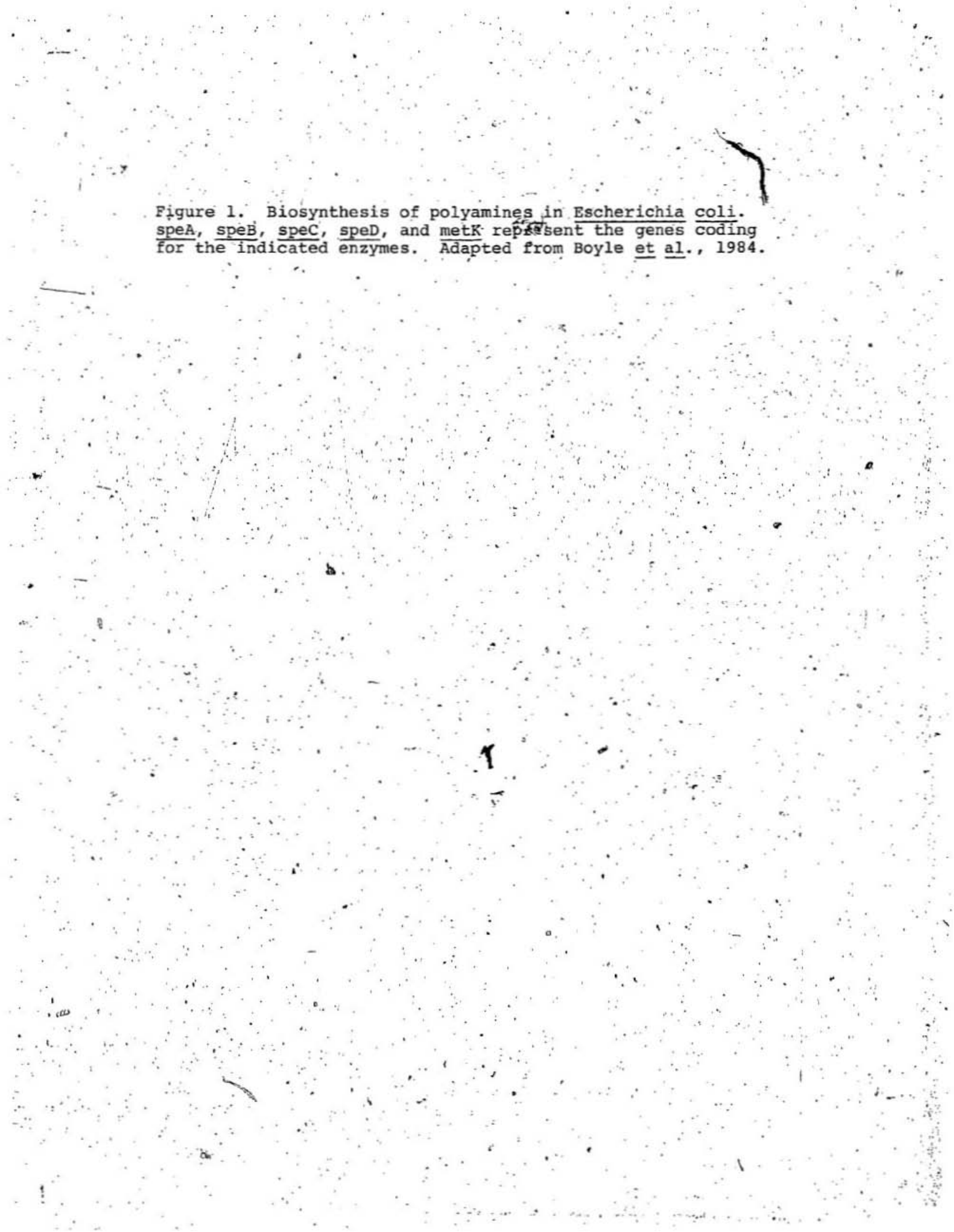
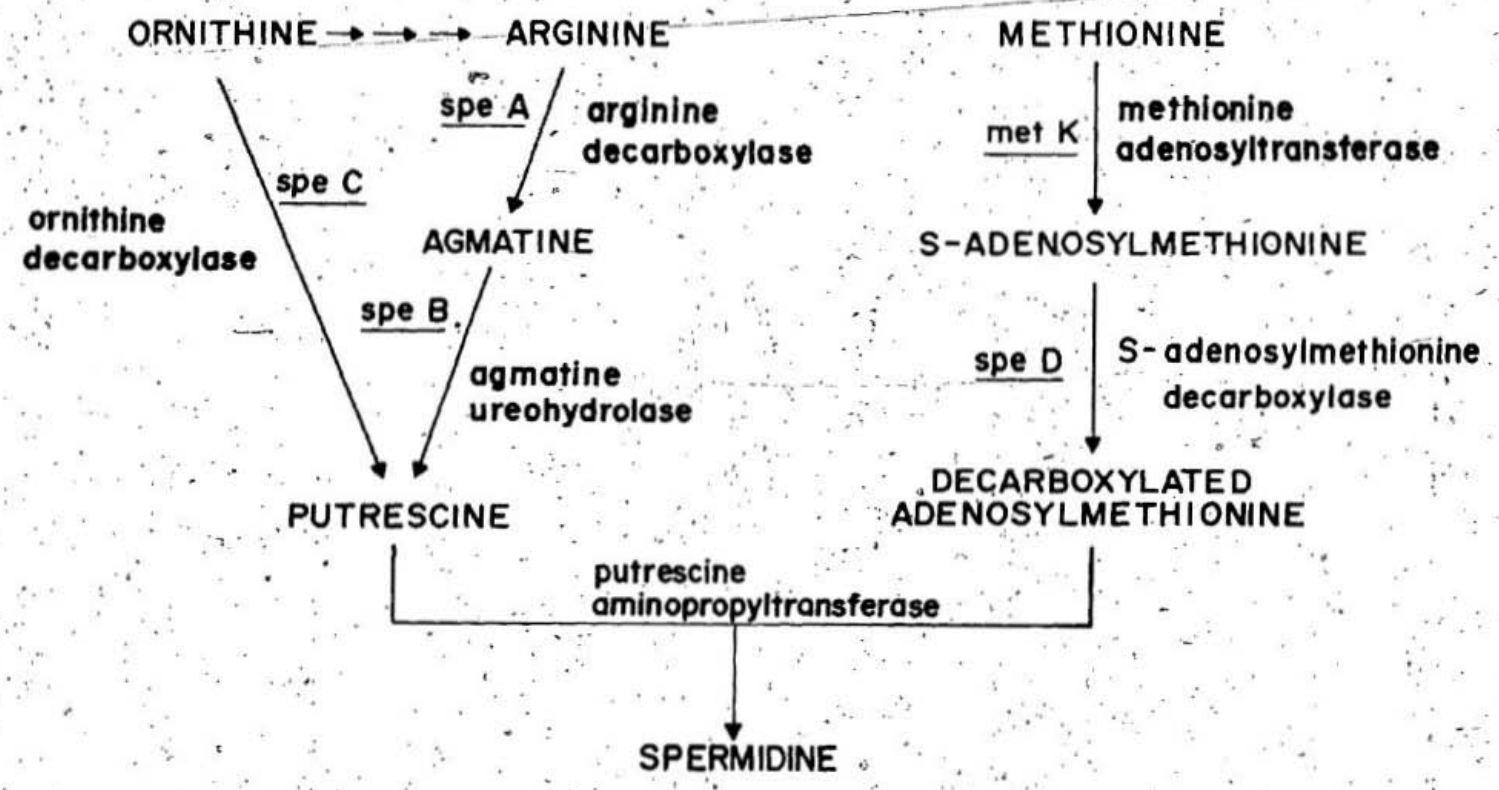


Figure 1. Biosynthesis of polyamines in Escherichia coli. speA, speB, speC, speD, and metK represent the genes coding for the indicated enzymes. Adapted from Boyle et al., 1984.


ORNITHINE
ARGININE
METHIONINE
spe A
arginine decarboxylase
met K
methionine adenosyltransferase
spe C
ornithine decarboxylase
AGMATINE
S-ADENOSYLMETHIONINE
spe B
agmatine ureohydrolase
spe D
S-adenosylmethionine decarboxylase
PUTRESCINE
DECARBOXYLATED ADENOSYLMETHIONINE
putrescine aminopropyltransferase
SPERMIDINE

been purified to near homogeneity and possess many characteristics in common (Wu and Morris, 1973; Applebaum et al., 1977). Since only one in ten strains of E. coli possesses the biodegradative enzymes whereas all E. coli strains possess the biosynthetic enzymes, it has been suggested that the biodegradative enzymes may have arisen by divergence from their respective biosynthetic forms (Applebaum et al., 1977; Gale, 1946). The function of the biodegradative enzymes may be a homeostatic mechanism of proton elimination to neutralize the acidity of the surrounding medium (Morris and Fillingame, 1974).

In mammalian cells, putrescine is formed by the decarboxylation of ornithine. No other route is known. The enzyme, ornithine decarboxylase, that catalyzes this reaction is considered to be the rate-limiting step in polyamine synthesis in these cells (Tabor and Tabor, 1976). The mammalian ornithine decarboxylase, like the procaryotic and lower eucaryotic enzyme, requires pyridoxal phosphate as a cofactor.

In both bacterial and mammalian cells, an aminopropyl group of decarboxylated adenosylmethionine is transferred to putrescine by aminopropyl transferase to form spermidine (Tabor, 1958; Hannonen et al., 1972; Raina and Hannonen, 1971). A second aminopropyl transferase has been purified from animal tissue that catalyses the transfer of an aminopropyl group of decarboxylated adenosylmethionine to spermidine to form spermine (Hannonen et al., 1972; Raina

and Hannonen, 1971). No known cofactors are required for aminopropyl transferase activity.

Tabor (1962) and Tabor et al. (1958) studied the formation of decarboxylated adenosylmethionine from L-methionine in E. coli. The conversion of L-methionine to S-adenosylmethionine is catalyzed by the enzyme, methionine adenosyltransferase, and the adenosyl group is derived from ATP. Magnesium is required as a cofactor in this reaction. S-Adenosylmethionine decarboxylase catalyzes the conversion of S-adenosylmethionine to decarboxylated adenosylmethionine. Wickner et al. (1970, 1971) found that this enzyme contained pyruvate as a cofactor. A similar pathway to decarboxylated adenosylmethionine is found in eucaryotic cells.

The amine cadaverine and its aminopropyl derivative are found in both bacteria and animal tissue. In E. coli, a biodegradative lysine decarboxylase is induced by high substrate concentration and catalyzes the conversion of lysine to cadaverine. This inducible enzyme has been known for many years and has been well characterized (Sabo et al., 1974a,b). The existence of the biodegradative lysine decarboxylase did not explain the appearance of cadaverine and its aminopropyl derivative in putrescine-starved cells growing in minimal medium (Cunningham-Rundles and Maas, 1975; Dion and Cohen, 1972a,b; Srinivasan et. al., 1973). Recently, a biosynthetic lysine decarboxylase has been found in E. coli (Wertheimer and Leifer, 1983). It has been

suggested that cadaverine may substitute for putrescine under these conditions (Cunningham-Rundles and Maas, 1975; Dion and Cohen, 1972a,b; Hafner et al., 1775; Srinivasan et al., 1973).

## 1.3 Functions of polyamines

### 1.3.1 Are polyamines essential for growth?

The first evidence that polyamines might be essential for growth was obtained by Herbst and Snell (1948) studying Haemophilus parainfluenzae. This bacterium grew only when putrescine, spermidine or spermine was added to the synthetic medium. More recently, March and Boyle (1981) reported that this strain of Haemophilus is unable to synthesize putrescine due to the absence of the putrescine biosynthetic enzymes, ornithine decarboxylase, arginine decarboxylase, and agmatine ureohydrolase. Naturally occuring fungal mutants of Aspergillus nidulans (Sneath, 1955) and Neurospora crassa (Deters et al., 1974) have been shown to have an absolute requirement for putrescine.

Growth of some microorganisms is stimulated by the addition of polyamines to the growth medium. However, this effect was not specific as it could be be obtained by other diamines, 0.1M NaCl and KCl, or sucrose (Mager, 1955, 1959; Martin et al., 1962). Growth stimulation by polyamines and salts may be related, in these instances, to the stabilization of the bacterial membrane. Rozansky et al. (1954) examined the antimicrobial action of spermidine and

spermine and found that Gram positive cocci were more sensitive to the inhibitory action of the polyamines than Gram negative bacilli.

In recent studies two approaches have been taken to determine the effect of polyamine starvation of cells and resultant changes in cell growth. One is a genetic approach in which E. coli strains have been constructed carrying deletions in the genes for the polyamine biosynthetic enzymes. The other approach is biochemical where cells were treated with specific inhibitors of polyamine biosynthesis. Hafner et al. (1979) have constructed an E. coli strain carrying deletions in the speA, speB, speC, and speD, the genes for arginine decarboxylase, agmatine ureohydrolase, ornithine decarboxylase and S-adenosylmethionine decarboxylase, respectively. This strain which lacked putrescine and spermidine, grew indefinitely in the absence of polyamines with a growth rate one third of that found in the presence of polyamines. Since small amounts of cadaverine and its aminopropyl derivative are present in this strain, it was suggested that these amines may substitute for the polyamines and can, at least, partially replace their growth-promoting properties. However, a strain carrying a mutation in the cadA (biodegradative lysine decarboxylase) gene, in addition to the deletions in the speA, speB, speC and speD genes, still grew indefinitely at a reduced growth rate (Tabor et al., 1980). Supplementing cultures of this polyamine-starved strain with

cadaverine did not change the growth rate. It was concluded from these results that the polyamines are not absolute requirements for growth. It would also appear that cadaverine can not substitute for putrescine and spermidine to promote growth. However, evidence for a constitutive lysine decarboxylase has been reported (Wertheimer and Leifer, 1983). This enzyme may supply the cell with trace amounts of cadaverine, sufficient for growth in the absence of the other polyamines. Therefore, to unequivocally rule out that cadaverine might partially replace putrescine and allow restricted growth, a strain must be constructed carrying mutations in the constitutive lysine decarboxylase as well as the biodegradative lysine decarboxylase and the polyamine biosynthetic enzymes.

An interesting observation was that the transduction of certain strA (rpsL, streptomycin resistance) genes into a strain of E. coli with deletions in the speA, speB, and speC genes produced an absolute requirement for polyamines (Tabor et. al., 1981). Tabor (1981), and Tabor and Tabor (1984) suggest that since the rpsL mutation is in S12 ribosomal protein, a change in ribosomal structure or conformation is responsible for the polyamine requirement. Another possible explanation for the polyamine requirement is based on the fact that rpsL mutations are known to decrease ambiguity in translation (Gorni, 1969). If polyamine deficiency increases this effect, growth would be inhibitied due to loss of essential proteins that require ambiguity for their synthesis (Tabor and Tabor, 1984).

Using E. coli mutants defective in certain polyamine biosynthetic enzymes, several investigators have attempted to answer two important questions. 1) Are the roles of putrescine and spermidine unique or can they substitute for one another? 2) If putrescine and spermidine are necessary for normal growth, as is suggested by previous work, what structural constraints are there for the amine requirement? Under conditions which led to the depletion of putrescine and a slightly elevated level of spermidine, cells grew at the normal wild-type rate (Morris and Jorstad, 1970). In contrast, cells depleted of spermidine due to a mutation in the speD gene have two times the wild-type content of putrescine, yet grew at three-quarters the rate of the wild-type strain (Tabor et al., 1978). This is approximately two times faster than a strain totally depleted of polyamines. These results imply that putrescine can substitute for the growth promoting effect of spermidine although less efficiently and, further, that cells with wild-type spermidine levels, but no putrescine, are physiologically normal; i.e. putrescine is not required for normal growth.

It is important to note that aberrant spermidine to putrescine ratios result in physiological abnormalities. For example, the cell size of strains with a high spermidine to putrescine ratio were 50% larger than cells with reduced spermidine to putrescine ratio, i.e. wild-type strains (Jorstad et al., 1980).

To answer the second question of structural constraint on the amine requirement, Jorstad et al. (1980) examined the effect of a homologous series of spermidine analogues on the growth of an E. coli strain depleted of putrescine and spermidine . The structure of these analogues was $NH_2-(CH_2)_X-NH(CH_2)_3-NH_2$, where X varied from 3 to 8 (X=4 is spermidine). Where X=3, the growth rate of the speA mutant was stimulated to the same extent as for spermidine supplemented cultures. Increasing the length of the four methylene chain of spermidine reduced the ability of the spermidine analogue to stimulate growth, such that the analogue with X=8 had no effect on the growth of the polyamine-starved strain. Clearly, there are structural constraints on the growth-stimulating activity of this triamine. The nature of this structural constraint and its potential interaction with DNA remain elusive.

Inhibitors of polyamine biosynthesis have been effective in limiting the rapid proliferation of mammalian cells and of parasitic protozoa (reviewed in Morris, 1978b; Pegg and McCann, 1982; Tabor and Tabor, 1984). Initial studies with bacteria in which irreversible inhibitors of ornithine decarboxylase and arginine decarboxylase were used show little or no effect on growth rate (Kallio et al., 1982). This may have been due to not sufficiently depleting cells of polyamines. Putrescine levels must become undetectable and spermidine levels must be reduced to 25% of wild-type levels before an effect on growth rate is seen (Morris, 1981).

In a later study, E. coli and Pseudomonas were treated with a combination of inhibitors which included DL-α-monofluoromethylornithine and DL-α-difluoromethylarginine, irreversible inhibitors of ornithine decarboxylase and arginine decarboxylase, respectively, and with dicyclohexylammonium sulphate, a competitive inhibitor of spermidine synthetase (Betonti et al., 1982). Incubation of cells in the presence of these inhibitors caused a marked decrease in the intracellular putrescine and spermidine levels as well as a reduction in growth rate. Addition of putrescine and spermidine to cultures of inhibitor-treated cells increased the intracelluar level of putrescine by 50% and spermidine to 100% of the control value and restored the growth rate to normal.

Despite subtle physiological changes brought about by aberrant spermidine to putrescine ratios (see above), polyamine-starved cells have been shown to be normal with respect to relative levels of stable RNA, the degree of methylation of RNA, the stability of stable RNA, and the control of RNA synthesis during amino acid starvation. Futhermore, the stability of β-galactosidase mRNA is slightly increased, and there is no change in the rate of protein turnover (Morris, 1973; Morris and Jorstad, 1973; Srinivasan et al., 1973)

Several conclusions can be drawn from the studies of polyamine starved cells. First, it seems that putrescine and spermidine are not absolute requirements for growth of

E. coli, but are required for normal growth. This suggests that the action of the polyamines at their cellular site(s) is probably stimulatory. Quite likely other amines and cations substitute for the polyamines in polyamine-starved cells. Second, spermidine can replace putrescine, although certain subtle changes are apparent, i.e. cell size; and optimal growth is maintained by spermidine and not any analogue of spermidine.

1.3.2 Polyamines and nucleic acids

Numerous studies have shown that polyamines bind tightly to nucleic acids (reviewed in Bachrach, 1973; Cohen, 1971; Tabor and Tabor, 1976). The association of spermidine and DNA was characterized as a strong non-covalent interaction between the positively charged base and the negatively charged phosphate of the nucleic acid. The binding of the polyamines to nucleic acids may explain the ability of these cations to protect nucleic acids against enzymatic degradation and thermal denaturation. Also, early studies described the precipitation of DNA by polyamines from a variety of sources.

The packaging of DNA in various bacteriophage particles seems to be dependent on the polyamines. For example, spermidine is required for packaging bacteriophage lambda DNA in vitro (Kaiser et al., 1975). Circumstantial evidence suggests that spermidine may be required for packaging bacteriophage T7 DNA as the DNA exists in a compact form in

the presence of this polyamine (Gosule and Schellman, 1976). Furthermore, approximately 40% of the cations associated with the DNA of bacteriophage T4 are polyamines (Ames and Dubin, 1960). Since the bacteriophage particle is impermeable to external polyamines, the polyamines associated with the DNA of the virion could not have been taken up following packaging. This is an important point as most studies that attempted to determine the intracellular location of the polyamines have been plagued by redistribution of these polycations among polyacid components of subcellular fractions following cell disruption. Therefore, the finding of polyamines associated with bacteriophage DNA has led to the idea that the polyamines, and spermidine in particular, are necessary for condensation of the DNA to permit proper packaging. However, polyamine-starved strains of E. coli serve as suitable hosts for a number of bacteriophage, including T4 and T7, albeit at a slower rate of production and with a smaller burst size (Hafner et al., 1979; Tabor, 1981; Tabor and Tabor, 1984). These bacteriophage contain no amines, indicating that some other cation is able to substitute for the polyamines (Hafner et al., 1979; Tabor and Tabor, 1984). Cells depleted of polyamines do not serve as hosts for bacteriophage lambda (Tabor, 1981). The molecular basis for this defect is not known. Nash (1975) reported an absolute requirement for spermidine during integration of bacteriophage lambda in vitro, suggesting a role for this

cation in this recombination event. Excision of lambda is stimulated by spermidine (Gottesman and Gottesman, 1975).

Several lines of evidence indicate that spermine and spemidine influence DNA stability and tertiary structure. The transforming ability of Bacillus subtilis DNA is lost following heating at 75°C, whereas, in the presence of $10^{-4}$ M spermine the DNA can be heated to 90°C without loss of transforming ability (Tabor, 1961). Spermidine and diamines at higher concentrations also stabilize transforming DNA. The DNA of bacteriophage lambda is protected from shearing by spermine, suggesting a stabilization and possible condensation of the nucleic acid (Kaiser et al., 1963). Also of significance is the finding that spermidine maintains isolated nucleoids of E. coli in a folded configuration (Flink and Pettijohn, 1975).

Recently, Lipetz et al. (1980) and Liu and Wang (1978) reported that the activity in vitro of Micrococcus luteus and E. coli DNA topoisomerases are modulated by polyamines. DNA gyrase, which introduces negative supercoils into circular DNA, is stimulated by spermidine (Liu and Wang, 1978). The DNA topoisomerse 1, which relaxes supercoiled DNA, is inhibited by spermidine (Lipetz et al., 1980). Therefore, spermidine has the potential to increase the negative helical twist of the DNA in vivo. This is a provocative finding in that the degree of superhelicity of bacterial and viral DNAs influences gene expression as measured by patterns of protein and RNA synthesis (Botchan,

1976; Botchan et al., 1973; DeWyngaert and Hinkle, 1979; Gomez-Eichelman, 1981; Kano et al., 1981; Menzel and Gellert, 1983; Smith et al., 1978; Yang et al., 1979), and RNA polymerase binding to DNA (Richardson, 1975; Wang, 1974). Increased superhelicity may induce melting at the promoter region of genes, thereby stimulating formation of initiation complexes between RNA polymerase and the DNA (Botchan, 1976; Benham, 1979; Hsieh and Wang, 1975; Vollenweider et al., 1979).

Recently, Russell et al. (1983) reported the conversion of B-DNA to Z-DNA in the presence of low concentrations of polyamines. This effect is not specific as polyaminoacids, histones, and divalent cations at higher concentrations could elicit this effect. However, this may prove to be a significant observation because of the increasing importance that Z-DNA is thought to play in biological systems (reviewed in Rich et al., 1984). Polyamines also interact with RNA. This was first deduced from studies in which RNA viruses, total RNA and synthetic polyribonucleotides were precipitated by the polyamines (reviewed in Bachrach, 1973). Cohen et al. (1971, 1978) were the first to show that under appropriate isolation conditions, spermidine is bound to tRNA. Crystallographic studies have shown that there are two classes of spermidine binding sites on various tRNAs. Approximately two spermidine molecules bind very strongly and in a cooperative manner to a tRNA molecule. One molecule binds in the major groove near the anticodon stem

and the other near the variable loop. The cooperativity of binding is lost if the tRNA molecule is cleaved into large fragments. The other class of binding sites is less well defined with approximately 16 molecules of spermidine binding weakly per tRNA molecule. Cohen (1978) proposed that spermidine might serve to stabilize the helical regions of the tRNA molecule.

Little attention has been paid to the role of polyamines in the organization and stability of the RNA in bacterial viruses. It is known that approximately 1000 spermidine molecules are complexed with one viral genome of bacteriophage R17 (Fukuma and Cohen, 1975). The viral RNA from a variety of sources is condensed by spermidine (Cohen, 1978). Also, infection of E. coli by RNA bacteriophage MS2 is greatly stimulated by this polyamine. Based on the studies mentioned above, Cohen (1971, 1978) has proposed that the viral RNA and mRNA may exist in alternating linear and looped forms with the former structure required for replication and translation, and the latter required for storage and transport. The association and dissociation of spermidine would mediate these structural modulations.

### 1.3.3 Polyamines and macromolecular synthesis

The major thrust of polyamine research in recent years has been towards understanding the function of these compounds in the regulation of macromolecular synthesis. Two experimental approaches have generally been applied to

this question. One approach has been to compare changes in polyamine accumulation and the activities of their biosynthetic enzymes with changes in the rate of DNA, RNA, and protein synthesis under various conditions. The other approach has been the study of polyamine effects on macromolecular synthesis *in vitro* using crude, partially purified, and purified systems. As Morris (1981) has noted, both approaches have definite limitations. The correlative approach suffers from the difficulty of distinguishing between a cause-effect and a coincidental relationship. Often overlooked in these types of studies is the inherent problem of RNA, DNA and protein synthesis being physically and temporally linked in *E. coli*. The problem with the *in vitro* approach is that extrapolation of results *in vitro* to the situation *in situ* is often meaningless. Polyamines exhibit many non-specific interactions *in vitro* that become insignificant at physiological salt concentrations (see Morris, 1981, for examples). Also, other cations, such as magnesium, can mimic the effects of polyamines *in vitro*, although higher concentrations may be needed. With these points in mind, the discussion which follows examines some of the data gained from these types of experiments.

### 1.3.4 DNA synthesis *in vitro*

O'Brien *et al.* (1966) reported that spermine inhibited the activity of partially purified DNA-dependent DNA polymerase in the presence of native DNA primer. DNA polymerase from KB cells was likewise inhibited by spermine (Bach, 1964). In contrast, Brewer and Rusch (1966) observed stimulation by spermine of DNA polymerase activity in nuclei isolated from *Physarum polycephalum* supplied with an exogenous source of DNA and deoxynucleotide triphosphates. The discrepency in these reports was partially explained by Schwimmer (1968) who found that putrescine and cadaverine inhibited DNA-dependent synthesis by *E. coli* polymerase using DNA from various sources. By contrast, these polyamines stimulated DNA polymerase activity with nucleohistone or chromatin for template. Schwimmer proposed that the diamines displaced the histones, and further, that the histones exert a greater inhibitory effect than do the amines on DNA polymerase activity. However, this concept of simple competition between diamines and histones for the DNA is not supported by the data. At sufficiently high concentrations of polyamines a reversal of the stimulatory effect on the nucleohistone-supported reaction would be predicted. No such reversal was observed at concentrations that caused 70–80% inhibition of polymerase activity in the free DNA-primed reaction.

Polyamines stimulate partially purified DNA polymerase B, but not DNA polymerase A, from rat brain (Chui and Sung,

1972); the order of effectiveness for stimulating DNA polymerase activity was spermine>spermidine>putrescine. Spermidine stimulated polymerase activity only in the presence of magnesium. Since the template used in these studies was native DNA, not nucleohistone or chromatin, these workers explain the difference between their results and those of others (O'Brien *et al.*, 1966; Bach, 1964; Schwimmer, 1968) as due to species specificity or the preparation of the DNA polymerase.

In a series of experiments, Kornberg and coworkers (see Schekman *et al.*, 1974) showed that either spermidine or *E. coli* DNA-binding protein stimulates DNA synthesis of primed single-stranded template *in vitro*. A complete replicative form of ϕX174 was synthesized in the presence of DNA-binding protein, whereas only small fragments were made if spermidine was substituted (Geider and Kornberg, 1974; Tomizawa *et al.*, 1974). DNA synthesis of unprimed ϕX174 template seemed to require both DNA-binding protein and spermidine.

1.3.5 RNA synthesis *in vitro*

A variety of experimental work has shown that polyamines promote RNA synthesis *in vitro*. *E. coli* RNA polymerase activity was more efficiently stimulated by spermidine, than putrescine and cadaverine (Fuchs *et al.*, 1967). Polyamines promoted RNA synthesis at low ionic strengths (between 0.02 and 0.12); at high ionic strengths

purified E. coli RNA polymerase dissociates into smaller subunits. Magnesium was also effective in stimulating RNA polymerase activity, however, higher concentrations were required than of polyamines (Fuchs et al., 1967). Even in the presence of polyamines, RNA polymerase activity required small amounts of magnesium. Treadwell et al. (1976) observed that the magnesium requirement for optimal synthesis of β-galactosidase in a DNA-directed in vitro system could be reduced from 16 to 9 mM by the addition of spermidine. Using the antibiotic rifampicin to uncouple transcription from translation, the primary effect of spermidine was shown to be at the level of transcription. High concentrations of polyamines inhibit RNA synthesis in vitro possibly by causing aggregation, precipitation, or a change in the conformation of the DNA template (reviewed in Bachrach, 1973). The mechanism of polyamine-promoted RNA synthesis is not understood. However, it is possible that polyamines bind to RNA to counteract the inhibitory effect of RNA produced or to protect the RNA from nuclease activity. Many studies have shown that polyamines protect nucleic acids from thermal denaturation and enzymatic degradation (see section 1.3.2).

Polyamines may also influence RNA synthesis in a qualitative as well as a quantitative manner. Gumport and Weiss (1969) observed that RNA polymerase from Micrococcus lysodeikticus transcribed both strands of double-stranded RF ϕX174 DNA, whereas the homologous E. coli polymerase

transcribed only one strand. In the presence of spermidine, the Micrococcal enzyme copied the RF DNA asymmetrically (Gumport, 1970; Gumport and Weiss, 1969). Spermidine may also determine selective transcription of specific regions (i.e. promoter) of the DNA probably by reducing, or eliminating, initiation from non-specific regions (Ascoli, 1968).

### 1.3.6 Protein synthesis *in vitro*

Although polyamines stimulate transcription of DNA and thus enhance protein synthesis, they have more specific effects on protein synthesis than just increasing the available mRNA for translation. Results clearly show that polyamines can stimulate the synthesis of individual polypeptides to different degrees in bacterial and eucaryotic cell-free systems. In a T7 DNA-directed protein synthesis system, spermidine stimulated the synthesis of polypetides of 105,000 daltons and 42,000 daltons about 10- and 3.5-fold, respectively. The synthesis of a polypeptide of 13,500 daltons was not promoted significantly by spermidine (Watanabe *et al.*, 1983). Spermidine stimulated the synthesis of the two higher molecular weight polypeptides at the level of both transcription and translation, but the effect was most pronounced at the level of translation. Putrescine caused differential stimulation but to a smaller degree. A similar differential stimulation by polyamines of phage RNA- directed synthesis of polypetide

has also been observed (Watanabe et al., 1981). Spermine, spermidine, and putrescine stimulate the translation in vitro of adenovirus mRNA, globin 9S mRNA, RNA from the bacteriophage R17, QB and MS2 (Atkins et al., 1975). Significantly, the addition of polyamines to the system altered the relative amounts of ten adenovirus polypeptides synthesized to relative amounts similar to those synthesized in vivo. This was mainly due to the preferential increased synthesis of the higher molecular weight proteins. Therefore, these studies suggest that polyamines are necessary for the completion of larger polypeptides in vitro, if not in vivo. This effect may be related to protection of RNA by polyamines to nuclease digestion.

Studies in vitro show that polyamines stimulate many of the intermediate steps in protein synthesis. The observations of some of these studies are summarized below:

a) Aminoacyl-tRNA synthesis. Magnesium is required for both the pyrophosphate exchange (the first step in the reaction) and for the overall aminoacylation. Spermidine can completely substitute for magnesium in the overall reaction, but not in the first reaction step. (Choursterman and Chapeville, 1973; Kayne and Cohn, 1972; Igarashi et al., 1972; Takeda and Igarashi, 1969). Some studies have suggested that these results are artifactual due to addition of magnesium as a contaminant in the tRNA added after the first reaction step (Chakraburtty et al., 1975; Santi and Webster, 1975).

b) Ribosome aggregation. Polyamines can replace much of the ribosomal magnesium and, like magnesium, cause 30S and 50S subunit association in vitro (Cohen, 1971, p 179ff; Zitomer and Flaks, 1972)

c) Peptide initiation. Polyamines reduce the requirement for magnesium during initiation complex formation between aminoacyl-tRNA, mRNA and ribosomes (Igarashi et al., 1972, 1974; Konecki et al., 1975; Teraoko and Tanaka, 1973a).

d) Peptide elongation. Spermine, in the presence of low magnesium concentrations, promotes peptidyl transferase activity of ribosomes (Teraoko and Tanaka, 1973b).

e) The fidelity of the translation process is increased in the presence of polyamines in vitro (Abraham et al., 1979; Abraham and Pihl, 1979; Jelenc and Kurland, 1976; reviewed in Tabor and Tabor, 1976; 1984). At high temperatures (37 °C) and high magnesium concentrations the effect on fidelity of translation is lost (Abraham et al., 1979). The fidelity of translation seems to be increased by polyamines in vivo, also (Tabor and Tabor, 1984 and refesences therein).

### 1.3.7 In vivo studies

Numerous studies have shown that increases in the accumulation of polyamines and the activities of their biosynthetic enzymes parallel or precede increases in the rates of DNA, RNA, and protein syntheses (reviewed in

Bachrach, 1973; Cohen, 1971; Tabor and Tabor, 1976). Others have examined polyamine levels in relaxed and stringent strains of E. coli mainly in an attempt to implicate guanosine-5'-diphosphate-3'-diphosphate (ppGpp) in the regulation of polyamine synthesis (see section 1.4.4). Unfortunately, these studies have been unsuccessful in demonstrating a direct control of macromolecular synthesis by the polyamines in vivo.

The discovery and synthesis of specific inhibitors of the polyamine biosynthetic enzymes has proven to be a powerful tool to examine the role of polyamines in macromolecular synthesis. However, the results of inhibitor studies have been equivocal. Eucaryotic cells treated with the ornithine decarboxylase inhibitor, α-hydrazino-ornithine, or by the adenosylmethionine decarboxylase inhibitor, methylgyloxal bis(guanylhydrazine), decreased polyamine levels, but had no effect on RNA synthesis (Fillingame et al., 1975; Harik et al., 1974; Kay and Pegg, 1973). Fillingame et al. (1975) observed that the block in spermidine synthesis caused a decrease in DNA replication that could be reversed by exogenous spermidine. In a similar study, inhibition of ornithine decarboxylase activity by α-methylornithine depleted rat hepatoma cells of putrescine and spermidine, and caused arrest of cell proliferation and DNA synthesis (Mamont et al., 1976). Based on these results, it was proposed that spermidine and spermine are necessary for enhanced DNA replication in

eucaryotic systems. One report found that there was no change in DNA synthesis following inhibition of polyamine biosynthesis (Harik et al., 1974).

Little use has been made of specific inhibitors of procaryotic polyamine biosynthetic enzymes to unravel possible role(s) of the polyamines in macromolecular synthesis. Most studies that have used these inhibitors have restricted the physiological parameters examined to growth rate (see section 1.3.1). A great deal of experimental work has taken advantage of conditional polyamine auxotrophs. Polyamine depletion was produced by exogenous arginine in an E. coli mutant defective in agmatine ureohydrolase. Exogenous arginine represses the arginine biosynthetic pathway such that putrescine is not formed from ornithine (Morris et al., 1970). Since the arginine to putrescine pathway is blocked due to the mutation in agmatine ureohydrolase, the cells are starved for polyamines. Dion and Cohen (1972b) observed that polyamine depletion in these cells caused an inhibition of both growth rate and nucleic acid synthesis. Growth and nucleic acid synthesis were restored to wild-type levels by exogenous putrescine and spermidine. Bacteriophage T4 infection of these polyamine-depleted cells resulted in a reduction in phage DNA synthesis and maturation which was reversed by addition of polyamines 15 min prior to infection (Dion and Cohen, 1972a). Using a similar conditional putrescine auxotroph and exogenous arginine to deplete the

culture of polyamines, Young and Srinivasan (1972) found that addition of putrescine caused a rapid increase in protein synthesis. Approximately 1-2 h later RNA and DNA syntheses were stimulated, and these were coincident with an increase in the rate of cell division. Experiments from the laboratory of Morris have confirmed these observations. Geiger and Morris (1980) found that the temporal response of macromolecular synthesis was faster than that reported by Young and Srinivasan (1972), but attributed the differences to the strains used and to the different polyamines provided. Also, during steady-state, polyamine-limited growth of E. coli, the elongation rates for β-galactosidase mRNA and protein as well as DNA replication fork movement were reduced in proportion to growth rate i.e. two-fold (Geiger and Morris, 1978, 1980; Jorstad and Morris, 1974; Morris and Hansen, 1973). The effect of polyamine depletion on the rate of movement of the DNA replication fork indicates an effect independent of the decreased growth rate. Usually, changes in the growth rate affect initiation rather than movement of the replication fork (Geiger and Morris, 1978, 1980).

These studies have revealed that significant molecular events occur following polyamine addition to polyamine depleted cells prior to changes in nucleic acids, i.e. stimulation of protein synthesis appears to be the primary effect of polyamines on polyamine starved cells. Therefore, the stimulation of RNA and DNA synthesis, and the dramatic

increase in viral DNA replication caused by addition of polyamines to polyamine starved cells may be either an indirect effect, or merely a general response to resumption of active protein synthesis (Young and Srinivasan, 1972).

Tabor and Tabor (1976) summarized the results of the most important studies as follows:

a) Polyamines and their biosynthetic enzymes are ubiquitous.
b) Microbiological mutants have been described in which there is a definite requirement of polyamines for growth.
c) The concentrations of polyamines and their biosynthetic enzymes increase when the growth rate increases. These increases usually precede or are simultaneous with increases in RNA, DNA and protein levels.
d) Ornithine decarboxylase has a remarkably fast turnover rate in animal cells, and the level of this enzyme rapidly changes after a variety of growth stimuli.
e) Polyamines have a high affinity for nucleic acids and stabilize their secondary structure. They are found associated with DNA in bacteriophages and have a variety of stimulatory effects on DNA and RNA biosynthesis *in vitro*.
f) Polyamines stimulate protein synthesis *in vivo* and *in vitro*.
g) Polyamines protect spheroplasts and halophilic organisms from lysis, indicating their ability to stabilize membranes.
Despite these observations, no specific mechanism has been firmly established for the action of the polyamines *in vivo*. It is clear that these compounds are physiologically important, however, and further work is necessary to establish the mechanism of their action.

After almost a decade of intensive investigation the physiological role of polyamines still remains elusive.

## 1.4 Regulation of the putrescine biosynthetic enzymes in E. coli

The regulation of the putrescine biosynthetic enzymes in E. coli involves a complex set of mechanisms acting at transcriptional and post-translational levels. Since most studies have relied upon measurement of the biological activities of these enzymes in vitro, it is often difficult to evaluate the true role of various effector molecules in vivo.

### 1.4.1 Feedback inhibition and repression

The polyamines putrescine and spermidine competitively inhibit ornithine decarboxylase and arginine decarboxylase of E. coli in crude and purified preparations. A mixture of competitive and non-competitive inhibition of a partially purified preparation of ornithine decarboxylase by the polyamines has been described by Morris et al. (1970). They concluded that the competitive inhibition was the result of competition between the polyamines and ornithine for the active site. Non-competitive inhibition, however, was due to interaction of polyamines with free pyridoxal- phosphate. Further purification of ornithine decarboxylase eliminated the apparent non-competitive inhibition by polyamines (Applebaum et al., 1977). At concentrations of 7.4 mM ornithine, slightly above the $K_m$ of 5.6 mM, 20 mM putrescine or 5 mM spermidine inhibited 50% of the activity of purified ornithine decarboxylase (Applebaum et al., 1977).

At saturating concentrations of arginine (7.4 mM arginine; $K_m$ =0.03 mM) and 1 mM magnesium, 50% of the activity of purified arginine decarboxylase was inhibited by 15 mM putrescine or 1.5 mM spermidine (Wu and Morris, 1973). Increasing concentrations of magnesium, a cofactor of arginine decarboxylase activity, fully reversed the inhibition by the polyamines. The competition between magnesium and the polyamines suggests that the ratio of intracellular magnesium to polyamines may be one mechanism for regulating arginine decarboxylase in vivo. Assuming that intracellular polyamines are not bound to intracellular components, the levels of putrescine and spermidine that are inhibitory to ornithine decarboxylase and arginine decarboxylase are in the physiological range, i.e. 25 mM putrescine and 2.5 mM spermidine (Morris et al., 1970). This suggests that feedback inhibition may play an important role in regulating the putrescine biosynthetic enzymes. Feedback inhibition occurs in vivo as evidenced by the rapid reduction in the release of $^{14}CO_2$ from [$^{14}$C]-arginine or [$^{14}$C]-ornithine following supplementation of a chemostat culture with polyamines (Tabor and Tabor, 1969).

Tabor and Tabor (1969) were the first to suggest that synthesis of ornithine decarboxylase and arginine decarboxylase are regulated by feedback repression in E. coli. The specific activity of both ornithine decarboxylase and arginine decarboxylase, assayed under optimal conditions, decreased following addition of either

putrescine or spermidine. Work by Morris et al. (1970) corroborated these findings using a mutant carrying a partial block in arginine decarboxylase. A putrescine deficiency is produced in this strain by addition of arginine to the growth medium as a result of repression of the arginine biosynthetic pathway (Maas, 1961), and hence there is a limitation of ornithine. Under these conditions, the specific activity of ornithine decarboxylase and the mutant arginine decarboxylase are derepressed. Cells with normal levels of putrescine did not show derepression of the enzyme activites following arginine supplementation. Since the interpretation of these results was potentially ambiguous due to the partial block in arginine decarboxylase, Satishchandran and Boyle (1984) repeated these experiments using mutants deleted in either the arginine decarboxylase or agmatine ureohydrolase genes. These authors also concluded that putrescine repressed the synthesis of ornithine decarboxylase and arginine decarboxylase.

The regulation of the activity and synthesis of agmatine ureohydrolase is probably very different from that of ornithine decarboxylase and arginine decarboxylase. Putrescine and spermidine have no effect on either the synthesis or the activity of agmatine ureohydrolase in E. coli (Morris et al., 1970; Satishchandran and Boyle, 1984). However, the specific activity of agmatine ureohydrolase is increased by agmatine. This increase in the specific

activity of agmatine ureohydrolase can be blocked by chloramphenicol suggesting that agmatine induced the synthesis of agmatine ureohydrolase (Satishchandran and Boyle, 1984). Therefore, the metabolic flux through the putrescine biosynthetic pathway II may regulate this enzyme.

1.4.2 Protein activators, inhibitors and anti-antizyme

The direct physiological role of putrescine and spermidine in regulating the putrescine biosynthetic enzymes in E. coli is obscured by the finding of specific protein inhibitors and activators of ornithine decarboxylase, and possibly arginine decarboxylase and agmatine ureohydrolase. A protein of 15,000 daltons inhibits ornithine decarboxylase activity non-competitively has been isolate from E. coli (Kyriakidis et al., 1978). The enzyme-inhibitor complex can be dissociated by salt to provide active ornithine decarboxylase and inhibitor. The inhibitor, termed antizyme to ornithine decarboxylase or ODC-antizyme, has many characteristics in common with an inhibitor of ornithine decarboxylase activity isolated from eucaryotic cells. Like the eucaryotic ODC-antizyme, the ODC-antizyme of E. coli is induced twenty-fold by polyamines and levels appear to vary with the growth phase (Kyriakidis et al., 1978). At late exponential growth, levels of ODC-antizyme as well as ornithine decarboxylase activity are maximal. Upon entering stationary phase, the activity of ornithine decarboxylase and antizyme decrease dramatically.

A thermostable, non-dialyzable factor that is resistant to trypsin has been reported to activate ornithine decarboxylase activity in E. coli (Kyriakidis et al., 1978). The amount of activator, like antizyme, is stimulated 1.6-fold in response to increased polyamine levels in the growth medium. Its levels also vary with the growth phase of the cells in a similar manner as ornithine decarboxylase and antizyme. The ratio of ODC- activator units to ODC-antizyme units drops from 50:1 to 4:1 when cells are grown in the presence of polyamines.

Recently, Heller et al. (1983a,b) reported that ODC-antizyme activity could be resolved into at least three distinct proteins, one acidic and two basic proteins. Approximately 90% of the total antizyme activity is associated with the basic proteins. The molecular weight of 43,000 was determined for the acidic protein by sodium dodecylsuphate-polyacrylamide gel electrophoresis and by gel filtration in the presence of Nonidet P-40 as 49,000. The molecular weight of this acidic antizyme differs markedly from the molecular weight estimate of 15,000 for the acidic antizyme originally determined by gel filtration in the absence of Nonidet P-40. The authors offer no explanation for this discrepancy (Heller et al., 1983a). The molecular weights of the two basic proteins were estimated from their mobilities on sodium dodecylsulphate-polyacrylamide gels to be 11,000 and 9,000. The basic antizymes not only inhibited ornithine decarboxylase but, to a lesser extent, arginine

decarboxylase. These antizymes had no effect on the activities of the biodegradative arginine or ornithine decarboxylases. During purification of agmatine ureohydrolase, Satishchandran and Boyle (Personal communication,1984) found the presence of a factor, possibly a protein, that inhibited agmatine ureohydrolase activity which may possess characteristics similar to the other antizymes.

An additional factor that may regulate ornithine decarboxylase activity has been isolated from E. coli that inhibits the activity of ODC-antizyme (Heller et al., 1983a). This factor is very similar to the anti-antizyme of ornithine decarboxylase isolated from rat liver by Fujita et al. (1982). Addition of this macromolecule to a mixture of ornithine decarboxylase and antizyme(s) reverses the inhibition of ornithine decarboxylase activity by antizyme, possibly by dissociating the complex of enzyme-antizyme. This factor also inhibits antizyme activity prior to addition of ornithine decarboxylase, which suggests a reversibility of antizyme action. It is possible that anti-antizyme is an inactive form or degradation product of ornithine decarboxylase. Anti-antizyme has no ornithine decarboxylase activity, itself. On gel filtration columns the anti-antizyme elutes immediately following (E.coli) (Heller et al., 1983a), or at about the same position (rat liver) (Fujita et al., 1982) as ornithine decarboxylase. Thus, the factor is of similar molecular weight to ornithine

decarboxylase. However, the factor shows little affinity for pyridoxal phosphate and a much greater affinity for antizyme than ornithine decarboxylase.

Although Canellakis and coworkers argue that antizyme(s) functions to regulate putrescine biosynthesis (Heller et al., 1983b), it is difficult to discern how, or why, the cell produces five proteins acting in concert as activators, antizymes, and anti-antizyme to regulate one, or possibly two enzymes, i.e. ornthine decarboxylase and arginine decarboxylase. It is tempting to speculate that antizyme is not involved in the regulation of putrescine biosynthesis, per se, but has some other function when complexed to ornithine decarboxylase. This is especially intriguing considering the histone-like properties of the basic antizymes and that eucaryotic ornithine decarboxylase stimulates transcription (Manen and Russell, 1975, 1977). It will be interesting to see if the antizyme(s) exhibit DNA-binding properties.

1.4.3 Pathway selection

As mentioned above, E. coli can synthesize putrescine by two pathways that branch off the arginine biosynthetic pathway. In unsupplemented medium, 75% to 90% of putrescine is formed by the decarboxylation of ornithine (pathway I). In minimal medium supplemented with arginine, pathway II is mainly utilized with no change in the nett production of putrescine (Morris et al., 1970). Since the specific

activities of the putrescine biosynthetic enzymes do not change following arginine supplementation, it was proposed that pathway selection by arginine was related to the intracellular pools of ornithine and arginine (Morris et al., 1970). Maas (1961) had previously shown that arginine represses the formation of most of the enzymes of the arginine biosynthetic pathway, as well as inhibiting the activity of acetylglutamate synthetase. Thus, as a result of exogenously supplied arginine, the intracellular pool of ornithine is diminished. This would explain the marked decrease in putrescine production by pathway I (ornithine to putrescine) in cells growing in arginine supplemented medium.

The increased utilization of pathway II by cells in arginine supplemented minimal medium could not be explained by induction of arginine decarboxylase and agmatine ureohydrolase as the specific activities of these enzymes remained unchanged (Morris et al., 1970). Futhermore, the intracellular concentration of arginine was ten-fold higher than the $K_m$ for arginine decarboxylase and increased by only 25% following arginine supplementation. Therefore, activation could not readily be explained by substrate limitation unless the substrate was inaccessible to the enzyme. Morris et al. (1970) proposed that there might be a specific channelling of exogenously supplied arginine into putrescine and little of the arginine synthesized endogenously would be accessible to arginine decarboxylase.

The idea that arginine decarboxylase was compartmentalized in the E. coli cell was also suggested by studies of Tabor and Tabor (1969). An E. coli mutant, blocked between acetylornithine and ornithine, was grown in a chemostat on limiting amounts of radioactively labelled citrulline and arginine. Based on the amount of radioactivity in the polyamines and protein, it was estimated that between 40% to 60% of putrescine was formed from the exogenously supplied arginine prior to the amino acid equilibrating with endogenously derived arginine. It has since been shown that arginine decarboxylase resides in the inner periplasmic space (Buch and Boyle, personal communication, 1984). Thus, compartmentalization of arginine decarboxylase in the inner periplasm further supports the contention that exogenous arginine can be preferentially channeled into polyamines and accounts for the increased utilization of pathway II.

1.4.4 Nucleotide effects and the stringent response

The activity of ornithine decarboxylase is influenced by certain nucleotide phosphates (Applebaum et al., 1977; Holtta et al., 1972). At subsaturating concentrations of ornithine, the ribonucleotide triphosphates, GTP and ATP, the deoxyribonucleotide phosphate, dGTP, and some other ribonucleotide mono- and diphosphates stimulated the activity of a partially purified preparation of ornithine decarboxylase (Holtta et al., 1972). In particular, 1 mM GTP stimulated the enzyme activity by four to five-fold and

reduced the $K_m$ for ornithine from 2 mM to 0.2 mM. This reduction of the $K_m$ by GTP explained the dilemma that the apparant $K_m$ in the absence of GTP is almost one hundredfold higher that the estimated intracellular level of ornithine (Applebaum et al., 1977; Holtta et al., 1972). The low concentrations of GTP required to stimulate ornithine decarboxylase activity ($K_m$ < 1 μM) suggests that this, or some similar nucleotide, may be an integral part of the enzyme molecule (Holtta et al., 1972). Because GTP can reverse the inhibitory effect of high ionic strength, Holtta et al. (1972) further suggest that GTP might be involved in subunit interactions. However, the mechanism of activation of ornithine decarboxylase by this and other nucleotides is still unknown.

A number of studies have attempted to implicated the nucleotide, guanosine 5'-diphophate 3'-diphosphate (ppGpp) in the regulation of ornithine decarboxylase. When E. coli cells are starved for an essential amino acid there is coordinate cessation of RNA and protein synthesis with a concomittant rise in the level of ppGpp (Gallant, 1979). This phenomenon has been termed the stringent response. E. coli strains carrying a lesion in the relA gene exhibit a relaxed respone to amino acid starvation, that is cessation of protein synthesis without the coordinate cessation of RNA synthesis. Moreover, these strains fail to accumulate ppGpp, since the relA gene is required for its synthesis. Several studies indicatethat ppGpp inhibits transcription of

ribosomal RNA and some mRNA species as well as stimulates the transcription of certain other mRNA species (Gallant, 1979). Much of this work, however, has only shown a correlation between ppGpp levels and RNA synthesis and not a direct transcriptional effect on gene expression by ppGpp.

Cohen et al. (1967) have shown that there was a 90% inhibition of RNA synthesis and little synthesis or accumulation of spermidine in a stringent strain (15 TAU $rel^+$) when starved for the amino acid arginine. In an isogenic strain (15 TAU $rel^-$) there was no cessation of RNA synthesis nor inhibition of synthesis and accumulation of spermidine. These results suggested that ppGpp is involved in the regulation of polyamine biosynthesis. However, these experiments have been criticized for the choice of the 15 TAU$^-$ strain, because of its complex nutritional requirements of arginine, glutamate and uracil (Edlin and Broda, 1968). In another study using an isogenic pair of stringent and relaxed strains, CP78 ($rel^+$) and CP79 ($rel^-$), the activity of ornithine decarboxylase and the accumulation of putrescine and spermidine were determined during amino acid starvation (Holtta et al., 1974). The activity of ornithine decarboxylase, assayed in crude lysates, decreased following amino acid starvation of the stringent strain. The intracellular accumulation of putrescine and spermidine also ceased. In contrast, the relaxed strain did not exhibit the decrease of ornithine decarboxylase activity and continued to accumulate putrescine and spermidine intracellularly.

Significantly, the inhibition of ornithine decarboxylase activity caused by amino acid starvation of the stringent strain could be reversed by the addition of GTP to the crude lysates. Since GTP is an activator of ornithine decarboxylase, it was postulated that ppGpp may inhibit ornithine decarboxylase activity by analogue competition with GTP (Holtta et al., 1974). A partially purified preparation of ornithine decarboxylase was inhibited by millimolar concentrations of ppGpp even in the presence of 1 mM GTP. The concentration of the nucleotides used in this study reflect physiological conditions. The $K_m$ for ornithine increased from 0.36 mM in the absence of ppGpp to 3.5 mM in the presence of the nucleotide. This value approaches the $K_m$ in the absence of GTP. Thus, it seemed reasonable to conclude that the regulation of polyamine synthesis involved ppGpp. Holtta et al., (1974) speculated that the regulation of polyamine synthesis is controlled by changes in the affinity of ornithine decarboxylase for the substrate. These changes in turn are mediated by the intracellular levels of ppGpp and GTP.

In experiments designed to test this hypothesis, Sakai and Cohen (1976) confirmed the respective action of ppGpp and GTP on partially purified ornithine decarboxylase from several strains of E. coli. One of these strains, E. coli B-207, is potassium dependent. Transfer of the strain B-207 from a potassium rich medium to a potassium poor, sodium rich medium elicits a relaxed response. During the relaxed

response there is a five to eight-fold stimulation of putrescine synthesis, most of which is excreted into the medium. There is no change in intracellular or extracellular spermidine. According to the authors, however, there was no consistent correlation between the ratio of ppGpp:GTP and ornithine decarboxylase activity as measured by putrescine levels. Boyle and Adachi (1982) using a different experimental approach also attempted to to address this problem. The activities and rates of synthesis of both ornithine decarboxylase and arginine decarboxylase were determined in E. coli during exponential growth and nutritional shift-up. Changes in the activity and rate of synthesis of ornithine decarboxylase directly correlated with changes in ppGpp levels, whereas the activity and rate of synthesis of arginine decarboxylase was inversely related to ppGpp levels. Clearly there is a conflict as to whether or not ppGpp has role in the regulation of ornithine decarboxylase at either the transcriptional or post-translational level.

1.4.5 Osmoregulation

There is evidence to suggest that cellular osmolarity may influence putrescine biosynthesis in procaryotic and eucaryotic cells (Munro et al., 1972, 1975; Peter et al., 1978). The putrescine content of E. coli and various marine bacteria is inversely related to the osmolarity of the medium in which cells are grown (Munro et al., 1972; Peter

et al., 1978). Experiments in which marine bacteria were transferred to a medium of low ionic strength revealed that there was a rapid and transient rise in intracellular putrescine and spermidine (Peter et al., 1978). Maximal accumulation was reached within 30 minutes and thereafter the intracellular polyamine levels declined. By 60 minutes a new, slower rate of increase in polyamine accumulation was seen. The interpretation of these results was that the increased polyamine accumulation was the result of increased polyamine synthesis and possibly increased synthesis of their biosynthetic enzymes. The subsequent decrease in polyamine accumulation prior to the new rate of synthesis being established was explained by feedback inhibition. These authors that suggest the polyamines may take part in membrane stability and phospholipid synthesis in marine bacteria.

In similar studies with marine bacteria, it was shown that addition of a variety of charged and uncharged solutes to cells growing in low salts medium caused a rapid loss of intracellular putrescine. The excretion of putrescine was an energy dependent process requiring potassium. These observations suggest a role for polyamines in osmoregulation (Munro and Sauerbier, 1973).

The activity of ornithine decarboxylase from E. coli is inhibited in vitro by 0.1 M to 0.2 M concentrations of NaCl, KCl and acetate salts. Also, the activity of ornithine decarboxylase and polyamine accumulation in several

eucaryotic cells is stimulated by low osmolarity and inhibited by specific cations and hypertonic conditions (reviewed in Bachrach, 1973). Recently, it was reported that in E. coli treated with D,L-α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, the intracellular concentrations of putrescine and spermidine were reduced by 60%, whereas intracellular concentrations of sodium and potassium were not significantly changed regardless of the osmolarity of the medium (Günther and Peter, 1979). Therefore, it seems that polyamines are not involved in long-term steady-state osmoregulation in E. coli. However, this does not prove that osmolarity does not influence polyamine synthesis. Whether putrescine and spermidine are involved in osmoregulation, or whether osmoregulation regulates polyamine biosynthesis, remains to be elucidated.

## 1.5 Cyclic AMP-a regulator of gene activity

### 1.5.1 Positive regulation

E. coli and other members of the Enterobacteriaceae are able to utilize a wide range of compounds as carbon and nitrogen sources. Many of these compounds are catabolized by inducible pathways. In addition to substrate, cAMP and the cAMP receptor protein (CRP) are required for activation of the catabolite-sensitive operons which code for these pathways.

The currently accepted model for cAMP stimulation of gene activity was first proposed by Pastan and Perlman (1970) with respect to the lactose operon of E. coli. Subsequently, more detailed analysis has refined this model (reviewed in Adhya and Garges, 1982; Botsford, 1981; de Crombrugghe et al., 1984; Rickenberg, 1974; Ullmann and Danchin, 1983). The addition of lactose, or a non-metabolizable analogue such a isopropyl-β-thio-D-galactoside, to a culture of E. coli induces the coordinate synthesis of large amounts of three proteins: β-galctosidase, galactoside permease, and thiogalactoside transacetylase. Subsequent addition of glucose to the culture immediately inhibits expression of the lactose operon. This phenomenon has been observed with other catabolite-sensitive operons and has been termed "catabolic repression". Catabolic repression can be overcome by the addition of cAMP to the culture. An explanation for this

effect was given by Makman and Sutherland who reported that E. coli contained cAMP, and the intracellular concentration of the nucleotide decreased in response to glucose addition. It has since been shown that the intracellular concentration of cAMP varies according to the carbon source available to the cell (Buettner et al., 1973; Epstein et al., 1975).

A detailed understanding of the mechanism by which cAMP stimulates the synthesis of inducible enzymes, such as those encoded by the lac, ara, and gal operons, has been deduced from a variety of physiological, genetic, and biochemical data.

The lactose operon consists of three structural genes, (Z, Y, and A) which code for β-galactosidase, galactoside permease and thiogalactoside transacetylase, respectively (Beckwith, 1970). There is one regulatory gene (I) and two regulatory sites (P and O) in the lactose operon. The lac I gene codes for a repressor protein that binds to the O (operator) region of the DNA and blocks synthesis of the lac enzymes. In the presence of the substrate or substrate analog, the affinity of the repressor for the operator is reduced. For efficient transciption of the lactose operon, cAMP and its receptor protein (CRP) must bind at the promoter region. The cAMP receptor protein is a dimer of two identical subunits (Eilen and Krakow, 1977). Both subunits possess a domain in the N-terminal portion of the molecule that binds cAMP. In the presence of cAMP, CRP undergoes a large allosteric transition that increases the

affinity of CRP for specific DNA sequences in the promoter ($K_d$ = $10^{-10}$ M to $10^{-13}$ M) relative to non-specific DNA sequences ($K_d$ = $10^{-7}$ M to $10^{-8}$ M) (Aiba and Krakow, 1981; Eilen et al., 1978; Kumar et al., 1980). The binding of CRP to the P region stimulates transcription by RNA polymerase. The DNA binding domain is in the C-terminal portion of the CRP molecule. According to Guiso and Blazy (1980) the amount of CRP, unlike cAMP, does not depend on the generation time of E. coli nor does it depend on the nature of the carbon source.

The regulation of the arabinose and galactose operons is more complex than regulation of the lactose operon. However, the function of cAMP-CRP in stimulating transcription of these operons is essentially the same.

The regulon that controls assimilation of L-arabinose is comprised of the araBAD operon which codes for three enzymes that convert L-arabinose to D-xylulose-5-phosphate, and in addition the araE gene codes for the permease, and the araF gene for the L-arabinose binding protein (Boos, 1972; Heffernan et al., 1976; Hogg and Englesberg, 1969; Parsons et al., 1974). The araE and araF genes are seperated and unlinked to the araBAD operon. A regulatory gene, araC, is contiguous with the araBAD genes. Transcription from araBAD and araC are from two divergent promoters situated approximately 150-170 base pairs apart (Greenfield et al., 1978; Lee et al., 1981; Smith and Schleif, 1978)). The araC protein has a dual function in

control of the arabinose operon. In the absence of L-arabinose, the *araC* protein interacts with an operator site (*araO*) to decrease expression of the *araBAD* and *araC* genes. In the presence of L-arabinose, *araC* protein is removed from *araO*, and binds to a new site, *araI*. The binding of *araC* protein to the *araI* site stimulates transcription of the *araBAD* operon. Maximal expression of *araC* and *araBAD* requires the cAMP-CRP complex that binds at one (Lee *et al.*, 1981) or possibly two (Ogden *et al.*, 1980) sites located between the divergent promoters. It has been proposed that the CRP binding site overlaps the operator (*araO*) gene and repression by the *araC* protein is due to exclusion of CRP from the promoter region (Ogden *et al.*, 1980).

Indole derivativies, imidazole and imidazole derivatives can circumvent the necessity for cAMP in the induction of the arabinose operon in adenylate cyclase-deficient (*cya*) strains of *E. coli* (Kline *et al.*, 1979; 1980a,b). The *araC* protein and CRP are essential for induction of the arabinose operon by these compounds. These indole and imidazole derivatives cannot substitute for cAMP in the induction of the *lac* and *gal* operons. The significance of this finding is that small metabolites other than cAMP may control expression of specific genes via CRP. It is not known, however, if CRP binds these indole and imidazole derivatives.

Three enzymes are required for catabolism of

D-galactose; UDP-glucose 4-epimerase, galactose 1-phosphate uridylyl transferase, and galactokinase. These enzymes, encoded by the galETK genes, are coordinately induced when D-galactose is the primary carbon source. Transcription of the galactose operon is initiated from two overlapping promoters. One promoter (P1) requires cAMP-CRP for activity. Activity from the other promoter (P2) is inhibited by the cAMP-CRP complex (Musso et al., 1977). Promoter P2 initiates transcription five nucleotides downstream from the initiation start site for promoter P1 with transcription from P1 being more efficient than from P2 (Queen and Rosenberg, 1981). There is a physiological rationale for the presence of two promoters in the galactose operon with diametrically opposed modes of regulation. Since the enzyme, UDP-glucose 4-epimerase is required for cell-wall synthesis, the galactose operon has both catabolic and biosynthetic functions. When D-galactose is the primary carbon source available to the cell, intracellular cAMP is high, therefore, cAMP-CRP stimulates P1 promoter activity and inhibits P2 promoter activity. Under conditions where cAMP levels are low, such as growth on glucose, transcription is initiated from the P2 promoter rather than P1 promoter. Thus, a low demand for epimerase activity required for cell-wall synthesis is met.

Although the specific DNA sequences that bind CRP are known for a number of genes (e.g. lac, ara, cat, pBR-P4 and deo) (reviewed in Adhya and Garges, 1982; de Crombrugghe et

al., 1984; Ullmann and Danchin, 1983), it is not clear how the cAMP-CRP complex stimulates transcription. The CRP binding site has been found to vary not only with respect to DNA sequence and symmetry but also with respect to the distance of the binding site to the initiation start site. For example, the CRP binding sites in the lac and galP1 promoters were determined by genetic studies of promoter mutations and by protection of relevant sequences by CRP from DNase digestion, methylation and UV irradiation of 5'-bromouracil substituted DNA (Schmitz, 1981; Simpson, 1980; Taniguchi et al., 1979). These data indicate that CRP recognizes a region of symmetry approximately 50 to 70 base pairs upstream of the lac initiation site (Schmitz, 1981; Simpson; 1980), whereas CRP recognizes a site approximately 30 to 50 base pairs upstream from the galP1 initiation site (Taniguchi et al., 1979). Sequences prior to position -60 in the galP1 promoter are not necessary for cAMP-CRP stimulation of gal transcription. Similar studies have shown that CRP binds around position -40 in the deoP2, cat and pBR-P4 promoters, and between -106 and -82 in the ara promoter (de Crombrugghe et al., 1984). There is another CRP binding site in deoP2 promoter at position -95 (Valentin-Hansen, 1982). Several consensus sequences have been derived from these CRP binding sites: the palindromic sequence, TGTGN CACA (Queen and Rosenberg, 1981); GTGA (Taniguchi et al., 1979); AAAGTGTGACA (O'Neill et al., 1981); and recently, AANTGTGANNTNNNNCA (Ebright, 1982).

Overall all these promoters exhibit some homology to other bacterial and phage promoters in that sequences in the -10 and -35 region are conserved. These later sequences are thought to be involved in RNA polymerase recognition and binding (Rosenberg and Court, 1979).

In addition to the primary sequence the tertiary structure of the DNA seems to be important for CRP recognition and binding. X-ray data suggest that two symmetrically related a-helices (one in each of the two subunits) interact with two symmetrical DNA sequences in the major grooves of right-handed DNA (de Crombrugghe et al., 1984). Similar models have been suggested for the interaction of the lambda Cro protein and lambda cI protein with DNA (reviewed in de Crombrugghe et al., 1984; Takeda et al., 1983).

Two models have been proposed to account for cAMP-CRP stimulation of transcription. One model requires direct interaction between CRP and RNA polymerase (Gilbert, 1976), and the other requires a CRP-induced destabilization of the DNA in the region of the promoter (Reznikoff and Abelson, 1978). Both models suggest stabilization of RNA polymerase at the promoter resulting in increased probability of the initiation of transcription. Only the latter model would easily reconcile the differences in distance between CRP and RNA polymerase binding sites seen in various CRP-stimulated genes. Evidence to support the model in which CRP induces destabilization of the promoter DNA will be considered first.

Circular dichroism studies of a synthetic undecamer containing 10 base pairs of the CRP consensus sequence as proposed by Ebright (1982) indicate that CRP, in the presence or absence of cAMP, induces a conformational change in the DNA characteristic of a B to C transition (Martin et al., 1983). This B to C transition would result in an increase in the helical twist of the undecamer while maintaining the right-handedness of the DNA. This does not, however, preclude long-range melting of the DNA.

Differences in the mobilities of CRP-lac promoter DNA complex and complexes between CRP and mutant lac promoter DNA (L8-UV and L305) in polyacrylamide gels have been offered as evidence that CRP either alters DNA conformation, or that CRP forms a transient intramolecular bridge between two domains at the promoter (Fried and Crothers, 1983). These workers also suggest that binding of a single CRP dimer to the promoter stimulates subsequent polymerase binding. Futhermore, functional CRP molecules are not readily released from the promoter following polymerase binding.

Ebright and Wong (1981) postulated that the adenyl moiety of cAMP directly intercalates with a thymine residue within the CRP binding site causing melting of promoter DNA. In this model CRP functions to position the adenyl moiety of cAMP in the correct orientation and does not necessarily influence the structure of DNA itself, but this model is probably wrong. A class of mutations in the crp gene (CRP )

that make CRP activity independent of cAMP have been characterized (Harman and Dobrogosz, 1983). In the absence of cAMP, wild-type CRP is resistant to digestion with protease, whereas in the presence of cAMP, wild-type CRP is sensitive to protease digestion. This is evidence that CRP undergoes a large allosteric transition following binding of cAMP. Protease digestion of the mutant CRP* generates a peptide pattern identical to that of the wild-type protein digested in the presence of cAMP. This suggests that the conformation of CRP* is similiar to the conformation of wild-type CRP-cAMP. Since CRP* enables expression of catabolic operons in a strain carrying a lesion in the adenylate cyclase gene, cAMP is not necessary for activation of transcription and Ebright's model is refuted.

Evidence for direct interaction between CRP and RNA polymerse is more scant. Takeda et al. (1983) have compared the characteristics of several DNA binding proteins that influence gene expression. The lambda repressor is thought to activate transcription by direct contact with RNA polymerase, and thereby stabilize the initiation start complex. Mutants of lambda repressor that are deficient in positive control but do not interfere with DNA binding have amino acid alterations at positions 34(glu-lys), 38(asp-asn) or 43(gly-arg). X-ray crystallographic studies indicate that these acidic amino acid residues are clustered together on the surface of the molecule and come into close contact with a positively charged patch of residues on RNA

polymerase. Acidic residues are found at a position in CRP that corresponds to that of the acidic residues in the lamdba repressor. Also, the recognition sequence for lambda repressor is at a similar position as that of the CRP recognition sequence relative to the RNA polymerase binding site in the promoters of the gal, araC and cat genes, and placing CRP close to RNA polymerase. Based on the similarities between CRP and lambda repressor, Takeda et al. (1983) suggest that CRP stimulates initiation by protein-protein ionic interaction between CRP and RNA polymerase."

Evidence for direct contact between RNA polymerase and CRP-cAMP has been derived from studies using antibodies against the sigma subunit of RNA polymerase. Stender (1980) showed that CRP enhanced the complement fixation response by anti-sigma of RNA polymerase in the presence and absence of cAMP and DNA. Antibodies against $\alpha$, $\beta$, $\beta'$ subunits elicited no response in the presence of CRP. This result indicates that stable complexes form in solution between CRP and RNA polymerase. However, the model for stimulation of transcription initiation by CRP-RNA polymerase interaction does not accommodate the differences in the position of the CRP and RNA polymerase binding sites in various CRP-activated operons. At present it remains to be determined whether CRP facilitates transcription by either making contact with RNA polymerase or if activation occurs by some other mechanism such as destabilization of the DNA in the region of the initiation start site.

1.5.2 Is CRP a transcriptional termination factor?

Expression of the galactose operon from the cAMP-CRP dependent promoter, P1, produces approximately equimolar amounts of the gal polypeptides. In the absence of cAMP-CRP, expression is from the P2 promoter and produces less of the distal polypeptides than of the proximal ones. Ullmann et al. (1979) have reported that cAMP-CRP mediated polarity by abolishing rho-dependent termination. Similar results were seen with a cAMP-independent lac promoter (L8UV) which ruled out possible cAMP-CRP stimulated initiation of transcription. The control of termination by cAMP-CRP is not understood. The cAMP-CRP complex may interact with RNA polymerase in an analogous manner as the N protein in bacteriophage lambda, such that termination of transcription is overcome (Campbell, 1979). Alternatively, cAMP-CRP may influence termination indirectly by changing the levels of termination factors. It has been noted by Botsford (1981) that the interpretation of the results of Ullman et al. (1979) are ambiguous because the rho-15(TS) mutation has pleiotropic effects (Adhya and Gottesman, 1979). Furthermore, termination of transcription in the lactose operon requires the product of the nusA gene (L-factor) (Greenblatt et al., 1980). Also, it has been argued that the observed polarity in the lactose operon can be accounted for by differential decay rates of the proximal and distal regions of the lac mRNA (Kennell and Reizman, 1977).

Queen and Rosenberg (1981) have offered a different explanation for the differing amounts of gal polypeptides produced in the presence or absence of cAMP than that of Ullmann et al. (1979). This is based on the differential translational efficiencies of the galP1 and galP2 mRNAs. Transcripts initiated from the P1 and P2 promoters were prepared in vitro in the presence and absence of cAMP and CRP. Hybridization experiments showed that both transcripts traversed the entire operon indicating no significant premature termination. Equal quantities of the transcripts were translated in a cell-free system. The promoter distal genes, T and K, were translated with equal efficiency by both galP1 and galP2 mRNAs. However, the promoter proximal gene, E, was translated four times more efficiently by galP2 than from galP1 mRNA. Since this four-fold difference in translational efficiency was maintained with transcripts initiating at P1 or P2 and extending just beyond the E gene, it was concluded that the mechanism responsible for differential translation of the E gene acts entirely within the 5'-proximal region of the gal mRNA. The different efficiency of translation of the E gene on the P1 and P2 mRNAs does not result from differences in the ribosome binding site for E gene on these two transcripts. However, the five additional nucleotides of the P2 transcript may increase the translational efficiency of the galP2 mRNA by affecting RNA secondary structure.

Significantly, the ratios of the galP1 and galP2

polypeptides correspond precisely to the discoordinate change in epimerase to kinase ratio observed *in vivo* during catabolic repression. Therefore, if cAMP switches transcription initiation from the *galP2* to the *galP1* promoter, the discoordinate expression of the galactose operon can be explained by differential translational efficiencies of the mRNAs rather than by termination of transcription.

1.5.3 Negative control

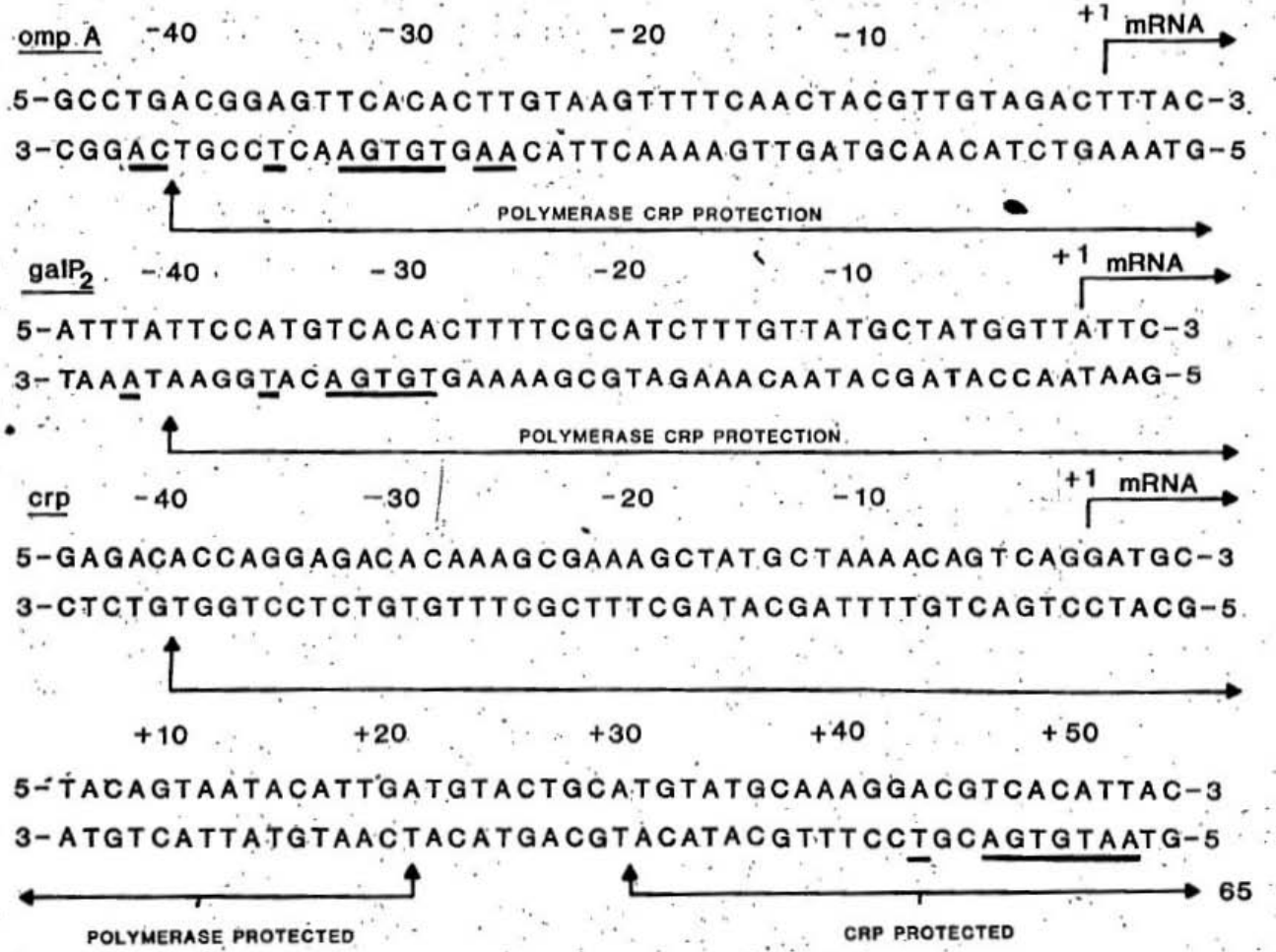
Mallick and Herrlich (1979) reported that cAMP exerts coordinate negative control on various *E. coli* genes. Cyclic AMP represses as many genes as it regulates positively. Both positive and negative control require CRP. Mallick and Herrlich have proposed that negative control of gene expression might be established at concentrations of cAMP below those required for positive control. Based on the observation that the outer membrane protein III is maximally repressed at concentrations of cAMP twenty-fold below concentrations required for maximal induction of lambda receptor (*lamB* gene product), it was suggested that two conformational states may exists for CRP or its DNA adduct. One conformation saturated with cAMP at low concentrations and exerting negative control, the other saturated at higher concentration of cAMP and mediating positive transcriptional regulation. An alternate mechanism was suggested in which cAMP-CRP induces the synthesis of a

"super repressor" that coordinates negative transcriptional control of many genes.

In addition to outer membrane protein III, several other genes have been identified that are negatively regulated by cAMP. These include the genes for glutamate synthase, glutaminase A (Prusiner *et al.*, 1972), adenylate cyclase (Majerfeld *et al.*, 1981), CRP (Aiba, 1983), galactose P2 (Musso *et al.*, 1977), *rrnB* (Glaser *et al.*, 1980)), spot 42 RNA (Sahagan and Dahlberg, 1979), and possibly the *ompA* gene (Movva *et al.*, 1981). Negative control by cAMP of several of these genes has been examined in detail. Initiation of transcription from the *galP2* promoter is inhibited by CRP excluding RNA polymerase access to the promoter due to overlapping recognition sites (Musso *et al.*, 1977) (Figure 2). Negative transcriptional control of the *ompA* gene *in vitro* appears to be mediated by a similar exclusion mechanism, although repression by CRP is not strictly dependent on cAMP (Movva *et al.*, 1981). Also, there is no evidence that the *ompA* gene is regulated by cAMP-CRP *in vivo*.

The *crp* gene is autogenously regulated by cAMP and CRP (Aiba, 1983). DNase footprinting studies have revealed that CRP recognises a sequence at position +26 to +67 in the 167 base pair leader sequence of the *crp* gene (Aiba, 1983). A second site weakly protected by cAMP-CRP from DNAse digestion was positioned around -65. Significantly, CRP and RNA polymerase bind to the *crp* gene simultaneously.

Figure 2. CRP binding sites in negatively regulated genes. The figure shows the sequences specifically recognized by cAMP-CRP in the promoter regions of the ompA, galP2 and crp genes. Numbers above the sequences indicate distances in base pairs from the corresponding start site of transcription. Horizontal arrows above the sequences indicate the direction of transcription. Bold lines below nucleotides represent sequences homologous with the consensus CRP-binding sequence (Ebright, 1982). Horizontal arrows below the sequences indicate the regions protected by CRP and RNA polymerase from DNase I digestion. Adapted from De Crombrugghe et al., 1984.


omp A
-40
-30
-20
-10
+1 mRNA
5-GCCTGACGGAGTTCACACTTGTAAGTTTTCAACTACGTTGTAGACTTTAC-3
3-CGGACTGCCTCAAGTGTGAACATTCAAAAGTTGATGCAACATCTGAAATG-5
POLYMERASE CRP PROTECTION
galP2
-40
-30
-20
-10
+1 mRNA
5-ATTTATTCCATGTCACACTTTTCGCATCTTTGTTATGCTATGGTTATTC-3
3-TAAATAAGGTACAGTGTGAAAAGCGTAGAAACAATACGATACCAATAAG-5
POLYMERASE CRP PROTECTION
crp
-40
-30
-20
-10
+1 mRNA
5-GAGACACCAGGAGACACAAAGCGAAAGCTATGCTAAAACAGTCAGGATGC-3
3-CTCTGTGGTCCTCTGTGTTTCGCTTTCGATACGATTTTGTCAGTCCTACG-5
+10
+20
+30
+40
+50
5-TACAGTAATACATTGATGTACTGCATGTATGCAAAGGACGTCACATTAC-3
3-ATGTCATTATGTAACTACATGACGTACATACGTTTCCTGCAGTGTAATG-5
65
POLYMERASE PROTECTED
CRP PROTECTED

Therefore, exclusion of polymerase from the promoter is probably not the mechanism of negative control for this gene. Since the CRP binding site is relatively far from the initiation start site, it is assumed that CRP prevents elongation of transcription rather than initiation.

There is striking homology among the DNA sequences that CRP recognizes in negatively and positively controlled genes (Figure 2). The consensus sequence for the CRP binding site in positively regulated genes (lac, ara, gal, cat, deoP) is found on the non-coding strand, whereas, the consensus sequence in negatively regulated genes (galP2, ompA, crp) is found on the coding strand. This correlation suggests that there may exist some relationship between the orientation of CRP binding site and CRP function (Aiba, 1983)

1.5.4 Protein kinases

Many regulatory processes in eucaryotic cells are mediated by cAMP-dependent protein kinases (reviewed in Krebs and Beavo, 1979: Rubin and Rosen, 1975). Of particular significance to the study here is the report that the activity of ornithine decarboxylase in S49 lymphoma cells is regulated by a cAMP-dependent protein kinase (Insel and Fennel, 1978). Both E. coli and Salmonella typhimurium possess protein kinase activity. Except for one early report of cAMP-dependent phosphorylation of histone by an E. coli protein extract (Kuo and Greengard, 1969), rigorous experimentation has shown that protein kinase activity in E.

coli and Salmonella is independent of cAMP (Ferro-Lazzui Ames and Nikaido, 1981; Malloy and Reeves, 1983; Manai and Cozzone, 1982; Wang and Koshland, 1980).

1.6 Statement of purpose

Two independent observations led to the undertaking of this study: 1) Over a ten-fold increase in growth rate of E. coli, obtained either by varying the carbon source of the culture medium or limiting glucose uptake, there is an approximately three-fold increase in the intracellular concentration of putrescine and a 3.5-fold increase in the intracellular concentration of spermidine (Boyle et al., 1977); 2) The intracellular concentration of cAMP is inversely related to the growth rate of E. coli as determined by the carbon source available to the cell; the greater the limitation of the carbon source the greater the cAMP concentration (Alpher and Ames, 1977; Buettner et al., 1973; Epstein et al., 1975; Pastan and Adhya, 1976) It was of interest, therefore, to determine whether cAMP plays a direct role in the regulation of the putrescine biosynthetic enzymes, and to determine whether cAMP exerts control of ornithine decarboxylase, in particular, at the transcriptional, translational, or post-translational level.

# Chapter 2

# METHODS

## 2.1 Bacterial strains used

The bacterial strains used in this study are shown in Table 2.

## 2.2 Bacterial growth

For the determination of biosynthetic ornithine decarboxylase, biosynthetic arginine decarboxylase, agmatine ureohydrolase, S-adenosylmethionine decarboxylase, glucose-6-phosphate dehydrogenase and β-galactosidase activities in wild-type, cya and crp deletion strains of E. coli, the following growth conditions were used. All cultures were started from single colony isolates which had been phenotypically characterized on separate MOPS-minimal plates (Neidhardt et al., 1974) containing either lactose, arabinose, maltose, glycerol or glucose. Strains were grown overnight in MOPS-minimal medium at 37°C in a reciprocal shaking water bath and used to inoculate experimental cultures to an optical density of 0.05-0.1 at 575 nm. Growth was followed spectrophotometrically (Lawrence and Maier, 1977) in cultures of 50-100 ml shaken at 37°C for 3-4

Table 2. Bacterial strains used

Escherichia coli strains:

| Organisms | Character | Source or reference |
|---|---|---|
| LS340 | trpA9605, his-85, metE70, trpR55 | Brinkman et al., 1973 |
| LS853 | trpA9605, his-85, Δcya-2, trpR55 | " |
| JW-1 | trpA9605, his-85, Δcya-2, trpR55, Gly$^+$ | This study |
| LS854.1 | trpA9605, his-85, Δcya-2, trpR55, Δcrp, rspL | Satischandran and Boyle, 1984 |
| CA8000 | thi | Dr. J. Beckwith, Harvard University |
| CA8306A | Δcya, thi | " |
| CA8306B | Δcya, thi, Gly$^+$ | " |
| HY27 | Δcya, crp, thi | Fraser and Yamazaki, 1980 |
| DM22 | pro, his, metG, serA, Δ(speC, glc) leu, rpsL, thi, hsd, sup | Boyle et al., 1984 |
| C600 | thr, leu, thi | Bachman, 1972 |
| HB101 | pro, leu, thi, rpsL20, hsdR, hsdM, ara14, galK2, xyl5, mtl1, supE44, recA | Boyer et al., 1969 |
| MRE600 | glu, rna | Cammack and Wade, 1965 |
| MA135 | thi, argE, his, trp, pro, str$^R$, speB, mal | Dr. W. Maas, New York University |
| P678-54 | thr1, leuB6, minA1, minB2, thi1, ara13, lacY1, gal6, malA1, xyl7, mtl2, rpsL135, tonA2, azi8. | Adler et al., 1967 |

Bacterial strains cont'd

| Organisms | Character | Source of Reference |
|---|---|---|
| UW44 | | Applebaum et al., 1975. |
| K-12 | Reference strain from Centre for Disease Control, Atlanta, Georgia | Dr. D. Brenner, Centre for Disease Control, Atlanta, Georgia. |

Enterobacteriaceae and other bacterial strains:

| Organism | Source |
|---|---|
| Edwardsiella tarda (4411-65) | Dr. D. Brenner Centre for Disease Control, Atlanta, Georgia. |
| Klebsiella pneumoniae II | " |
| Serratia marcescens | " |
| Morganella morganni (2325-70) | " |
| Yersinia enterocolitica (496-70) | " |
| Halobacterium halobium | Dr. W. F. Doolittle Dalhousie University |
| Halobacterium volcanii | " |
| Coccochloris peniocytis | " |
| Salmonella typhimurium | This laboratory |
| Citrobacter freundii | " |
| Enterobacter aerogenes | " |
| Proteus mirabilis | " |
| Erwinia carotovora | " |

Bacterial strains cont'd.

| Organism | Source |
|---|---|
| Pseudomonas putida | Dr. E. Barnsley Memorial University of Newfoundland |
| Bacterial DNA: | |
| Clostridium perfringens | Sigma Chemical Co. |
| Micrococcus lysodeikticus | " |

generations. Where indicated, nucleotides and isopropyl-β-thio-D-galactoside (IPTG) were included in the growth medium at 0.5-10.0 mM and 0.5 mM, respectively. Cells were harvested by centrifugation, washed with cold MOPS-minimal medium and resuspended in breakage buffer [0.1 M Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) pH 7.4; 5 mM $MgCl_2$; 5 mM dithiothreitol; 0.04 mM pyridoxal phosphate]. One twentieth of the volume of the original culture was used. All further manipulations were done on ice. Breakage of cells was achieved by passing the cell suspension twice through an Aminco pressure cell at 10,000 pounds per square inch, and cell wall debris and unbroken cells were sedimented by centrifugation at 10,000 x g for 10 min.

## 2.3 Enzyme Assays

The arginine decarboxylase assay was that of Wu and Morris (1973) and the ornithine decarboxylase assay was the same as that described for arginine decarboxylase except L-[1-$^{14}$C]ornithine was substituted for L-[U-$^{14}$C]arginine and magnesium was omitted. Biodegradative ornithine decarboxylase activity was determined according to Applebaum et al. (1975). Preparations of extracts and assay conditions were the same as for biosynthetic ornithine decarboxylase except that the breakage buffer and assay system were at pH 7.0. For decarboxylase assays the reaction was started by addition of the cell extract to the

reaction mixture and immediately placing the stoppered reaction vessel in a water bath at 37°C. Release of $CO_2$ was linear for more than 2 h and assays were routinely conducted for 30 min. Trichloroacetic acid was added to a final concentration of 5% (w/v) to terminate the reaction and convert $H^{14}CO_3^-$ to free $^{14}CO_2$. The $^{14}CO_2$ was trapped in filter papers saturated with Protosol (placed in a plastic centre well) and determined by liquid scintillation spectrometry. The enzyme activity was expressed as umole $CO_2$ released per h per mg protein, calculated from the specific activity of the L-isomer of ornithine or arginine in the assay mixture. The S-adenosylmethionine decarboxylase assay was that of Tabor et al. (1978) and $^{14}CO_2$ release was determined as described above. Agmatine ureohydrolase activity was determined according to Morris and Pardee (1966) with a slight modification. The urea formed during the reaction was assayed by the phenol-hypochlorite reaction following conversion of urea to ammonia (Wetherburn, 1967). β-galactosidase activity was determined according to Miller (1976), and glucose-6-phosphate dehydrogenase activity was determined according to Malamay and Horecker (1961). Protein was determined according to Bradford (1976).

## 2.4 Assay for tetracycline resistance

Cultures of E. coli strains transformed with plasmids pCOD, pTET, and pGA39 were grown in MOPS-glucose medium, or Luria-broth (Miller, 1976), to an optical density of 0.2 at 575 nm. A swab was dipped in the culture and streaked over the surface of a MOPS-glucose or Luria-agar plate with or without 5 mM cAMP. Filter discs of 1/4 inch diameter (Schleicher and Schuell) containing 1 to 75 µg of oxytetracycline (Sigma Chemical Co.) were placed on plates which were incubated for 18 h at 37 °C. The clear zones of growth inhibition around the discs were measured and indicated the degree to which the particular strain was sensitive to tetracycline, i.e. the larger the zone, the more sensitive the strain was to the antibiotic. Since the square of the radius of the zone of inhibition is proportional to the log of the initial concentration of the antibiotic on the disc, small differences in the zones of inhibition represent large differences in the concentration of tetracycline at the point of inhibition (Cooper, 1963).

## 2.5 Plasmids used

The plasmids used in this study are shown in Table 3.

Table 3. Plasmids used

| Plasmid | Description[a] | Reference |
|---|---|---|
| pBR322 | *tet*$^R$, *amp*$^R$ | Bolivar *et al.*, 1977 |
| pODC-1 | *speC*, *tet*$^R$ | Tabor *et al.*, 1983 |
| pKA5 | *speA*, *speB*, *metK*, *tet*$^R$, *amp*$^R$ | " |
| pKA8 | *metK*, *tet*$^R$ | " |
| pKA12 | *speA*, *metK*, *amp*$^R$ | " |
| pGA39 | *cm*$^R$, *tet*$^S$ | An and Friesen, 1979 |
| pTET | *cm*$^R$, *tet*$^R$ | This study |
| pCOD | *cm*$^R$, *speC:tet* | " |
| pUC8 | *lacZ*, *amp*$^R$ | Vieira and Messing, 1982. |

a Gene designations: *amp*$^R$, ampicillin resistance; *cm*$^R$, chloramphenicol resistance; *tet*$^S$ or *tet*$^R$, tetracycline sensitivity or resistance; *speC:tet*, fusion of the *speC* promoter region to the *tet* structural gene.

## 2.6 Isolation and manipulation of plasmid DNA

Plasmid DNA was amplified by addition of 170 µg/ml of chloramphenicol (Sigma Chemical Co.) to cultures growing in Luria broth as described by Clewell (1972). Extraction and purification of plasmid DNA was achieved by the alkaline extraction procedure described by Birnboim and Doly (1979). Further purification of supercoiled plasmid DNA for cell-free transcription-translation studies was done by dye-bouyant density centrifugation in CsCl gradients containing ethidium bromide (Maniatis et al., 1982).

E. coli cells were prepared for transformation with plasmid DNA by the method of Cohen et al. (1972) as modified by Dagert and Erhlich (1979). Cells were grown from single colony isolates in Luria-broth to an optical density of 0.2 at 575 nm, chilled on ice and harvested by centrifugation. The pellet was resuspended to one half the volume of the original culture in 0.1 M $CaCl_2$ and incubated at 4 °C for 20 min. The cells were sedimented by centrifugation at 10,000 x g for 5 min and resuspended in 0.1 M $CaCl_2$. One hundredth of the volume of the original culture was used. Approximately 0.1 to 0.5 µg of plasmid DNA was added to 0.2 ml of competent cells. This mixture was incubated on ice for 12 to 16 h, and then heat-shocked at 42 °C for 80 seconds. The cells were then added to 3 ml of Luria-broth and incubated at 37 °C for 2 to 5 h before plating on selective medium.

## 2.7 Purification of chromosomal DNA and total RNA

Chromosomal DNA from various procaryotes was isolated by the method of Marmur (1961). Total RNA was purified from *E. coli* by the method of Gopalakrishna *et al.* (1981).

## 2.8 Endonuclease digestion and ligation

Restriction endonuclease digestions of plasmid and chromosomal DNA and ligation of DNA fragments were performed as recommended by the manufacturer of the enzyme (Bethesda Research Laboratories, Boehringer Mannheim Canada Ltd., and Amersham Corporation).

## 2.9 Agarose and polyacrylamide gel electrophoresis

Electrophoresis of DNA was carried out in 0.8% or 1.0% (w/v) agarose or low melt agarose in 90 mM Tris base, 90 mM boric acid, and 2.5 mM disodium EDTA (pH 8.3). Ethidium bromide was added to the buffer at a final concentration of 0.5 µg/ml. Agarose gel electrophoresis was usually performed horizontally at 10 volts/cm for approximately 3 h at room temperature. After electrophoresis, the DNA bands were visualized using a UV transilluminator and photographed. Restriction endonuclease digests of bacteriophage lambda or pBR322 DNA were used as molecular weight markers for estimating the sizes of linear fragments.

Fractionation of protein by gel electrophoresis in 10% to 15% acrylamide and 0.1% sodium dodecylsulphate was performed as described by Laemmli (1970) and radioactively

labelled protein was detected by fluorography according to Bonner and Laskey (1974).

2.10 Recovery of DNA fragments from low-melt agarose

Restriction endonuclease fragments of DNA were isolated by the method of Schmitt and Cohen (1983). Following electrophoresis in low-melt agarose, a slab of the agarose containing the desired fragment was cut from the gel. The gel slice was placed in 10 ml of low salt buffer [0.2M NaCl; 20mM Tris HCl, pH 7.4; 1.0 mM EDTA] and heated at 65°C until the agarose was completely melted (approximately 30 min). An elutip-D column (Schleicher and Schuell, Inc.) was activated with high salt buffer [1.0M NaCl; 20mM Tris HCl, pH 7.4; 1.0mM EDTA] and then washed with low salt buffer. The melted agarose sample was passed over the column, which was washed with 10 ml of low salt buffer. The bound DNA was eluted from the column with 0.4 ml of high salt buffer. The DNA sample was precipitated by the addition of two volume of ethanol and storage at -70°C for one hour. Following collection of the precipitated DNA by centrifugation the DNA fragments were then used for molecular cloning.

2.11 Radiolabelling of protein in minicells

Minicells were purified by a modification of the methods of Roozen et al. (1971) and Schoemaker and Markovitz (1981). Plasmid bearing cells of E. coli strain P678-54 (Adler et al., 1966) were incubated at 37°C in MOPS-minimal

medium supplemented with 0.2% glucose, 0.5% casamino acids, 50 µg/ml of required amino acids, and 1 µg/ml of thiamine. Cells were harvested in early stationary phase by centrifugation. The pellet was resuspended in one twentieth the original volume of cold B.S.G. buffer (0.85% NaCl; 0.03% $KH_2PO_4$; 0.06% $Na_2PO_4$; and 100 µg/ml gelatin) (Curtiss, 1965). Samples were layered on top of 30 ml sucrose gradients (5-30% wt/vol in B.S.G.) and subjected to centrifugation at 4,500 r.p.m. for 20 min in a Sorvall HB-4 rotor at 4°C (Reeve, 1976). Minicell bands were removed from the gradient with a sterile Pasteur pipette, diluted with cold B.S.G. buffer, and sedimented by centrifugation at 10,000 x g for 15 minutes. The pellet was resuspended in 1 ml of cold B.S.G. buffer and the sucrose gradient centrifugation was repeated as described above. The purified minicells were resuspended in 1 ml of MOPS-minimal medium to an optical density of 1.0 at 575 nm, and 200 µg/ml of D-cycloserine (Sigma Chemical Co.) was added to reduce any parent cell contamination. The suspension was incubated at 37°C for 15 hour to allow degradation of chromosomally-encoded mRNA. The minicells were sedimented by centrifugation, washed with cold MOPS-minimal medium, and then resuspended in 1 ml of MOPS medium containing 0.2% glucose, and 50 µg/ml of each of 19 L-amino acids (except L-methionine) plus 1 µg/ml thiamine. In kinetic experiments, 1-10 mM cAMP was added at this time and incubation continued at 37C for 2 h prior to addition of

radiolabelled methionine. Under these experimental conditions, labelling was terminated after 20 min. [$^{35}$S]methionine was added at 20 µCi/ml (5 µg/ml). In experiments where minicells were qualitatively labelled [$^{14}$C]lysine was used at 10 µCi/ml (5 µg/ml), and non-radioactive lysine was omitted from the labelling medium instead of methionine. Incubated was at 37°C for 1 hour. Radioactively labelled minicells were washed with 0.85% NaCl prior to analysis of protein by gel electrophoresis.

2.12 Cell-free transcription and translation

The coupled transcription-translation system was ~~that~~ described by Zubay (1973) as modified by Pratt et al. (1981). In this protocol plasmid pODC-1 and pBR322 DNA was used to direct protein synthesis in a reaction mixture that contained a S-30 extract prepared from E. coli strains, MRE600 or LS854.1. The S-30 extract is a crude cell extract prepared by sedimenting lysed cells at 30,000 x g for 30 min as described by Zubay (1973). The standard reaction mixture (30 µl) usually contained 2 to 5 µg DNA in 5 µl, 5 µl of S30 extract, 2 µl of [$^{35}$S]methionine (7 µl/ul), 3 µl of 0.1 M magnesium acetate, and 7.5 µl of low molecular weight mix as described by Pratt et al. (1981). Where indicated purified CRP, a generous gift from Dr. J. Krakow, Hunter College, was added to reaction mixtures at a concentration of 1 µg/reaction. Radioactive protein was analyzed by gel electrophoresis in 10%-15% polyacrylamide and 0.1% sodium dodecylsulphate.

2.13 Quick blot analysis of messenger RNA

Ornithine decarboxylase mRNA levels were determined by the quick blot method of Bresser et al. (1983a,b) and Gillespie and Bresser (1983). In the presence of 12.2 molal NaI and non-ionic detergents, and at temperatures below 25 °C, mRNA binds to nitrocellulose while DNA and rRNA does not. In the presence of NaI and absence of detergents at temperatures that denature DNA (>95 °C), DNA selectively binds to nitrocellulose. Reagents and mRNC™ paper were supplied by Schleicher and Schuell. The protocol developed for immobilization of E. coli mRNA to nitrocellulose was essentially the same as that described by Bresser et al. (1983a,b). Twenty ml cultures were grown in MOPS medium in the presence and absence of 2 mM cAMP and 0.5 mM IPTG for greater than five generations. At an optical density of 0.5 at 575 nm, cells were rapidly chilled by adding 15 ml of culture to 10 ml of ice-cold MOPS medium containing chloramphenicol that gave a final concentration of 170 µg/ml. The cells were sedimented by centrifugation and washed in ice-cold MOPS medium containing 170 µg/ml chloramphenicol and 10 mM vanadium ribonucleosides. The pellet was resuspended in 200 µl of lysis buffer [2 mg/ml lysozyme; 25% sucrose; 10 mM Tris-HCl, pH 7.5] and the suspension divided into two equal parts. Following incubation for 5 min at room temperature, one aliquot was analyzed for mRNA and the other for DNA.

2.13.1 Messenger RNA analysis

Vanadium ribonucleosides were added to one of the aliquots at a final concentration of 10 mM to inhibit RNase activity. The mixture was then subjected to three cycles of freeze-thawing. One twentieth of the volume of 4 mg/ml proteinase K (Boehringer Mannheim) was added and the suspension incubated for 15 min at 37 °C. One twentieth of the volume of 10% Brij-35 was added and mixed, followed by addition of one twentieth of the volume of 10% sodium deoxycholate. The preparation was incubated on ice for 5 min at which time one volume of supersaturated NaI was added followed by the same volume of NaI- plus™ (Schleicher and Schuell). Dilutions of the lysate were made in NaI-plus™ solution.

2.13.2 DNA analysis

Disodium EDTA was added to the other aliquot at a final concentration of 1 mM to inhibit DNase activity. The DNA preparation was subjected to the same freeze-thawing and deproteinization steps as the RNA preparation. The volume was maintained by the addition of $H_2O$ instead of detergents. One volume of hot supersaturated NaI solution was added followed by an equal volume of saturated NaI solution. Dilutions were made in saturated NaI, and the lysate and dilutions were heated to 95-100 °C for at least 10 min to denature DNA.

2.13.3 Nucleic acid immobilization on nitrocellulose

The mRNA and DNA samples were filtered through nitrocellulose that had been soaked in RNase-free $H_2O$ followed by six fold concentrated SSC (SSC = 0.15M NaCl, 0.015M NaCitrate, pH 7.0) for at least 5 min. The RNA samples were filtered at a temperature of less than 25°C, while the DNA samples were filtered at a temperature of greater than 50°C. A ninety-six well filtering manifold (Bio-Rad) was used for support of the filter. Following immobilization of the nucleic acid on the nitrocellulose, the filter was washed in $H_2O$ and then 70% ethanol to remove excess NaI. Any co-immobilized proteins that might interfere with molecular hybridization were acetylated with acetic anhydride. The filter was baked at 80°C for 90 min.

2.14 Southern blot

To detect the presence of homologous sequences to the E. coli speC gene in the genomes of Gram-negative and Gram-positive bacteria, the Southern transfer and hybridization technique was used (Southern, 1975). The hybridization probes were the plasmids pBR322 and pODC-1. Plasmid DNA was made radioactive in vitro with [$\alpha$-$^{32}$P]dATP (3000 Ci/mmol) using a nick-translation system purchased from either New England Nuclear or Amersham Corporation. The $^{32}$P-labelled probes were hybridized to replicas (blots) of agarose gel electropherograms prepared under the following conditions: Restriction endonuclease reactions

containing 2.5-3.0 µg of chromosomal DNA and 10 units of restriction endonuclease were incubated overnight at 30°C. Molecular weight markers were prepared by digesting [$^{32}$P]-labelled adenovirus type-5 (a generous gift from Dr. Ban Younghusband) or pODC-1 DNA with restriction endonucleases. DNA samples were subjected to electrophoresis in 1% agarose and DNA was visualized as described above. The digested chromosomal DNA and molecular weight markers were denatured and transferred to nitrocellulose paper (Schleicher and Schuell, BA-85) from agarose gels as described by Wahl et al. (1979).

2.15 Molecular hybridization conditions

Prehybridization and hybridization conditions were essentially those of Rily and Anilionis (1980) with slight modification. The nitrocellulose paper was incubated at 42°C (quick blot analysis) or 37°C (heterologous hybridization) in a medium containing: 50% formamide; five fold concentrated SSC; five fold concentrated Denhardt's reagent (Denhardt's reagent is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone); 250 µg/ml herring sperm DNA; 0.1% sodium dodecylsulphate. The hybridization buffer was essentially the same except that the DNA carrier was at 100 µg/ml and one times Denhardt's reagent. Radioactively labelled DNA probes (specific activity $10^8$ cpm/µg DNA) were added to a concentration of 5-10 ng/ml of hybridization buffer and the mixture sealed in

a polyethylene bag. Hybridization reactions were routinely incubated at 37 °C for 19-48 hours followed by two washes of 15 minutes in a buffer containing two fold concentrated SSC and 0.1% sodium dodecylsulphate, two washes in a buffer containing one tenth diluted SSC and 0.1% sodium dodecylsulphate. The temperature at which these washes were conducted were 42 °C for the quick blot analysis or at 37 °C for heterologous hybridization. The nitrocellulose paper was air-dried and exposed to X-ray film (X-Omat AR film purchased from Kodak) at -70 °C for 1-5 days.

2.16 Densitometry

Autoradiograms were traced on a Corning scanning densitometer (Model 750) using the log inverse mode. The peaks were cut out and weighed. Peak weights determined from the same autoradiogram were taken as a measure of the relative amount of [$^{32}$P]-DNA that was hybridized, or radioactivity incorporated into protein. The weights of the peaks are expressed as scan units, i.e. one unit is equivalent to one mg.

2.17 Quantitative analysis of mRNA with a DNA reference

The hybridization signal was determined by densitometric scanning of the autoradiogram and expressed as scan units. The hybridization signal to mRNA and DNA at each dilution was plotted to give corrected mRNA and DNA values (Gillespie and Bresser, 1983). The corrected

hybridization signal to mRNA from a given number of cells was divided by the corrected hybridization signal to DNA from the same number of cells to give the relative mRNA level.

## 2.18 Spectrophotometry

Spectrophotometric readings were taken with a Beckman U.V.-visible spectrophotometer, model 24. and 1 cm quartz cuvettes were used.

# Chapter 3

# NEGATIVE CONTROL OF THE ACTIVITIES OF THE PUTRESCINE BIOSYNTHETIC ENZYMES BY CYCLIC AMP IN E. COLI

## 3.1 Introduction

Boyle and Adachi (1982) reported that the activity of ornithine decarboxylase was repressed, whereas the activity of arginine decarboxylase was derepressed, following nutritional shift-up of E. coli cells from poor growth medium to an enriched medium. Since intracellular concentrations of cAMP (Buettner et al., 1973; Epstein et al., 1975; Pastan and Adhya, 1976) and polyamines (Boyle et al., 1977) are known to vary with the growth rate as determined by the primary carbon source in the medium, the role of cAMP in the regulation of the putrescine biosynthetic enzymes was examined.

The activities of ornithine decarboxylase and arginine decarboxylase were determined in crude extracts prepared from cells grown under conditions known to modulate intracellular levels of cAMP. Enzymic activities were determined in wild-type, cya, and crp strains of E. coli grown on various carbon sources, in the presence and absence of cAMP.

## 3.2 Results

### 3.2.1 Effect of carbon source on ornithine decarboxylase and arginine decarboxylase activities

The intracellular concentration of cAMP is known to vary as a function of the primary carbon source of the medium in which cells are grown (Buettner et al., 1973; Epstein et al., 1975; Pastan and Adhya, 1976) For example, cells grown on glycerol exhibit high endogenous levels of cAMP, whereas cells cultured on glucose, which allows a faster growth rate, have reduced intracellular levels of cAMP.

By manipulating the primary carbon source, the activities of ornithine decarboxylase and arginine decarboxylase in cells grown on poor carbon sources, such as glycerol and acetate, were diminished relative to cells culture on glucose (Table 4).

These results suggest that since there is a direct correlation between growth rate and enzyme activities, there is an inverse correlation between endogenous cAMP levels and enzyme activities. Alternatively, it may be argued that the activities of these enzymes are growth rate dependent and the inverse relationship between cAMP and the activities of ornithine decarboxylase and arginine decarboxylase are coincidental. This is not the case as is demonstrated in the following experiments.

Table 4. Effect of carbon source on ornithine decarboxylase and arginine decarboxylase activities in E. coli

| Carbon source | Growth rate ($h^{-1}$) | Relative activity | |
|---|---|---|---|
| | | ODC | ADC |
| Glucose | 0.55 | 1.00 | 1.00 |
| Glucose - 6-$PO_4$ | 0.52 | 0.80 | 1.01 |
| Glycerol | 0.33 | 0.76 | 0.67 |
| Succinate | 0.31 | 0.45 | 0.55 |
| Acetate | 0.17 | 0.46 | 0.59 |

E. coli CA8000 cells were grown at 37°C in MOPS medium supplemented with one of the following carbon sources: 0.2% glucose, glucose-6-$PO_4$, glycerol; 0.4% succinate or acetate. Cultures were harvested after 3-4 generations as described in section 2.2. Specific activity of ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) are expressed relative to specific activity in glucose grown cells, and are the average of two experiments.

### 3.2.2 Ornithine decarboxylase and arginine decarboxylase activities in *cya* mutants

The effects of endogenous and exogenous cAMP on ornithine decarboxylase and arginine decarboxylase activities were examined in three isogenic strains of *E. coli*: LS340 (wild-type), LS853 (a *cya* deletion strain) and JW-1 (a spontaneous revertant of LS853). Strain JW-1 was capable of growth on glycerol but not on lactose, arabinose, or maltose as cAMP is required for the utilization of these carbohydrates. The ability of *cya* strains to utilize glycerol in the absence of cAMP is thought to be due to a mutation in the *glpK* promoter (Fraser and Yamazaki, 1980).

Ornithine decarboxylase activity was 34% higher, and arginine decarboxylase was 42% higher in LS853 (*cya*) than in LS340 when grown on glucose in the absence of cAMP (Tables 5 and 6).

The enzymic activities were similarly elevated in JW-1 which grows on glycerol and has a *cya* deletion, suggesting that the adenylate cyclase deficiency in these cells allows derepression of ornithine decarboxylase and arginine decarboxylase activities. After growth of the parent strain (LS340) on glycerol the activites of ornithine decarboxylase and arginine decarboxylase were 53% and 67%, respectively, of those measured after growth on glucose. In contrast, ornithine decarboxylase and arginine decarboxylase activities were not repressed in JW-1 (*cya*, Gly$^+$) when it was cultured on glycerol. The diminished activities of

Table 5. The effect of *cya* and exogenous cAMP on ornithine decarboxylase activity

| Strain | Character | Carbon source | Specific activity -cAMP | Specific activity +cAMP |
|---|---|---|---|---|
| LS340 | *cya*$^+$, *crp*$^+$, Gly$^+$ | glucose | 1.36 | 0.69 |
| LS853 | Δ*cya*, *crp*$^+$, Gly$^-$ | glucose | 1.82 | 0.75 |
| JW-1 | Δ*cya*, *crp*$^+$, Gly$^+$ | glucose | 2.09 | 0.73 |
| LS340 | *cya*$^+$, *crp*$^+$, Gly$^+$ | glycerol | 0.72 | 0.39 |
| JW-1 | Δ*cya*, *crp*$^+$, Gly$^+$ | glycerol | 2.03 | 0.72 |

Cells were grown for 3-4 generations in MOPS medium supplemented with the indicated carbon source in the presence or absence of 1 mM cAMP. Abbreviations: Gly$^+$, glycerol utilization; Gly$^-$, inability to utilize glycerol.

Ornithine decarboxylase activity is expressed as μmoles $CO_2$ released per h per mg protein. Specific activities are the average of three experiments. Standard deviations were less than 10% of the values shown.

Table 6: The effect of *cya* and exogenous cAMP on arginine decarboxylase activity

| Strain | Character | Carbon source | Specific activity -cAMP | Specific activity +cAMP |
|---|---|---|---|---|
| LS340 | *cya*$^+$, *crp*$^+$, Gly$^+$ | glucose | 0.95 | 0.60 |
| LS853 | Δ*cya*, *crp*$^+$, Gly$^-$ | glucose | 1.35 | 0.64 |
| JW-1 | Δ*cya*, *crp*$^+$, Gly$^+$ | glucose | 1.51 | 0.67 |
| LS340 | *cya*$^+$, *crp*$^+$, Gly$^+$ | glycerol | 0.64 | 0.41 |
| JW-1 | Δ*cya*, *crp*$^+$, Gly$^+$ | glycerol | 1.37 | 0.57 |

For procedure and explanation of abbreviations see Table 5. Arginine decarboxylase activity is expressed as μmoles $CO_2$ released per h per mg protein ($\times 10^{-1}$). Specific activities are the average of three experiments. Standard deviations are less than 10% of the values shown.

ornithine decarboxylase and arginine decarboxylase in the parent strain are, presumably, the result of endogenous cAMP levels elevated by growth on glycerol. This conclusion is supported by the response of the parent strain (LS340) and the isogenic cya carrying strain (LS853) to exogenously supplied cAMP. After growth of LS340 and LS853 in the presence of 1 mM cAMP the activities of ornithine decarboxylase were 50% and 60%, respectively, of those measured after growth in the absence of cAMP.

3.2.3 Cyclic AMP receptor protein is required for repression

The role of the cAMP receptor protein (CRP) in the repression of ornithine decarboxylase and arginine decarboxylase by cAMP is shown in Tables 7 and 8. Activities of ornithine decarboxylase and arginine decarboxylase were determined in a series of isogenic strains, with and without mutations in the cya and crp structural genes. These strains were cultured on glucose and glycerol in the presence and absence of 1 mM cAMP. Introduction of a mutated crp gene in a strain carrying the cya deletion (CA8306B) did not change the expression of ornithine decraboxylase and arginine decarboxylase in cells grown on glucose. When cAMP was added exogenously, however, ornithine decarboxylase and arginine decarboxylase activities were not repressed in the cya, crp-derivative strain (HY27), but they were repressed in the wild-type (CA8000) and cya (CA8306A) strains (Tables 7 and 8). This

indicates that a functional CRP is required for cAMP to exert its negative effect on ornithine decarboxylase and arginine decarboxylase expression.

Increasing the exogenous concentration of cAMP from 0 to 1.0 mM did not significantly change the growth rate of the wild-type strain (CA8000), but it did repress ornithine decarboxylase and arginine decarboxylase activities (Figure 3). If the concentration of cAMP was increased above 1 mM, the growth rate was significantly inhibited and the corresponding ornithine decarboxylase and arginine decarboxylase activities were further repressed; maximal repression was obtained at 2.5 mM for arginine decarboxylase and 10 mM cAMP for ornithine decarboxylase. The effect of exogenous cAMP on the growth rate of CA8306A (cya) was dramatic (Figure 3). The growth rate constant increased from 0.30 $h^{-1}$ in the absence of cAMP to 0.53 $h^{-1}$ in the presence of 0.5 mM exogenous cAMP, a rate equivalent to that maintained by the parent strain cultured on glucose in the absence of exogenous cAMP. It may be inferred from the growth rates that an exogenous concentration of 0.5 mM cAMP is sufficient to restore intracellular concentrations of cAMP in this cya strain to a physiologically functional level. Even at the faster growth rate, ornithine decarboxylase and arginine decarboxylase activities were repressed by exogenous cAMP. In the cya strain (CA8306A) the maximum repression was about 50% for both ornithine decarboxylase and arginine decarboxylase, and was obtained

Table 7. The effect of *crp* and exogenous cAMP on ornithine decarboxylase activity.

| Strain | Character | Carbon source | Specific activity -cAMP | Specific activity +cAMP |
|---|---|---|---|---|
| CA8000 | *cya*$^+$, *crp*$^+$, Gly$^+$ | glucose | 1.03 | 0.63 |
| CA8306A | Δ*cya*, *crp*$^+$, Gly$^-$ | glucose | 1.33 | 0.75 |
| CA8306B | Δ*cya*, *crp*$^+$, Gly$^+$ | glucose | 1.34 | 0.75 |
| HY27 | Δ*cya*, *crp*$^-$, Gly$^+$ | glucose | 1.34 | 1.77 |
| CA8000 | *cya*$^+$, *crp*$^+$, Gly$^+$ | glycerol | 0.81 | 0.75 |
| CA8306B | Δ*cya*, *crp*$^+$, Gly$^+$ | glycerol | 1.27 | 0.83 |
| HY27 | Δ*cya*, *crp*$^-$, Gly$^+$ | glycerol | 1.56 | 1.59 |

For procedure and explanation of abbreviations see Table 5. Ornithine decarboxylase activity is expressed as μmoles $CO_2$ released per h per mg protein. Specific activities are the average of two experiments.

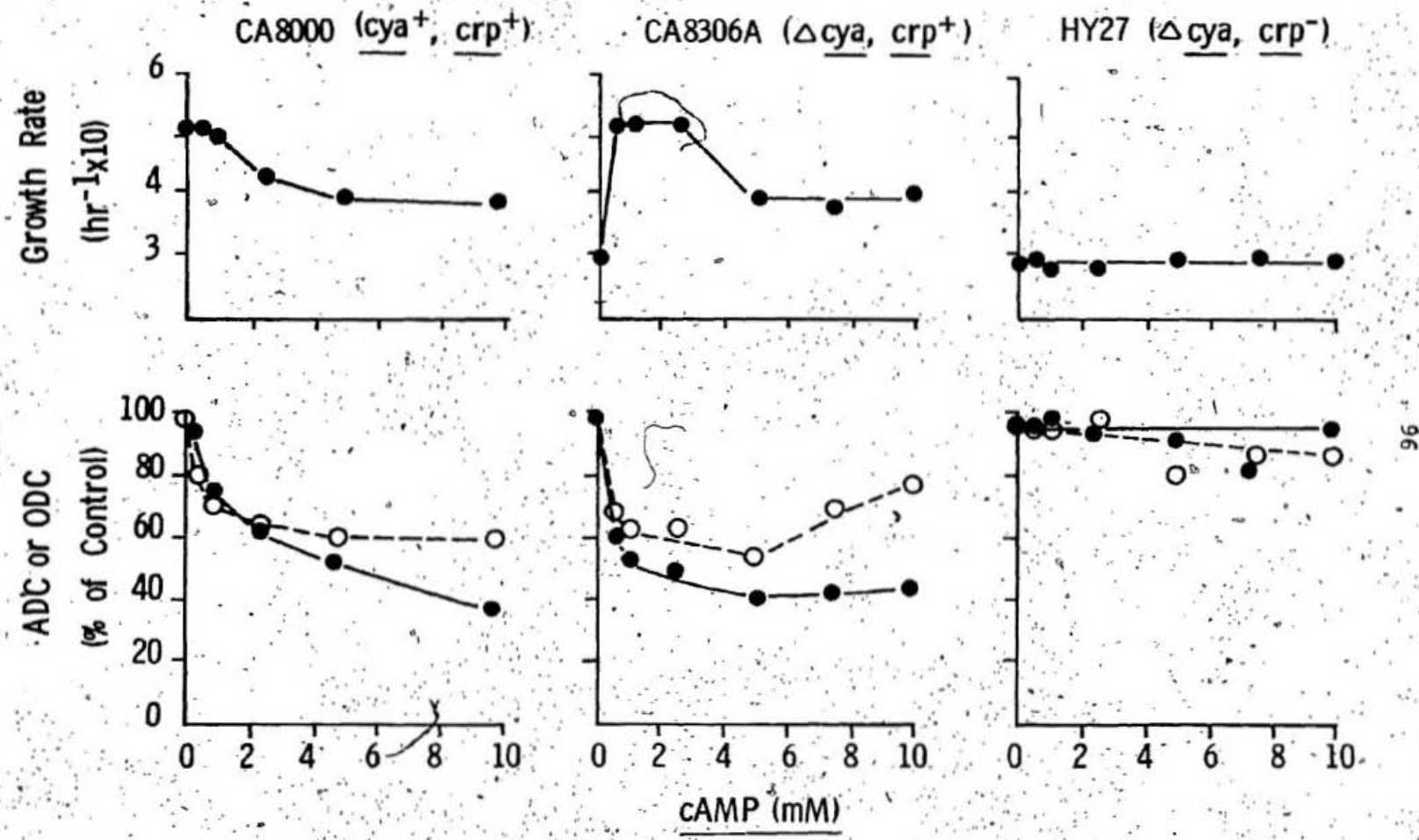
Table 8. The effect of crp and exogenous cAMP on arginine decarboxylase activity.

| Strain | Character | Carbon source | Specific activity -cAMP | Specific activity +cAMP |
|---|---|---|---|---|
| CA8000 | *cya*$^+$, *crp*$^+$, Gly$^+$ | glucose | 0.84 | 0.66 |
| CA8306A | Δ*cya*, *crp*$^+$, Gly$^-$ | glucose | 0.92 | 0.75 |
| CA8306B | Δ*cya*, *crp*$^+$, Gly$^+$ | glucose | 0.94 | 0.70 |
| HY27 | Δ*cya*, *crp*$^-$, Gly$^+$ | glucose | 1.02 | 1.24 |
| CA8000 | *cya*$^+$, *crp*$^+$, Gly$^+$ | glycerol | 0.57 | 0.68 |
| CA8306B | Δ*cya*, *crp*$^+$, Gly$^+$ | glycerol | 0.80 | 0.65 |
| HY27 | Δ*cya*, *crp*$^-$, Gly$^+$ | glycerol | 0.92 | 1.14 |

at 2.5 mM cAMP. The reason for the increase in arginine decarboxylase activity when the cya strain (CA8306A) was grown in the presence of greater than 5 mM cAMP is unclear. This effect was not seen in LS853, a strain also carrying a deletion in the cya structural gene.

In the CRP defective strain, HY27, neither the activities of ornithine decarboxylase and arginine decarboxylase nor the growth rate were affected by cAMP, even at concentrations as high as 10 mM cAMP (Figure 3). This result suggests that the repression of ornithine decarboxylase and arginine decarboxylase activities in the wild-type (LS340 and CA8000) and cya strains (LS853, JW-1, CA8306A and CA8306B) was not due to post-translational inhibition by cAMP. The following additional data support this conclusion. 1) Dialysis of crude extracts prepared from wild-type and cya strains to remove cAMP did not relieve the repression of ornithine decarboxylase and arginine decarboxylase activities by cAMP. 2) Addition of 0-10 mM cAMP to the enzyme assay reaction mixture had no effect on arginine decarboxylase activity, and inhibited ornithine decarboxylase activity only 10% at 1 mM and 28% at 10 mM cAMP. It should be noted that the concentrations of cAMP at which there is significant inhibition of ornithine decarboxylase activity in vitro are well above the in vivo levels of 1-10 μM. 3) Significant carry-over of cAMP from cultures growing in the presence of the nucleotide to the assay system was unlikely as there was a greater than

Figure 3. Growth rate, ornithine decarboxylase and arginine decarboxylase activities in wild-type, cya and crp strains as a function of cAMP concentration. Cells were grown for 3-4 generations in MOPSglucose medium supplemented with 0 to 10 mM cAMP. Ornithine decarboxylase (ODC, O----O), and arginine decarboxylase (ADC, O----O), activities are expressed as percent of control. Values are averaged for three experiments with standard deviations of less than 10% of the values shown.


CA8000 (cya+, crp+)
CA8306A (Δcya, crp+)
HY27 (Δcya, crp−)
Growth Rate (hr−1x10)
ADC or ODC (% of Control)
cAMP (mM)
6
4
3
100
80
60
40
20
0
0 2 4 6 8 10

thousandfold dilution during preparation of the crude extract. 4) Mixing equal aliquots of extracts prepared from acetate and glucose grown cells, or from cells grown in the presence and absence of cAMP, gave predicted average activities for the enzymes, indicating that a dissociable inhibitor was not responsible for the observed repression.

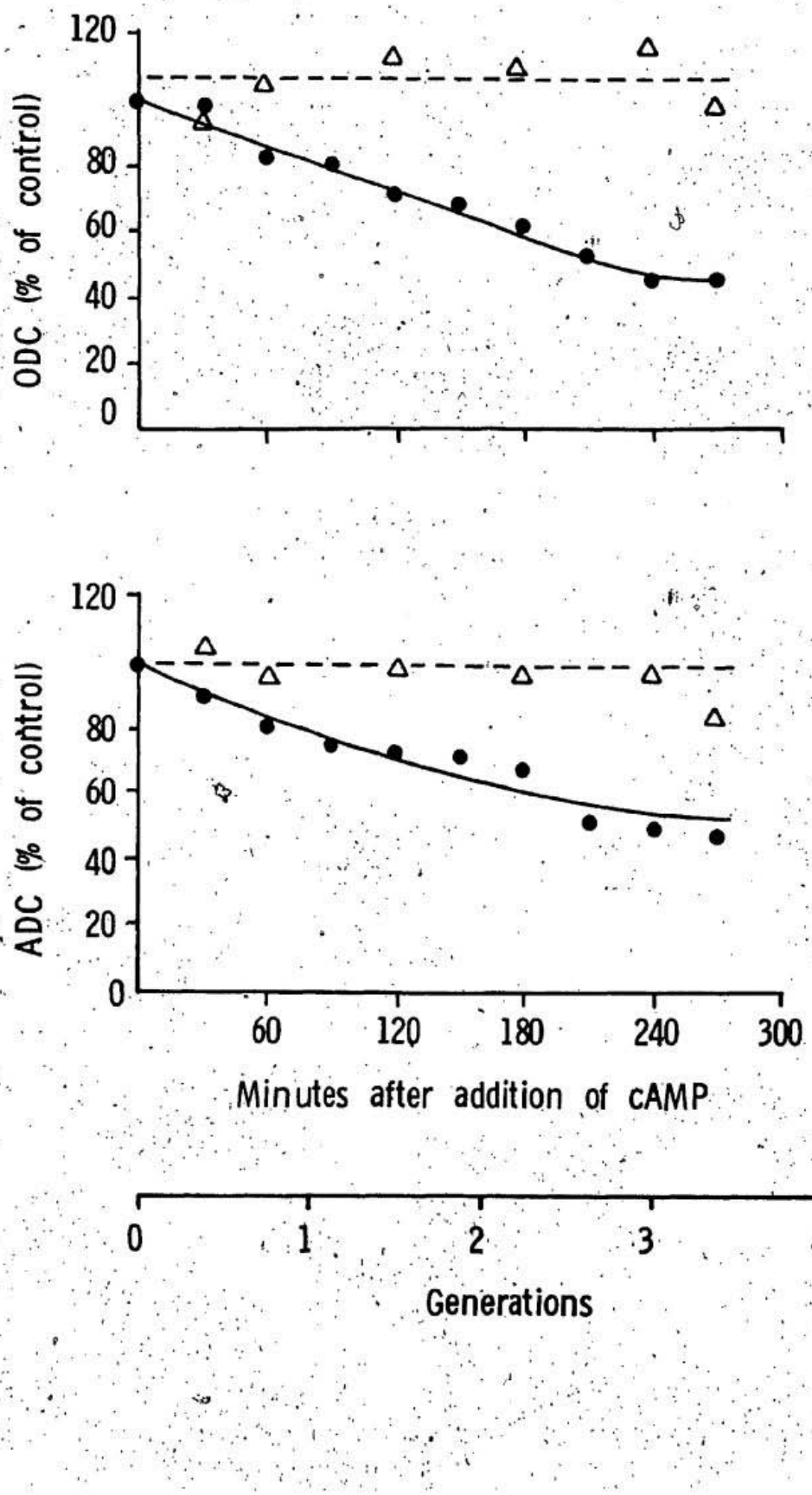
### 3.2.4 Temporal repression of ornithine decarboxylase and arginine decarboxylase activities following addition of cAMP

The temporal repression of ornithine decarboxylase and arginine decarboxylase activities by cAMP in a *cya* deletion strain (CA8306A) is shown in Figure 4. Maximal repression for both enzyme activities was approached 200-250 minutes (3 generations) following addition of cAMP to the growth medium. Thereafter, the activities of ornithine decarboxylase and arginine decarboxylase in cAMP-treated cells represent steady-state conditions.

### 3.2.5 Effect of cAMP on S-adenosylmethionine decarboxylase and agmatine ureohydrolase

S-Adenosylmethionine decarboxylase activity in wild-type (CA8000), *cya* (CA8306A) and *crp* (HY27) strains was unresponsive to changes in endogenous cAMP concentrations brought about by carbon source manipulation and cAMP supplementation. Agmatine ureohydrolase activity in the wild-type (CA8000) and *cya* strain (CA8306A) was diminished

Figure 4. Temporal repression of ornithine decarboxylase and arginine decarboxylase activities following addition of cAMP. A culture of E. coli CA8306A was grown for 4 generations in MOPS - glucose medium. The culture was divided into two equal parts, and at t = 0, 1mM cAMP was added to one of the cultures. At the indicated times aliquots were withdrawn from the culture and the specific activities of ornithine decarboxylase (ODC) and arginine decarboxylase were determined. The last aliquot was removed while the culture was still in the exponential phase of growth. Addition of cAMP, (●———●); no cAMP, (Δ———Δ). The specific activities are expressed as a percentage of activities at t = 0.


120
80
60
40
20
0
ODC (% of control)
ADC (% of control)
60
120
180
240
300
Minutes after addition of cAMP
0
1
2
3
4
Generations

by addition of cAMP to the growth medium, but only to about one half the extent seen for arginine decarboxylase at corresponding cAMP concentrations. The addition of cAMP to the CRP defective strain, HY27, had no effect on agmatine ureohydrolase (data not shown).

3.2.6 Effect of cAMP on glucose-6-phosphate dehydrogenase and β-galactosidase

While studying the influence of cAMP on enzymes other than those involved in polyamine biosynthesis, it was found that glucose-6-phosphate dehydrogenase, the first enzyme in the pentose phosphate pathway, was repressed by exogenously supplied cAMP to the same extent as ornithine decarboxylase and arginine decarboxylase activities in the wild-type (CA8000) and cya (CA8306A) strains. Repression was also mediated via CRP, as evidenced by the lack of repression in a strain carrying a crp lesion, strain HY27 (data not shown).

It is important for the interpretation of the results that the exogenous concentration of cAMP that accumulated intracellularly was sufficient to permit normal gene expression. The requirements for cAMP to stimulate β-galactosidase activity in E. coli have been well established (Pastan and Perlman, 1970; Pastan and Adhya, 1976). Therefore, IPTG induced β-galactosidase activity was assayed in cell-free extracts of strain CA8000 (wild-type) which had been grown in the presence of cAMP. In the

absence of the gratuitous inducer, IPTG, no β-galactosidase activity was detected at 1 mM cAMP and 1,583 units/mg protein were detected at 10 mM cAMP. In the presence of IPTG, 15,869 units/mg protein of β-galactosidase activity were detected at 1 mM and 24,765 units/mg protein at 10 mM cAMP. At these concentrations of cAMP both ornithine decarboxylase and arginine decarboxylase activities in the same extracts were repressed to the same extent as shown in Figure 3. This demonstrated that the exogenous cAMP concentrations used accumulated intracellularly in sufficient quantities to allow normal positive regulation of β-galactosidase, while simultaneously effecting negative regulation of ornithine decarboxylase and arginine decarboxylase activities.

3.2.7 Specificity of cAMP effect

In order to assess whether structurally similar nucleotides could elicit similar repressing effects on ornithine decarboxylase and arginine decarboxylase activities, 5 mM concentrations of 3'-AMP, 5'-AMP, ADP, and ATP were provided individually to cultures of CA8306A (cya). None of these nucleotide derivatives could substitute for cAMP in repressing ornithine decarboxylase or arginine decarboxylase activities (data not shown).

### 3.2.8 Effect of cAMP on ODC-antizyme

Ornithine decarboxylase has been shown to be inhibited by a specific inhibitor named ODC-antizyme (Kyriakidis et al. (1978). The possibility that cAMP supplementation was stimulating the production of this inhibitor was assessed. Cultures of cya strain, CA8306A, were grown for six generations in the presence and absence of 5 mM cAMP. Crude extracts of these cultures were assayed for differences in ODC-antizyme levels. Ornithine decarboxylase activity was measured in crude extracts that had been serially diluted. The specific acitivity of ornithine decarboxylase at each dilution was multiplied by the dilution factor to give a relative enzymic activity. According to Kyriakidis et al. (1978), the presence of ODC-antizyme in extracts is indicated by an increase in relative ornithine decarboxylase activity at higher dilutions. The relative ornithine decarboxylase activity in extracts prepared from cells grown in the presence and absence of cAMP increased identically with each dilution, but only to a maximum of 1.75 to 2-fold above undiluted extracts at a dilution of 1 in 128 (data not shown).

## 3.3 Discussion

The experiments described in this chapter demonstrate that the two polyamine biosynthetic enzymes, ornithine decarboxylase and arginine decarboxylase, are negatively controlled by cAMP in E. coli. It was essential in these

studies to show that intracellular concentrations of cAMP were elevated in cells grown in the presence of cAMP. As a direct assay of intracellular cAMP levels is technically difficult (Pastan and Adhya, 1976) and at best allows for 50% standard deviation (Primakoff, 1981), this problem was circumvented by assessing endogenous levels of cAMP physiologically. The increased growth rate of CA8306A (cya), when grown in the presence of 0.5 mM cAMP, to a rate equivalent to that maintained by the parent strain in the absence of exogenous cAMP, implies that 0.5 mM exogenous cAMP restored the intracellular concentration of the nucleotide to physiologically normal levels (Figure 3). Furthermore, the increase in β-galactosidase activity in the wild-type strain, CA8000, also indicates that endogenous concentrations of cAMP were elevated by 1 mM exogenous cAMP to a level that overcame catabolic repression mediated by glucose.

As dialysis of crude extracts prepared from cells grown in the presence of cAMP did not increase the activities of ornithine decarboxylase or arginine decarboxylase, and the effect of cAMP was negligible or absent upon ornithine decarboxylase and arginine decarboxylase activities in vitro, it is unlikely that cAMP is directly involved in post-translational regulation of these enzymes in E. coli. This conclusion is further supported by the finding that the activities of ornithine dcarboxylase and arginine decarboxylase in the crp strain (HY27) were unresponsive to changes in exogenous cAMP concentrations.

The role of other nucleotides, particularly GTP and ppGpp, both as potential activators or inhibitors of ornithine decarboxylase and arginine decarboxylase (Holtta et al., 1972, 1974) has also been ruled out by the dialysis experiments. The finding that the amount of ODC-antizyme is identical (albeit very low) in extracts from control cultures and from cultures supplemented with cAMP argues that cAMP is not repressing ornithine decarboxylase activity by stimulating the production of ODC-antizyme.

The negative control of ornithine decarboxylase and arginine decarboxylase by cAMP is apparently very specific as the activity of S-adenosylmethionine decarboxylase, another enzyme involved in polyamine biosynthesis, was unaffected by increased concentrations of cAMP. This result is evidence that cAMP plays an important role in regulating the synthesis of the putrescine biosynthetic enzymes. Since the formation of putrescine is considered the rate-limiting step in production of the polyamines, the control of ornithine decarboxylase and arginine decarboxylase activities by cAMP provides an explanation for changes in polyamine levels as a function of growth rate (Boyle et al., 1977), i.e. intracellular concentrations of cAMP.

The change in specific activities of ornithine decarboxylase and arginine decarboxylase as a function of growth rate (Table 4) correlates reasonably well with the growth rate dependent change in intracellular putrescine levels reported for E. coli (Boyle et al., 1977).

Intracellular putrescine levels per mg cellular protein are 44% less when E. coli K-12 strains are grown on acetate than when grown on glucose. The specific activities of ornithine decarboxylase and arginine decarboxylase in extracts prepared from cells cultured on acetate were 50% and 59%, respectively, of those grown on glucose. This correlation between the specific activities of ornithine decarboxylase and arginine decarboxylase, and putrescine levels as a function of growth rate suggests that the specific activities of ornithine decarboxylase and arginine decarboxylase mirror their functional activities in vivo. However, this assumes that putrescine levels reflect an aggregate change in two separate biosynthetic pathways. Moreover, it does not account for a significant but small proportion of putrescine normally excreted, or the conversion of putrescine to spermidine.

Published findings suggest that cAMP exerts it effect predominately at the level of transcription in the procaryotic cell. A few reports of translational control have not been confirmed or have been discounted (Rickenberg, 1974). Assuming that CRP acts solely at the promoter, as has been established for positively regulated operons (De Crombrugghe et al., 1984; Ullman and Danchin, 1983), the requirement for CRP suggests that negative control of ornithine decarboxylase and arginine decarboxylase may also be exerted at the level of transcription. In addition, cAMP may negatively regulate ornithine decarboxylase and arginine

decarboxylase in an indirect manner by stimulating the synthesis of an as yet uncharacterized repressor molecule which inhibits the enzyme activities or transcription of the *speC* and *speA* genes.

# Chapter 4

# EXPRESSION OF THE GENES ENCODING THE PUTRESCINE BIOSYNTHETIC ENZYMES OF *E. COLI* IN MINICELLS

## 4.1 Introduction

In order to accurately assess whether factors, such as cAMP-CRP, are involved in transcriptional control of the genes for the putrescine biosynthetic enzymes, advantage was taken of the availability of cloned copies of the *speA*, *speB*, and *speC* genes (Tabor *et al.*, 1983). These genes were cloned by isolating DNA restriction endonuclease fragments from plasmids of the Clarke-Carbon collection (Clarke and Carbon, 1976) which had been putatively assigned to carry the genes for the putrescine biosynthetic enzymes (Hafner *et al.*, 1979). Using recombinant DNA techniques, these fragments were inserted into the cloning vehicle, pBR322, resulting in the construction of two plasmids, pODC-1 and pKA5 (Tabor *et al.*, 1983) (Figures 5 and 6).

Wild-type strains of *E. coli*, transformed with either plasmid pODC-1 or pKA5 exhibited elevated ornithine decarboxylase or arginine decarboxylase activities, respectively; strains containing plasmid pKA5 also exhibited elevated agmatine ureohydrolase and methionine

Figure 5. Restriction map of plasmid pODC-1. The wide line indicates PBR322 sequences and the thin line represents an *E. coli* Pst 1 chromosomal fragment containing the *speC* gene.

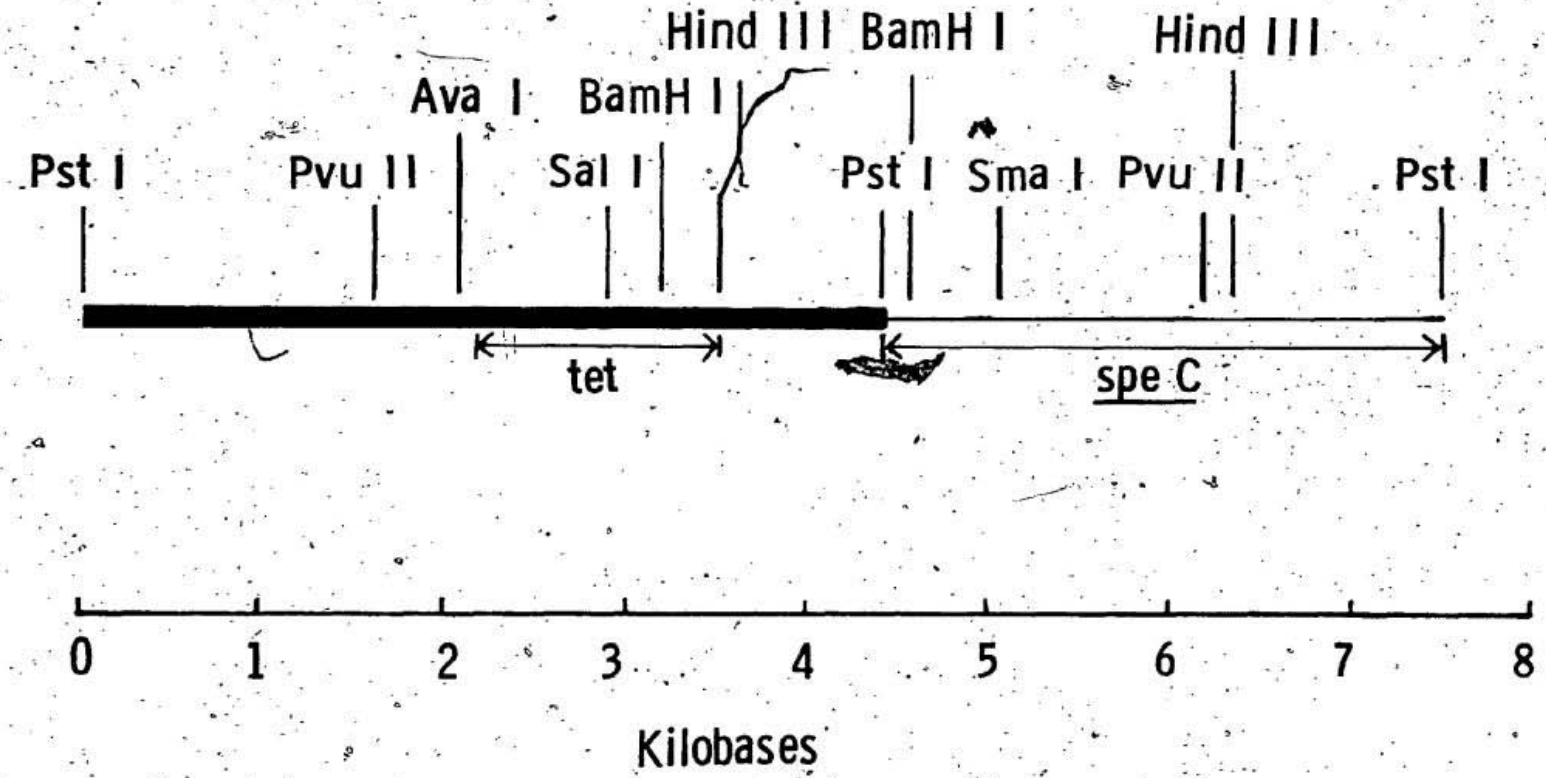
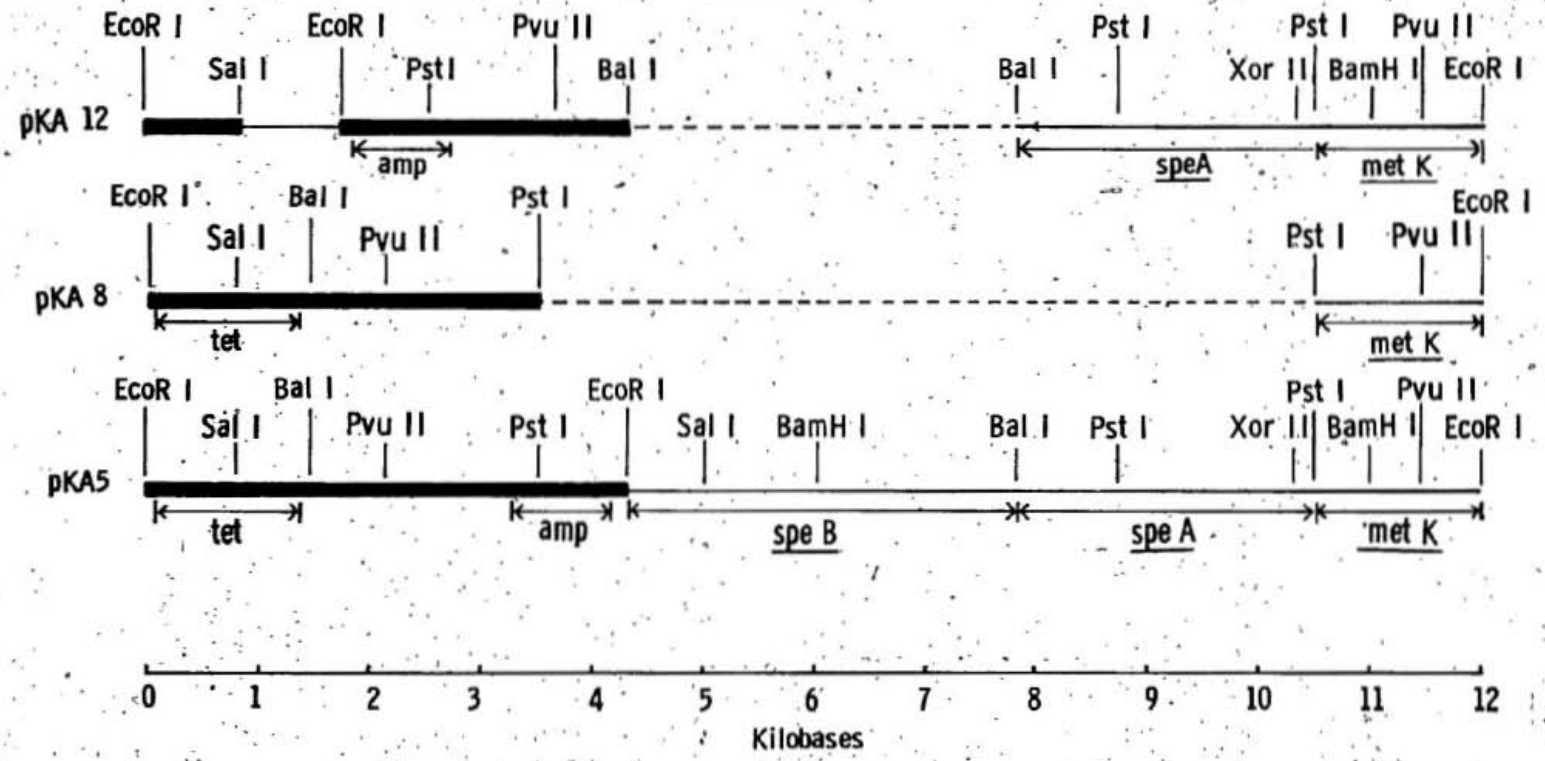

Hind III
BamH I
Hind III
Ava I
BamH I
Pst I
Pvu II
Sal I
Pst I
Sma I
Pvu II
Pst I
tet
spe C
0
1
2
3
4
5
6
7
8
Kilobases

Figure 6. Restriction maps of pKA plasmids. The plasmids pKA8 and pKA12 are derivatives of plasmid pKA5 (Tabor et al., 1983). The dashed line indicates sequences deleted from plasmid pKA5 during construction of pKA8 and pKA12. The thin line indicates the insert remaining from pKA5. The wide line indicates the pBR322 sequences. The sequences underscored by arrows do not represent the coding regions for the indicated gene, but rather the region containing the gene as deduced from the reconstructed plasmids (Adapted from Boyle et al., 1984).


EcoR I
Sal I
EcoR I
PstI
Pvu II
Bal I
pKA 12
amp
Bal I
Pst I
Pst I
Pvu II
Xor II
BamH I
EcoR I
speA
met K
EcoR I
Sal I
Bal I
Pvu II
Pst I
pKA 8
tet
Pst I
Pvu II
EcoR I
met K
EcoR I
Sal I
Bal I
Pvu II
Pst I
EcoR I
Sal I
BamH I
Bal I
Pst I
Pst I
Pvu II
Xor II
BamH I
EcoR I
pKA5
tet
amp
spe B
spe A
met K
0
1
2
3
4
5
6
7
8
9
10
11
12
Kilobases

adenosyltransferase activities (Boyle et al., 1984). As the elevated enzyme activities may have been the result of these plasmids carrying regulatory elements rather than structural genes, E. coli strains with deletions in the structural genes for these enzymes were transformed with the plasmids pODC-1 or pKA5. The plasmid pODC-1 restored and elevated ornithine decarboxylase activity in a speC deletion mutant. Similarly, the plasmid pKA5 restored and elevated arginine decarboxylase, agmatine ureohydrolase, and methionine adenosyltransferase activities in a strain carrying deletions in the speA, speB and metK genes (Boyle et al., 1984).

These results are strong evidence that plasmid pODC-1 carries the structural gene for ornithine decarboxylase (i.e. speC) and plasmid pKA5 carries the structural genes for arginine decarboxylase, agmatine ureohydrolase, and methionine adenosyltransferase (i.e. speA, speB, and metK, respectively). To substantiate this conclusion, I analyzed proteins synthesized in minicells containing plasmid pODC-1, pKA5 and two derivative plasmids of pKA5: pKA8, and pKA12. Plasmid pKA8 conferred on a host strain elevated methionine adenosyltransferase activity, only; whereas pKA12 conferred elevated arginine decarboxylase and methionine adenosyltransferase activities on host strains (Boyle et al., 1984).

## 4.2 Results

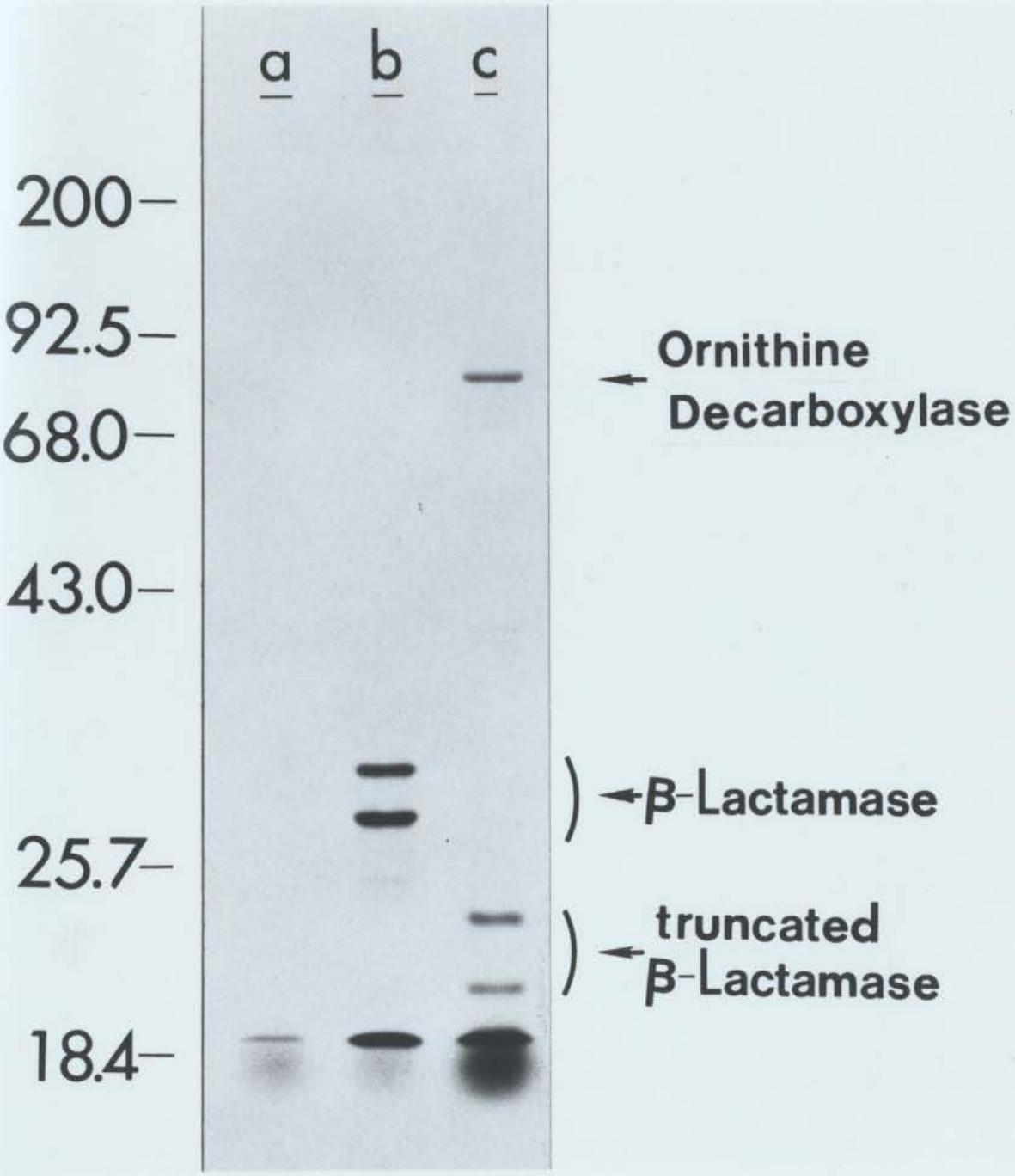
### 4.2.1 Expression of the *speA*, *speB*, *speC* and *metK* genes in minicells

The *E. coli* minicell-producing strain, P678-54 was transformed with the plasmids, pODC-1, pKA5, pKA8, and pKA12. Minicells were purified as described in section 2.11, and incubated for 15 hours at 37°C to allow for degradation of chromosomally-encoded mRNA. Shorter periods of preincubation resulted in production of polypeptides which were not specific for the plasmid. Following this preincubation period minicells were radioactively labelled with [$^{14}$C]-lysine.

In minicells containing plasmid pODC-1, a protein which corresponded to the reported molecular weight of ornithine decarboxylase (M =80,000) (Applebaum *et al.*, 1977) was radiochemically labelled (Figure 7). Due to insertion of the 3.2 kb fragment into the *Pst*1 site of the *amp* gene of pBR322, the precursor and mature forms of β-lactamase were truncated.

In mincells containing plasmid pKA5, four proteins, in addition to those encoded by plasmid pBR322, were radiochemically labelled. They had molecular weights of approximately 74,000, 70,000, 42,000 and 38,000 daltons (Figure 8). The two highest molecular weight proteins corresponded to the reported molecular weights of arginine

Figure 7. Polypeptides encoded by pODC-1 in minicells. The fluorogram of a sodium dodecylsulphate - polyacrylamide gel displays [$^{14}$C] - labelled polypeptides encoded by minicells containing pODC-1. Sample slots were loaded with extracts from minicells containing (a) no plasmid, (b) pBR322, and (c) pODC-1. The arrows indicate the position of ornithine decarboxylase or β-lactamases. The molecular weight markers used: myosin, 200,000 (200); phosphorylase b, 92,500 (92.5); bovine serum albumin, 68,000 (68); ovalbumin, 43,000 (43); α-chymotrypsinogen, 25,700 (25.7); and β-lactoglobulin, 18,400, (18.4).

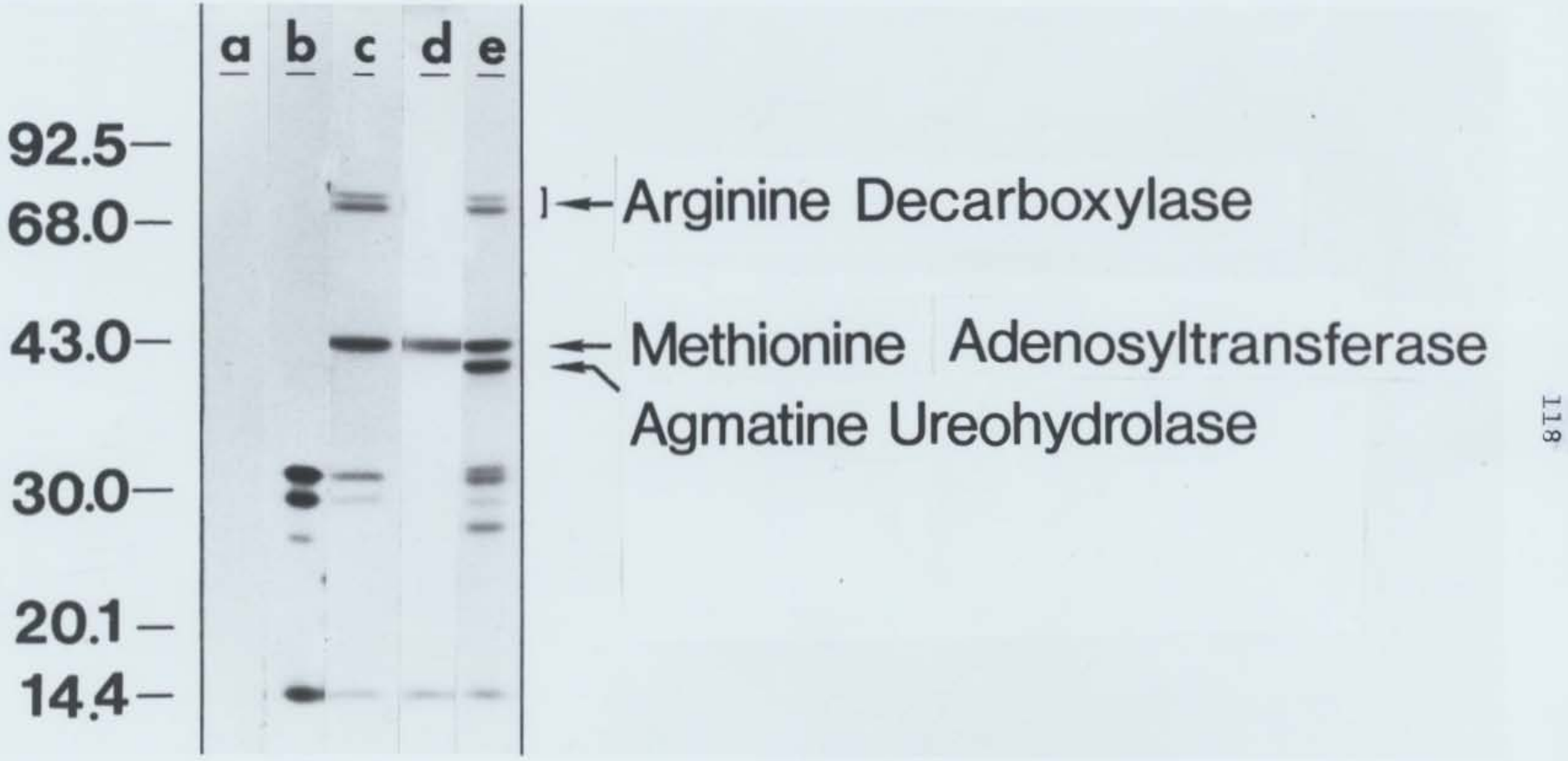

a
b
c
200
92.5
68.0
43.0
25.7
18.4
Ornithine Decarboxylase
β-Lactamase
truncated β-Lactamase

decarboxylase (Wu and Morris, 1973). The 42,000 dalton protein corresponds to methionine adenosyltransferase (Markham *et al.*, 1980). Comparison of the molecular weights of the proteins radioactively labelled in minicells containing pKA5 to those labelled in minicells containing either plasmid pKA8 or pKA12 (Figure 8) strongly suggests that the 38,000 dalton protein encoded by pKA5 and pKA12 is agmatine ureohydrolase.

4.2.2 Repression of ornithine decarboxylase synthesis by cAMP in minicells

Since minicells lack chromosomal DNA (Fraser and Curtiss, 1975 and references therein), they represent a potentially ideal system for the study of gene regulation in the absence of other genes. Therefore, the minicell system could be used to discriminate between the two hypotheses that either 1) cAMP and CRP inhibits expression of the *speC* gene directly or, 2) cAMP and CRP induces a repressor protein that inhibits *speC* expression. If repression of ornithine decarboxylase synthesis by cAMP occurs directly at the *speC* gene or *speC* mRNA, the amount of ornithine decarboxylase synthesized in minicells would be decreased by increasing concentrations of exogenously supplied cAMP. On the other hand, if negative regulation was mediated by a cAMP-induced repressor, cAMP would have no effect on the amount of ornithine decarboxylase synthesized in minicells since the repressor gene would be absent in these cells.

Figure 8. Polypeptides encoded by pKA plasmids in minicells. The fluorogram of a sodium dodecylsulphate-polyacrylamide gel displays [$^{14}$C] - labelled polypeptides encoded by minicells containing (a) no plasmid, (b) pBR322, (c) pKA12, (d) pKA8, and (e) pKA5. The arrows indicate the position of the arginine decarboxylase, methionine adenosyltransferase and agmatine ureohydrolase. The molecular weight markers used: phosphorylase b, 92,500 (92.5); bovine serum albumin, 68,000 (68.0); ovalbumin, 43,000 (43.0); carbonic anhydrase, 30,000 (30.0); trypsin inhibitor, 20,000 (20.0); and α-lactalbumin, 14,000 (14.0).


a
b
c
d
e
92.5
68.0
43.0
30.0
20.1
14.4
Arginine Decarboxylase
Methionine Adenosyltransferase
Agmatine Ureohydrolase

The amount of pODC-1 directed synthesis of ornithine decarboxylase in minicells was determined as a function of exogenously supplied cAMP. Following the 15 hour pre-incubation period, minicells harbouring plasmid pODC-1 were incubated in the presence of 0-10 mM cAMP for 2 h prior to the addition of [$^{35}$S]methionine. Since incorporation of [$^{35}$S]methionine into total protein was linear for more than 30 minutes in the presence or absence of cAMP, incubation was terminated after 20 minutes (Figure 9). These conditions were chosen to ensure that the radioactive amino acid, or some other factor was not limiting the incorporation. Figure 10 shows that the synthesis of ornithine decarboxylase was repressed by cAMP. At a concentration of 10 mM cAMP there was a 50% reduction in the amount of ornithine decarboxylase synthesized (Figures 10A and 11).

In several experiments the synthesis of β-lactamase, which is truncated due to insertion of the *speC* gene within the *amp* gene, was repressed by cAMP but not to the same extent as ornithine decarboxylase synthesis. The synthesis of β-lactamase, directed by plasmid pBR322 in minicells was not, however, repressed by cAMP (Figure 10B). As the synthesis of β-lactamase directed by pBR322 is not repressed by cAMP under the conditions used here, and those of others (Aiba, 1983), it is suspected that insertion of the *speC* gene into the *amp* gene may be responsible for the aberrant repression of β-lactamase by cAMP in minicells.

Figure 9. Incorporation of [$^{35}$S] - methionine into total protein in minicells. Minicells transformed with pODC-1 (●——●), pBR322 (▲-----▲), or no plasmid (■——■) were isolated, preincubated, and radiolabelled with [$^{35}$S] - methionine. At the indicated intervals, aliquots were withdrawn from the minicell suspension and the amount of radioactivity determined in hot Trichloroacetic acid precipitable material. Preincubation of minicells in 1-10 mM cAMP 2h prior to the addition of [$^{35}$S] - methionine gave identical kinetics of incorporation.

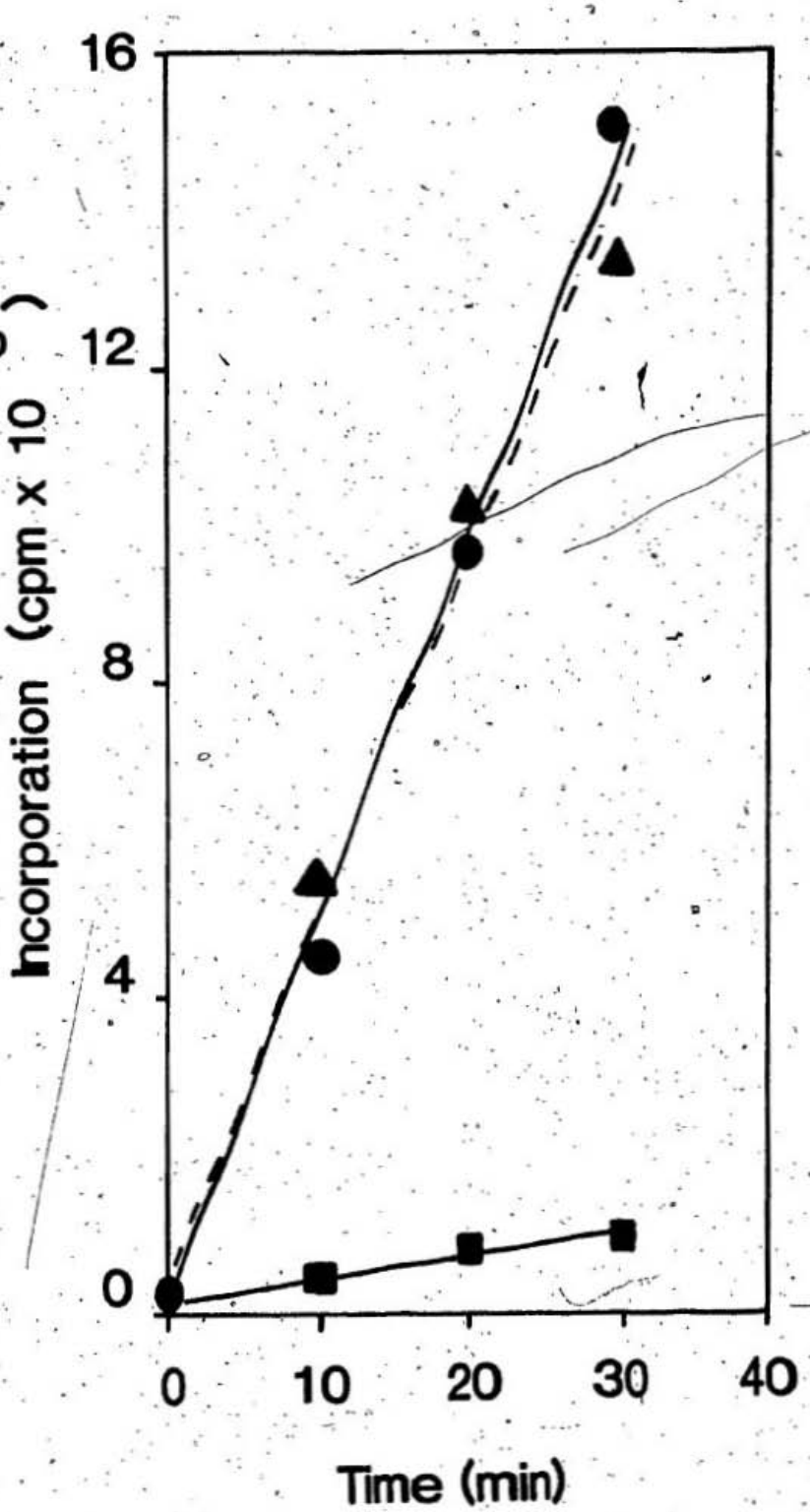

16
12
8
4
0
Incorporation (cpm x 10 -3 )
0
10
20
30
40
Time (min)

Figure 10. Inhibition of ornithine decarboxylase synthesis by cAMP in minicells. Minicells transformed with (A) pODC-1, or (B) pBR322, were isolated, preincubated and then incubated for 2h in the presence or absence of cAMP prior to the addition of [$^{35}$S] - methionine. After 20 min. incubation in the presence of [$^{35}$S] - methionine, extracts from equal aliquots of the minicell suspension were analyzed by electrophoresis in 10% polyacrylamide and 0.1% sodium dodecylsulphate.

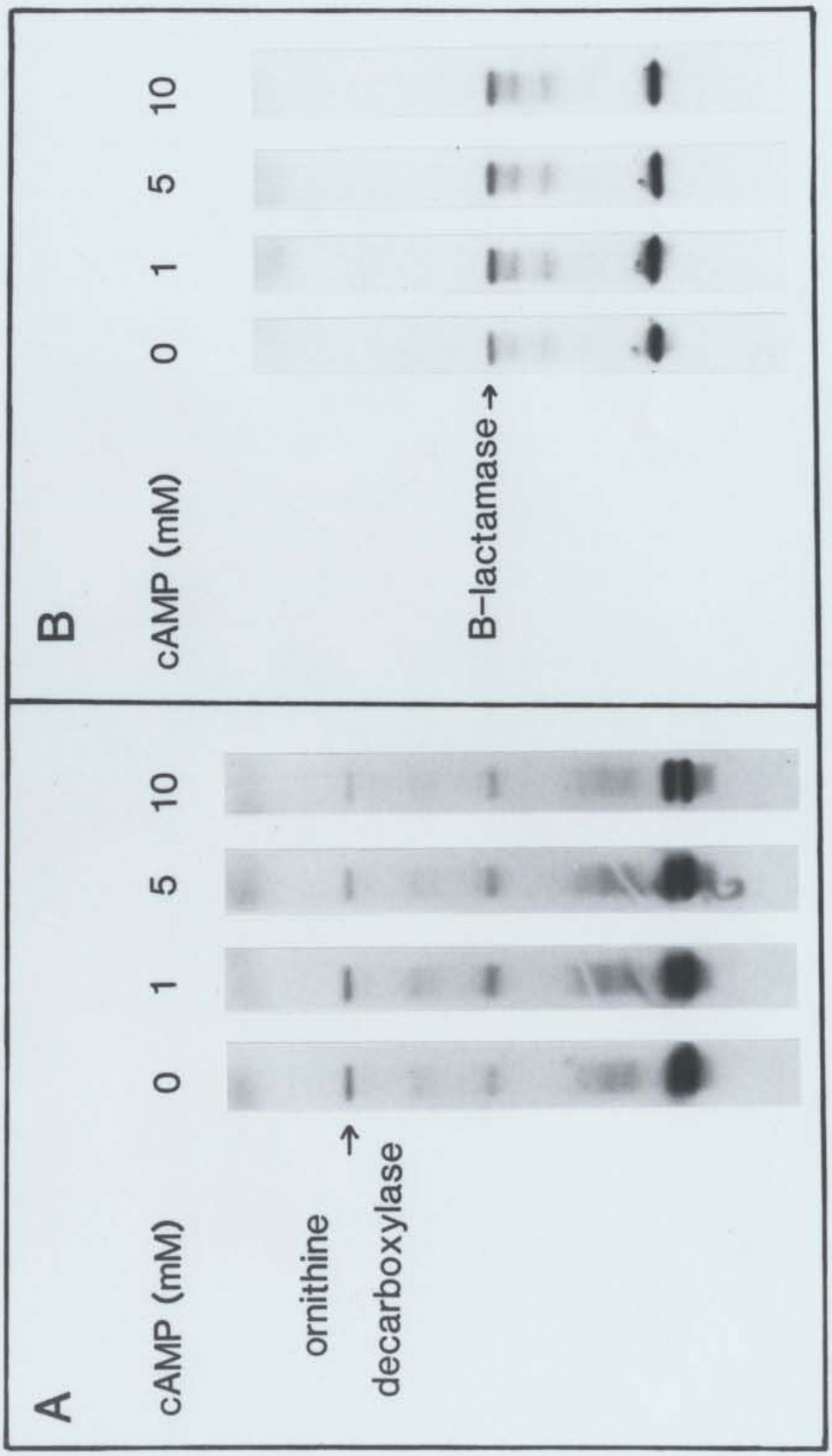
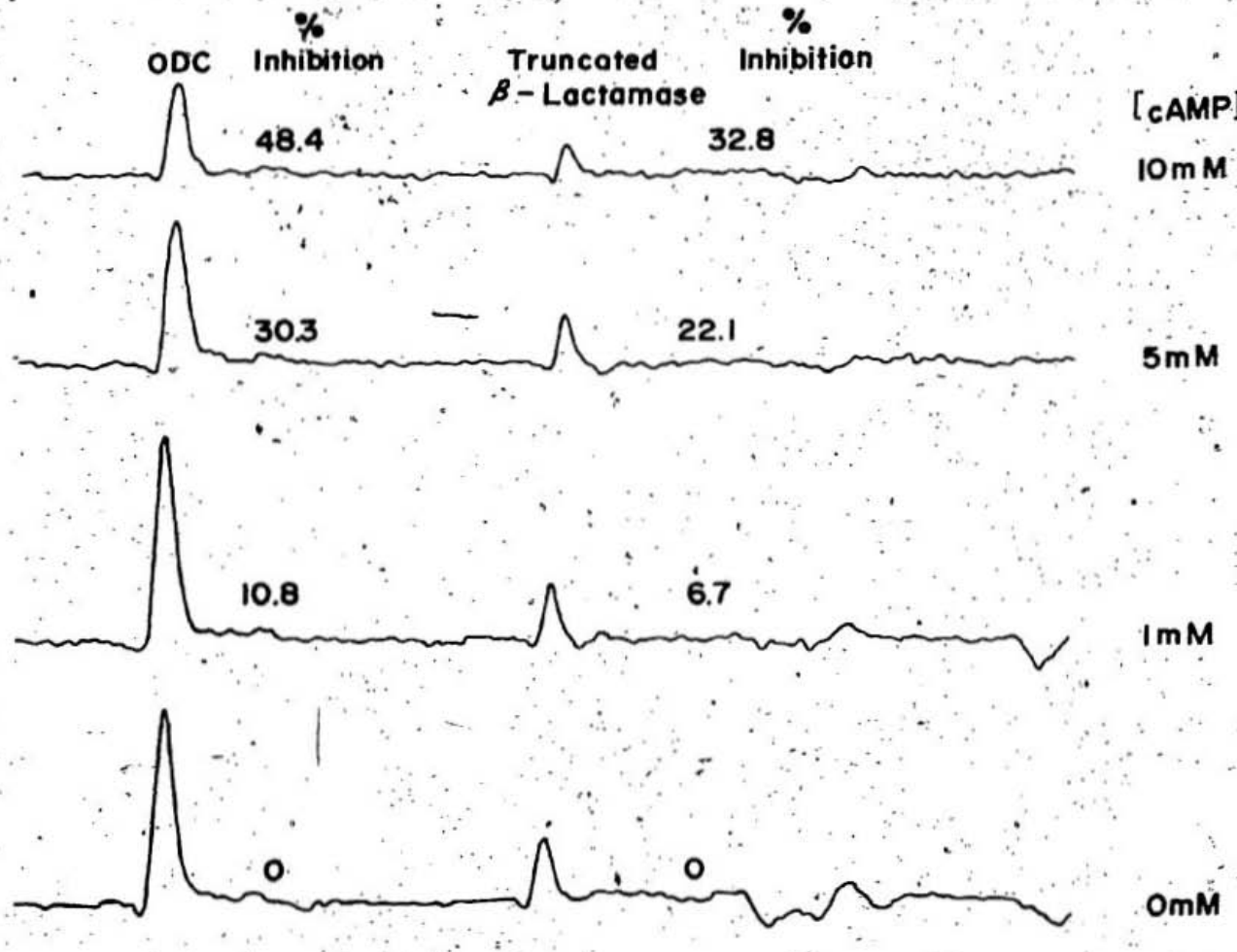

A
cAMP (mM)
0
1
5
10
ornithine
decarboxylase
B
cAMP (mM)
0
1
5
10
B-lactamase

Figure 11. Quantitation of repression of ornithine decarboxylase synthesis by cAMP in minicells. Minicells were isolated, preincubated and then incubated in the presence or absence of 0-10 mM cAMP for 2h prior to the addition of [$^{35}$S] - methionine. After 20 min. incubation in the presence of [$^{35}$S] - methionine, equal aliquots of radiolabelled extracts were analyzed by electrophoresis in 10% polyacrylamide and 0.1% sodium dodecylsulphate. The radiolabelled polypeptides were detected by fluorography. Each track of the autoradiogram was scanned under identical conditions so that they could be quantitatively compared. Beside each peak [ornithine decarboxylase, (ODC) and truncated β-lactamase] is the percentage of repression of synthesis at a given concentration of cAMP.


ODC
% Inhibition
Truncated β – Lactamase
% Inhibition
[cAMP]
48.4
32.8
10mM
30.3
22.1
5mM
10.8
6.7
1mM
0
0
0mM

## 4.3 Discussion

The expression of plasmids, pODC-1 and pKA5 in minicells confirms that these plasmids bear the structural genes for *speC* and the structural genes for *speA* and *metK*, respectively. The restoration and elevation of agmatine ureohydrolase activity in a *speB* deletion mutant transformed with pKA5 indicated that this plasmid also bears the structural gene for agmatine ureohydrolase (*speB*) (Boyle *et al.*, 1984). The 38,000 dalton subunit of agmatine ureohydrolase deduced from the polypeptides synthesized in minicells has been confirmed by purification of agmatine ureohydrolase to near homogeneity (C. Satishchandran and S.M. Boyle, personal communication). Therefore, the gene order in the 63 to 64 minute region of the *E. coli* chromosome is established as *glc speC metK speA speB serA*. Futhermore, restriction endonuclease analysis places the *speA* gene much closer to the *metK* gene than 0.2 minutes (8 Kb) estimated by $P_1$ transduction experiments (Hafner *et al.*, 1977).

Wu and Morris (1973) reported that purified arginine decarboxylase existed in two electrophorectically distinct species of 71,000 and 75-76,000 daltons estimated from their mobilities on polyacrylamide gels in the presence of sodium dodecylsulphate. The synthesis of two polypeptides of 70,000 and 74,000 daltons in minicells transformed with plasmid pKA5 indicate that these proteins are both coded by the same *speA* gene. This led to the idea that the 74,000

dalton species of arginine decarboxylase might represent a precursor of a mature 70,000 dalton arginine decarboxylase.

Peptide mapping of the 70,000 and 74,000-dalton species demonstrated that these polypeptides are identical except for one peptide (J. Buch and S.M. Boyle, personal communication). Both species of arginine decarboxylase, whether synthesized in whole cells or in minicells bearing pKA5, are immunoprecipitated by antiserum to the 70,000 dalton arginine decarboxylase. Moreover, the 74,000 dalton polypeptide accumulated in an E. coli strain that is defective in processing signal sequences of proteins destined for transport across the cytoplasmic membrane. Pulsing E. coli cells with radioactive amino acids followed by increasing periods of chase with non-radioactive amino acids showed that the 74,000 dalton polypeptide is processed to form the mature 70,000 dalton arginine dcarboxylase which resides in the inner periplasmic space (J. Buch and S.M. Boyle, personal communication). Thus, these results support the previously hypothesized compartmentalization of arginine decarboxylase (Tabor and Tabor, 1969; Morris et al., 1970), and may account for the preferential utilization of exogenous arginine for polyamine synthesis.

The repression of ornithine decarboyxlase synthesis by cAMP in minicells is not likely to be due to changes in plasmid copy number as the extent of DNA replication in plasmid bearing minicells is limited to 1-2 rounds of replication and would be complete prior to the commemcement

of the experiment described here (Inselburg, 1970, 1971). Since minicells lack chromosomal DNA, repression of ornithine decarboxylase cannot be mediated by a cAMP-induced repressor. Further, as there are no confirmed reports of cAMP-CRP influencing translation, these results indicate that cAMP-CRP negatively regulates *speC* transcription directly at the promoter.

# Chapter 5

# EXPRESSION OF THE *SPEC* GENE IN A CELL-FREE TRANSCRIPTION-TRANSLATION SYSTEM

## 5.1 Introduction

Cell-free protein-synthesizing systems have been used to determine the influence of various effector molecules on gene expression at the levels of transcription and translation (Zubay, 1973). Protein factors present in cell-free extracts prepared from wild-type strains of *E. coli* can be studied through a technique in which the cell-free extract (S-30) is prepared from mutants defective in the factor, e.g. *crp* mutants. The S-30 extract can then be reconstituted with the purified protein and its effect examined. Molecules of low molecular weight, such as cAMP, can be readily removed from the S-30 extract by dialysis and added to the reaction system in defined amounts.

Cell-free protein-synthesizing systems can also be used to determine the direction of transcription of a gene and localize the region around the initiation site (promoter). This is done by analysis of protein products whose synthesis was directed by restriction endonuclease fragments of the desired gene. Digestion at various points within the gene

will generate DNA fragments that will direct the synthesis of various truncated polypeptides. It is then possible to deduce from the truncated polypeptides the direction of transcription and, hence, the approximate start of transcription initiation. A similar approach has been used to locate genes on specialized transducing phages (Mackie, 1979) and plasmids (Chen and Zubay, 1983).

The synthesis of ornithine decarboxylase directed by pODC-1 DNA was examined in a cell-free protein synthesizing system with respect to the influence of putative regulatory molecules, arginine, ornithine, putrescine, spermidine, and cAMP and CRP. In addition, the cell-free protein-synthesizing system was used to localize the *speC* promoter region on plasmid pODC-1.

## 5.2 Results

### 5.2.1 Cyclic AMP represses the synthesis of ornithine decarboxylase *in vitro*

In order to determine the effect of cAMP on the synthesis of ornithine decarboxylase *in vitro*, the incorporation of [$^{35}$S]-methionine into total protein was determined as a function of time and amount of DNA template in the reaction system. Figure 12B shows that incorporation was maximal in reaction systems containing 2-3 ug of DNA template and S-30 extract prepared from *E. coli* strain MRE600. The kinetics of incorporation into total protein was linear for about 30 minutes when 2 µg of DNA was used

(Figure 12A). Incorporation of [$^{35}$S]-methionine into total protein in a reaction system prepared from an S-30 extract of LS854.1 was maximal when 5 µg of pODC-1 DNA was used (Figure 12D). Incorporation was linear for approximately 40 minutes (Figure 12C). Reaction systems containing a S-30 extract prepared from MRE600 incorporated ten fold more radioactivity into total protein than those containing extracts of LS854.1. Based on these results, reaction systems prepared from a MRE600 S-30 extract contained 2 µg of pODC-1 DNA and incorporation was terminated after 20 minutes. For systems prepared from a LS854.1 extract 5 µg of pODC-1 DNA was added and incubation was for 40 minutes. Using a cell-free transcription-translation system directed by pODC-1 DNA, it was found that increasing concentrations of cAMP in the reaction mixture progressivley inhibited the synthesis of ornithine decarboxylase. At a concentration of $10^{-5}$ M cAMP radiolabelling of ornithine decarboxylase was less than 10% of the control lacking cAMP (Figures 13 and 14).

As was seen in minicells, the synthesis of the truncated β-lactamase directed by pODC-1 was slightly repressed by cAMP *in vitro*. However, cAMP did not repress the synthesis of β-lactamase directed by pBR322 DNA *in vitro* (Aiba, 1983). This result suggests that the insertion of the *speC* gene into the *amp* gene allows for repression of β-lactamase synthesis by cAMP *in vitro*.

Figure 12. Incorporation of [$^{35}$S] - methionine into total protein in a cell-free transcription-translation system. Panels A and C show the time course of incorporation. The reaction mixtures contained: (A) a S-30 extract prepared from strain MRE600, and either 2μg of DNA (●——●) or no DNA (O----O); (C) a S-30 extract prepared from strain LS854.1 (crp), and either 4μg of DNA (●——●) or no DNA (O----O). Aliquots were removed from the mixture at the indicated intervals. Panels B and D show the dependence of the reaction on the amount of DNA. The reaction mixtures contained: (B) a S-30 extract prepared from strain MRE600 and 0-10μg of DNA; (D) a S-30 extract prepared from strain LS854.1 and 0-10μg of DNA. Equal aliquots were removed from the mixtures after 20 min. (B) or 40 min. (D) of incubation. Total protein synthesis was estimated by the amount of radioactivity in hot Trichloroacetic acid precipitable material.

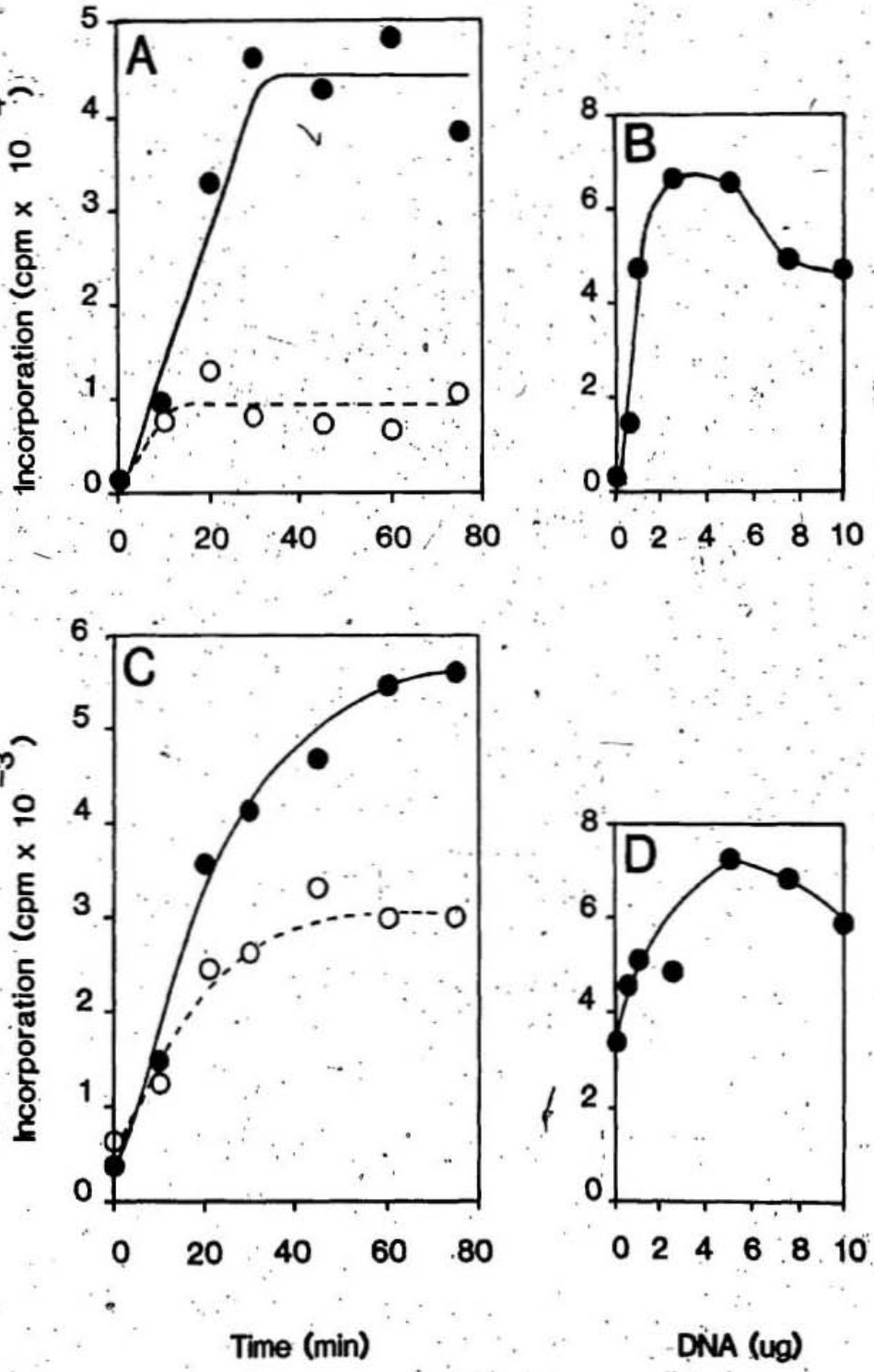

A
B
C
D
Incorporation (cpm x 10$^{-4}$)
Incorporation (cpm x 10$^{-3}$)
Time (min)
DNA (ug)
0
20
40
60
80
2
4
6
8
10
1
3
5

Figure 13. Effect of cAMP on the synthesis of ornithine decarboxylase in a cell-free transcription-translation system directed by pODC-1 DNA. Reaction mixtures contained 2 µg of pODC-1 DNA, S-30 extract prepared from strain MRE600, and either 0, 1, 10, or 100 µM cAMP. One reaction mixture contained no DNA template (NT). Equal aliquots of reaction mixtures were analyzed by electrophoresis in 10% polyacrylamide and 0.1% sodium dodecylsulphate. Polypeptides radiolabelled with [$^{35}$S]methionine were detected by fluorography. The molecular weight markers used were: phosphorylase b, 92,500 (93); bovine serum albumin, 68,000, (68); ovalbumin, 43,000 (43); α-chymotrypsinogen, 25,700 (26); β-lactoglobulin, 18,400 (18).

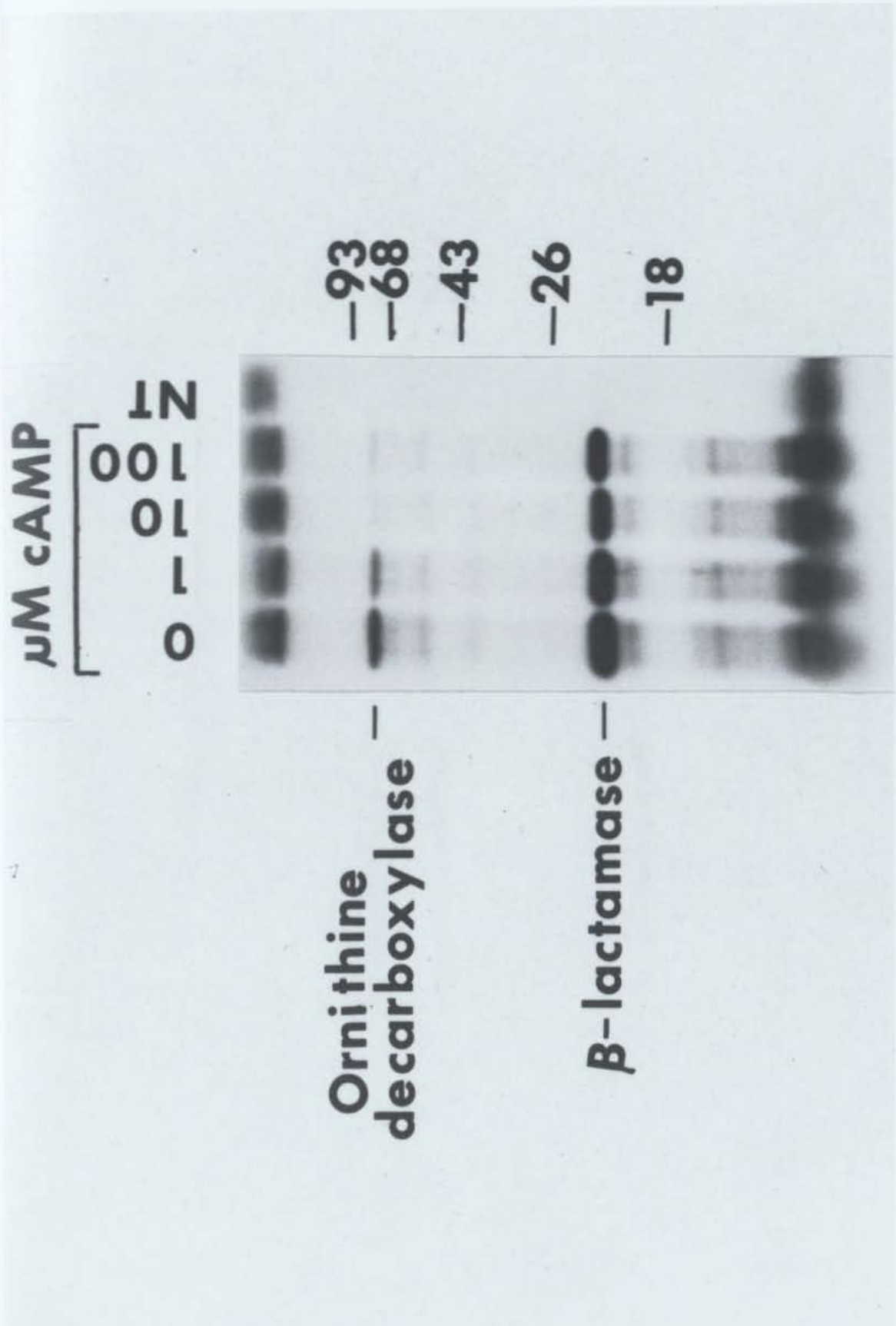
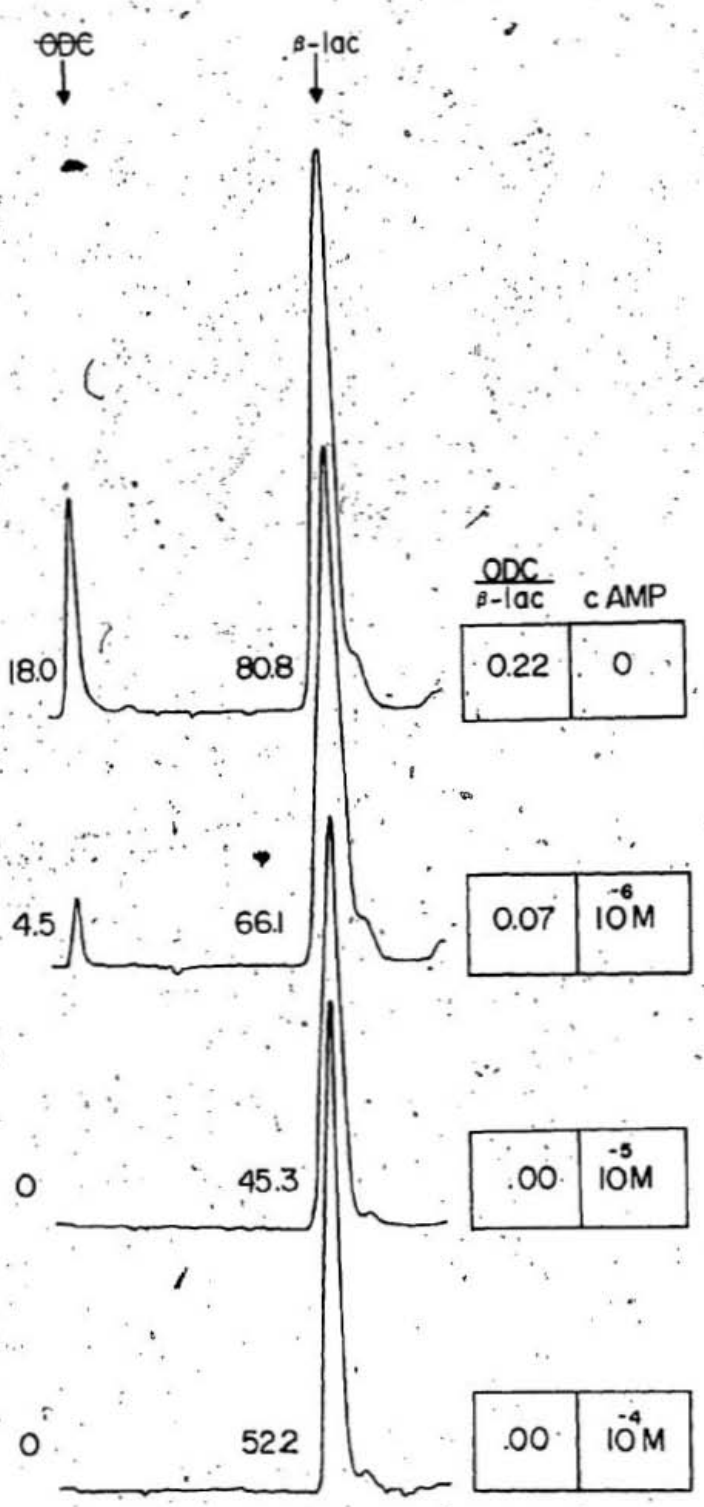

μM cAMP
0
1
10
100
NT
Ornithine decarboxylase
β-lactamase
93
68
43
26
18

Figure 14. Quantitiation of ornithine decarboxylase synthesis in a cell-free transcription-translation system as a function of cAMP concentration. The autoradiogram shown in figure 13 was scanned by densitometry. The peaks were cut out and weighed. The position of ornithine decarboxylase (ODC) and β-lactamase (β-lac) is shown by the arrows. It should be noted that the β-lactamse is truncated due to insetion of the *speC* gene within in the *amp* gene. The truncated β-lactamase may, therefore, be unstable *in vitro*. The value beside each peak represents the scan units defined in section 2.16. The ratio of ornithine decarboxylase to β-lactamase scan units at each concentration of cAMP is shown in the table at right.


ODC
β-lac
ODC / β-lac
c AMP
18.0
80.8
0.22
0
4.5
66.1
0.07
10^-6 M
0
45.3
.00
10^-5 M
0
52.2
.00
10^-4 M

5.2.2 CRP is required for repression of ornithine decarboxylase *in vitro*

Negative control of *speC* expression by cAMP in a cell-free protein-synthesizing system was shown to be dependent on CRP. Synthesis of ornithine decarboxylase was not repressed by cAMP in reaction mixtures containing a S-30 extract prepared from a *crp* deletion strain, LS854.1 (Figures 15 and 16). Addition of purified CRP restored the ability of cAMP to exert negative control in these reaction mixtures (Figures 15 and 17).

5.2.3 Effect of other putative regulatory molecules on ornithine decarboxylase

Addition of $10^{-4}$ M to $10^{-2}$ M ornithine or arginine to the cell-free protein synthesis system had no effect on the pODC-1 directed synthesis of ornithine decarboxylase (data not shown). However, it should be noted that the control reaction system contained $3.4 \times 10^{-5}$ M arginine.

At $10^{-6}$ M to $10^{-5}$ M, putrescine appears to inhibit the synthesis of ornithine decarboxylase relative to B-lactamase (Figure 18 and Table 9). At concentrations greater than $10^{-5}$ M, the synthesis of ornithine decarboxylase, as well as total protein, was stimulated by putrescine.

Spermidine showed a similar biphasic effect on ornithine decarboxylase synthesis *in vitro*. Addition of $10^{-6}$ M to $10^{-5}$ M spermidine to the *in vitro* system had no

Figure 15. Effect of cAMP on ornithine decarboxylase synthesis in a CRP deficient and CRP supplemented cell-free transcription-translation system directed by pODC-1 DNA. Reaction mixtures contained 4 ug pODC-1 DNA, a S-30 extract prepared from strain LS854.1, and either 0, 1, 10, or 100 uM cAMP, ($CRP^-$). An identical set of reaction mixtures were supplemented with 1 ug of purified CRP ($CRP^+$). One reaction mixture contained no DNA template (N.T.). Equal aliquots of the reaction mixtures were analyzed by electrophoresis in 10% polyacrylamide and 01% sodium dodecyl-sulphate. Polypeptides radiochemically labelled with [$^{35}$S]-methionine were detected by fluorography. The molecular weight markers (S) used were: phosphorylase b, 92,500 (93K); bovine serum albumin, 68,000 (68); ovalbumin, 43,000 (43K); α-chymotrypsinogen 25,700 (27K); β-lactoglobulin, 18,400 (18K).

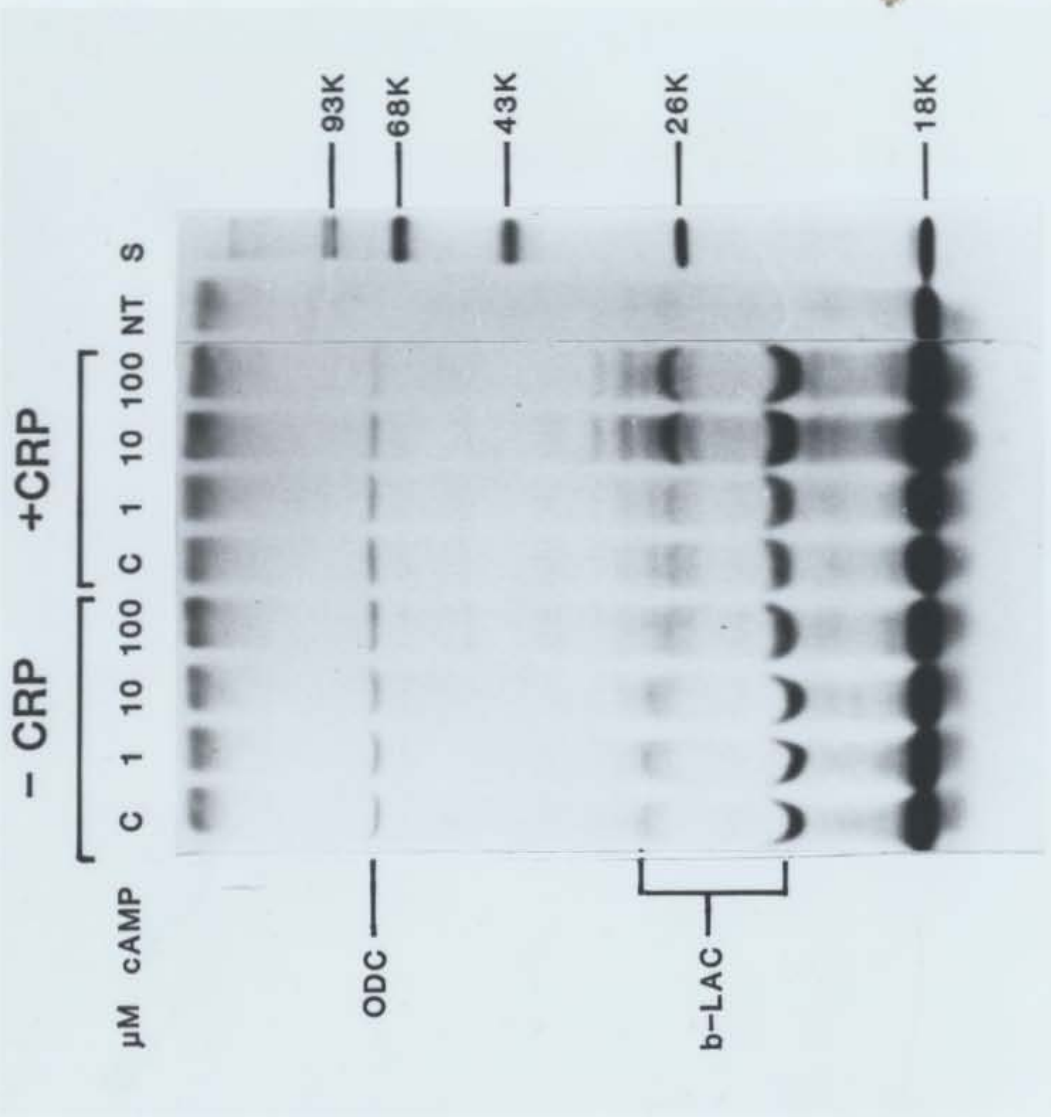

– CRP
+CRP
μM cAMP C 1 10 100 C 1 10 100 NT S
ODC
b-LAC
93K
68K
43K
26K
18K

Figure 16. Quantitation of ornithine decarboxylase synthesis in a CRP deficient cell-free transcription-translation system as a function of cAMP. The first four lanes from left of the autoradiogram shown in figure 15 were scanned by densitometry. The peaks were cut out and weighed. The position of ornithine decarboxylase (ODC) and β-lactamase (β-lac), are indicated by arrows. The value beside each peak represents scan units defined in section 2.16. The ratio of ornithine decarboxylase to β-lactamase scan units at a given concentration of cAMP is shown in the table at right.

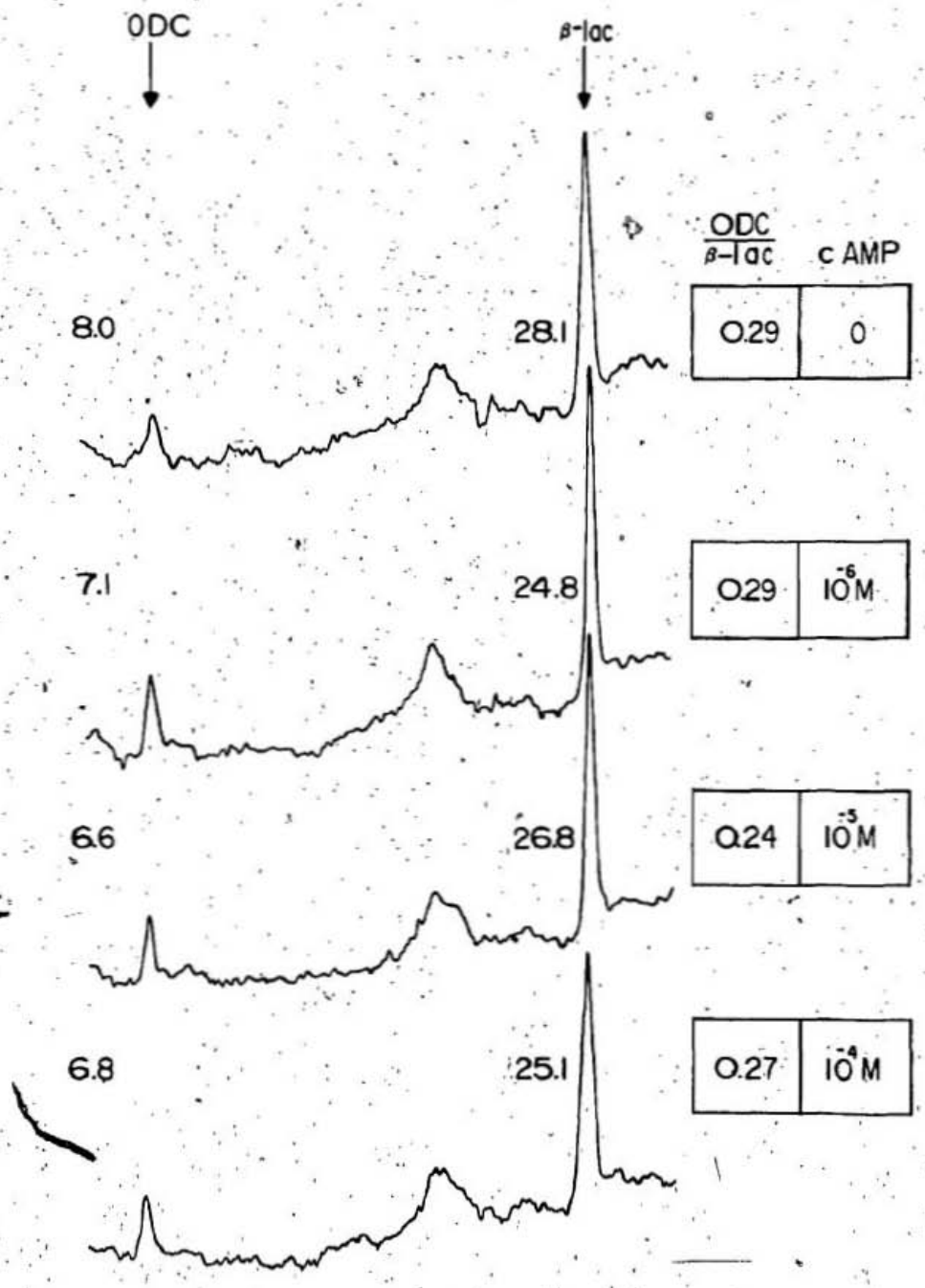

ODC
β-1ac
ODC / β-1ac
c AMP
8.0
28.1
0.29
0
7.1
24.8
0.29
10⁻⁶M
6.6
26.8
0.24
10⁻⁵M
6.8
25.1
0.27
10⁻⁴M

Figure 17. Quantitation of ornithine decarboxylase synthesis in a CRP supplemented cell-free transcription-translation system as a function of cAMP concentration. Lanes 5 to 8 from left on the autoradiogram shown in figure 15 were scanned by densitometry. The peaks were cut out and weighed. The position of ornithine decarboxylase (ODC) and β-lactamase (β-lac) are indicated by the arrows. The value beside each peak represents scan units defined in section 2.16. The ratio of ornithine decarboxylase to β-lactamase synthesis at a given concentration of cAMP is shown in the table at right..

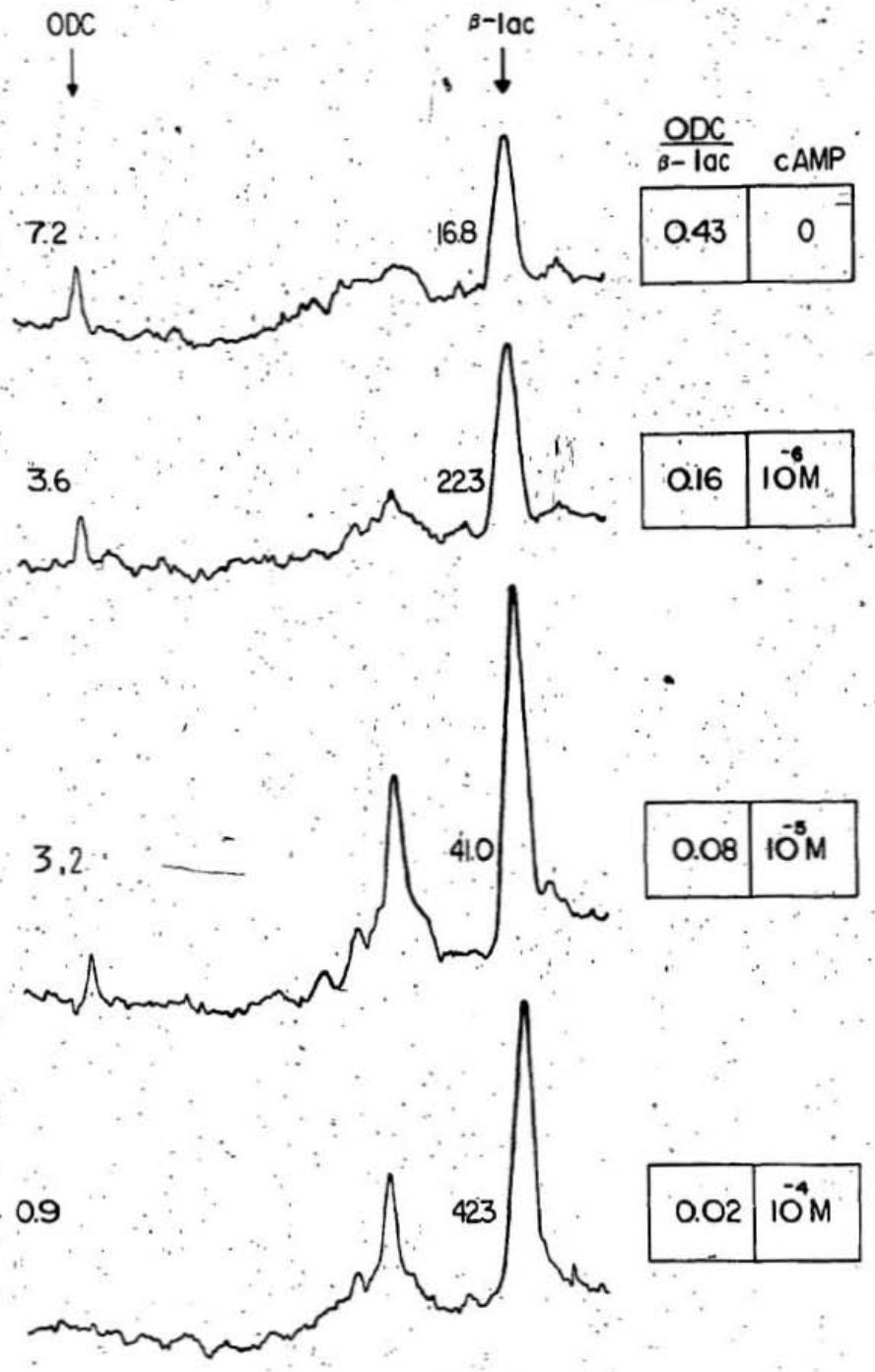

ODC
β-lac
ODC / β-lac
cAMP
7.2
16.8
0.43
0
3.6
223
0.16
10^-6 M
3.2
41.0
0.08
10^-5 M
0.9
423
0.02
10^-4 M

Figure 18. Effect of polyamines on the synthesis of ornithine decarboxylase in a cell-free transcription-translation system directed by pODC-1 DNA. Reaction mixtures contained 2 ug of pODC-1 DNA, a S-30 extract prepared from strain MRE600, and $10^{-6}$M to $10^{-2}$M putrescine (Put) or spermidine (Spd). The control reaction contained no polyamine supplement. Equal aliquots of reaction mixtures were amalyzed by electrophoresis in 10% polyacrylamide and 0.1% sodium dodecylsulphate. Polypeptides radiochemically labelled with [$^{35}$S]methionine were detected by fluorography.

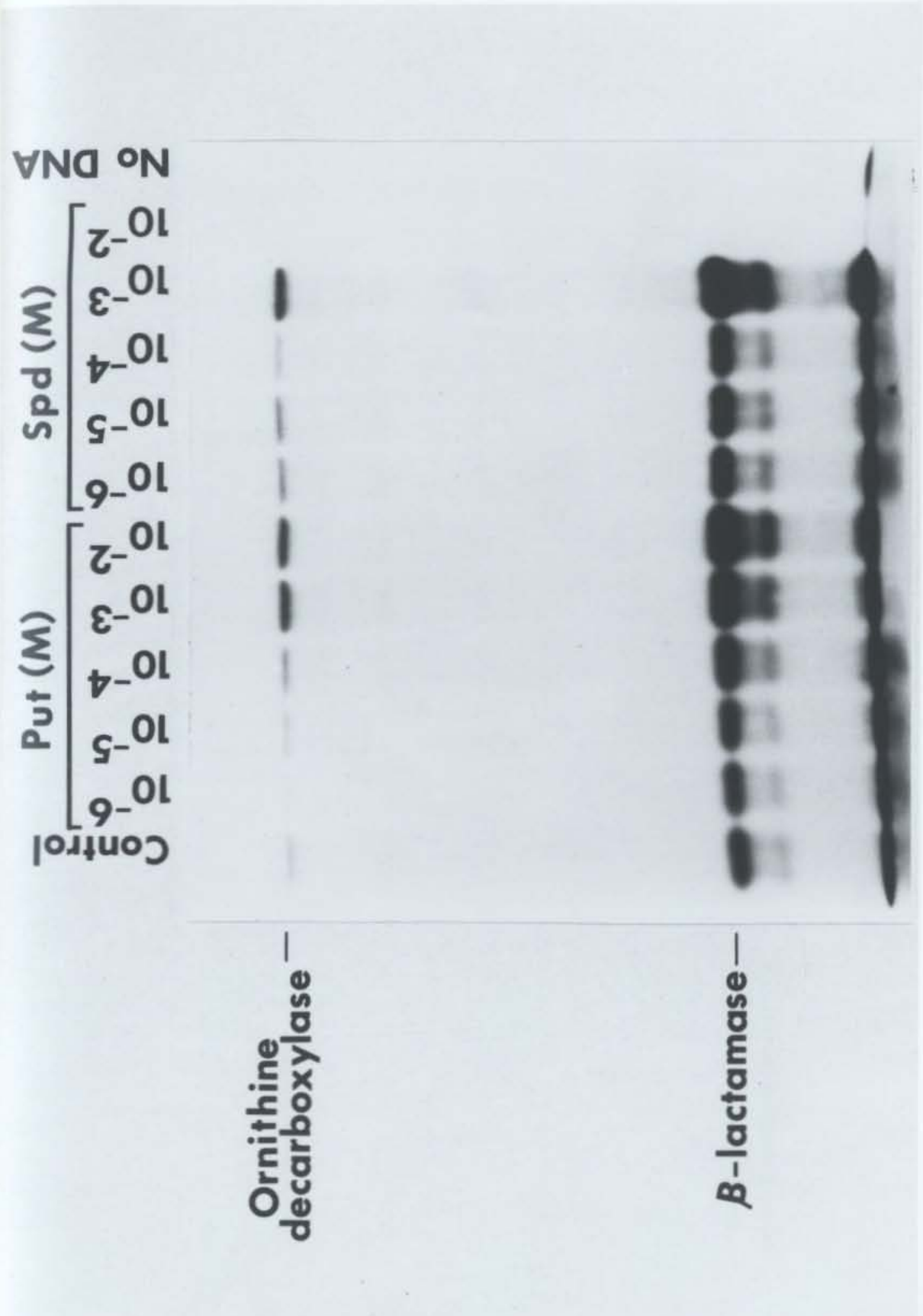

Ornithine decarboxylase
β-lactamase
Control
Put (M)
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²
Spd (M)
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²
No DNA

Table 9. Quantitation of the effect of polyamines on ornithine decarboxylase synthesis in a cell-free transcription-translation system.

| Polyamine | Scan Units[a] ODC | β-lac | ODC/β-lac |
|---|---|---|---|
| None | 1.91 (100) | 37.47 (100) | 0.051 (100) |
| $10^{-6}$M Put. | 0.00 (0) | 35.45 (96) | 0.000 ( 0) |
| $10^{-5}$M " | 0.44 (23) | 42.71 (113) | 0.010 (20) |
| $10^{-4}$M " | 1.63 (85) | 65.63 (175) | 0.025 (49) |
| $10^{-3}$M " | 14.67 (768) | 104.57 (279) | 0.140 (275) |
| $10^{-2}$M " | 14.06 (736) | 129.95 (348) | 0.108 (211) |
| $10^{-6}$M Spd. | 3.25 (170) | 65.60 (175) | 0.050 (98) |
| $10^{-5}$M " | 3.46 (181) | 66.55 (177) | 0.052 (102) |
| $10^{-4}$M " | 1.01 (52) | 59.68 (159) | 0.017 (33) |
| $10^{-3}$M " | 20.56 (1076) | 147.43 (393) | 0.139 (273) |
| $10^{-2}$M " | no polypeptides synthesized | | |

The amount of ornithine decarboxylase (ODC) and β-lactamase (β-lac) synthesized as a function of polyamines was estimated by densitometric scanning of the autoradiogram shown in figure 18. Scan units are defined in section 2.16. Values in parentheses are the percentage of the control.
Put=putrescine; Spd=spemidine.

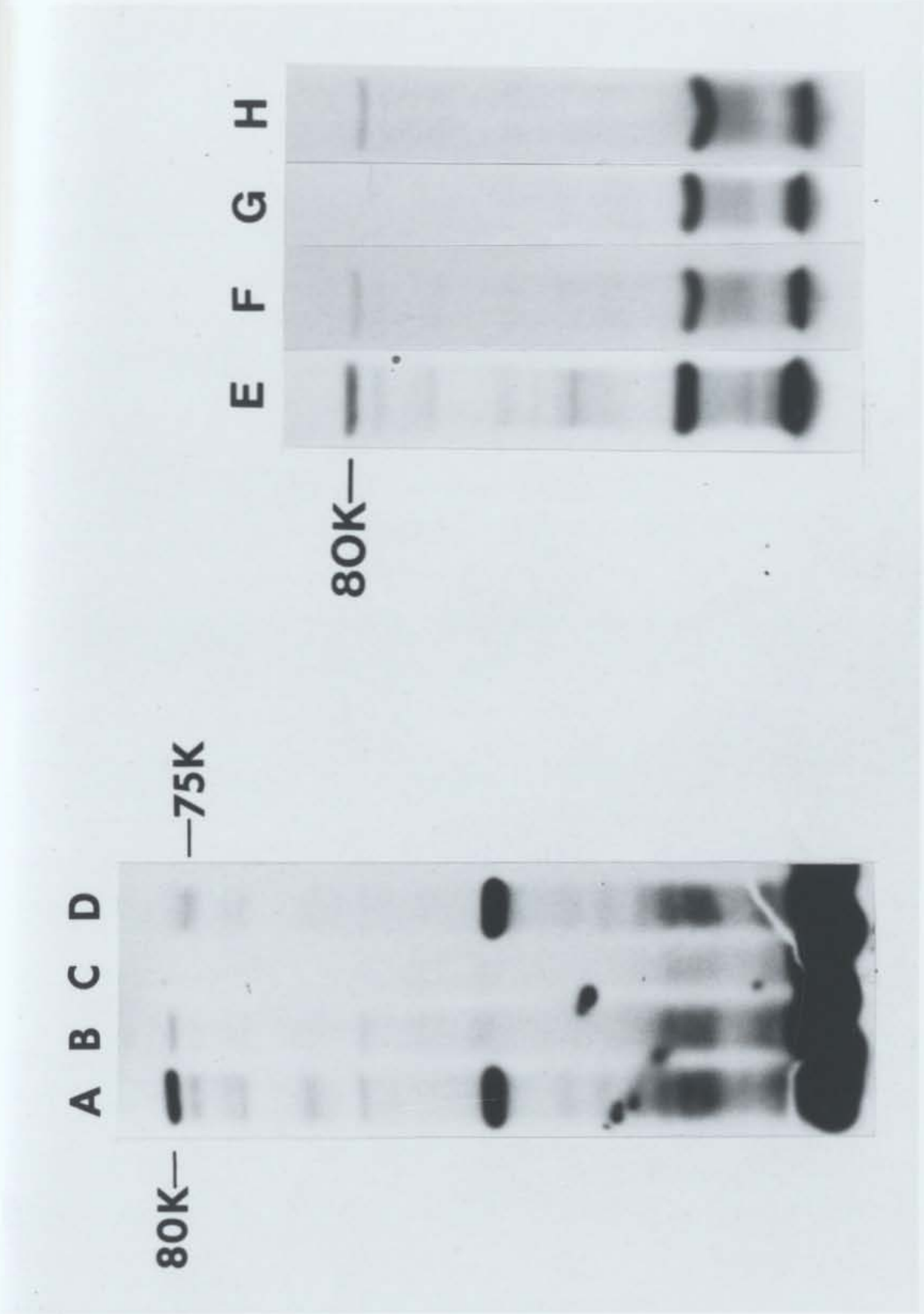
effect on ornithine decarboxylase synthesis, but did have a slight stimulatory effect on total protein synthesis (Figure 18 and Table 9). Synthesis of ornithine decarboxylase relative to β-lactamase was repressed when $10^{-4}$ M spermidine was added to the reaction system. At a concentration of $10^{-3}$ M spermidine, there was a dramatic stimulation of both ornithine decarboxylase and β-lactamase synthesis. Synthesis of ornithine decarboxylase and β-lactamase was totally abolished at $10^{-2}$ M spermidine, presumably due to precipitation of the DNA template.

5.2.4 Localization of the speC promoter

To determine the location of the speC promoter on plasmid pODC-1, DNA fragments of pODC-1 were generated by endonuclease digestion and used to direct the synthesis of ornithine decarboxylase in vitro. DNA fragments generated by Bam H1, Pst 1 or Eco R1 endonuclease digestion (Figure 5, pg. 106) directed the synthesis of an intact ornithine decarboxylase of 80,000 daltons. Fragments generated by Sma 1 endonuclease digestion directed the synthesis of a new polypeptide of approximately 70-75,000 daltons with the loss of the 80,000 dalton ornithine decarboxylase. However, fragments generated by Hind III endonuclease digestion did not direct the synthesis of an intact or truncated ornithine decarboxylase (Figure 19).

The linearized DNA was 60%-70% less efficient at directing protein synthesis in vitro than supercoiled DNA.

Figure 19. Polypeptides synthesized in a cell-free transcription-translation system directed by restriction endonuclease fragments of pODC-1 DNA. Plasmid pODC-1 DNA (5 ug) was digested with the following restriction endonuclease: (A) no enzyme; (B) Pst 1; (C) Hind III and Pst 1; (D) Sma 1; (E) no enzyme; (F) Eco R1; (G) Hind III; (H) Bam H1. Aliquots of the reaction mixtures were analyzed by electrophoresis in 10% polyacrylamide and 0.1% sodium dodecylsulphate. Polypeptides radiochemically labelled with [$^{35}$S]methionine were detected by fluorography. The position of ornithine decarboxylase of 80,000 daltons (80K) and truncated ornithine decarboxylase (75K) are indicated by the horizontal bars.


A B C D
80K—
—75K
E F G H
80K—

These results were interpreted to mean that the *speC* promoter is carried on the 1.1 kb *Pst* 1-*Hind* III restriction fragment of pODC-1 (Figure 5, pg. 106). The direction of transcription is deduced to be from the *Pst* 1-*Hind* III region toward the *Sma* 1 site with termination occuring downstream from the *Sma* 1 site.

## 5.3 Discussion

Using a coulped transcription-translation system, I have shown that the pODC-1 directed synthesis of ornithine decarboxylase is repressed by cAMP. Negative control of ornithine decarboxylase synthesis by cAMP, like ornithine decarboxylase activity *in vivo*, was dependent on a functional CRP being present in the reaction system.

The biphasic effect of putrescine and spermidine on ornithine decarboxylase synthesis *in vitro* can be interpreted as specific repression of *speC* transcription concommitant with a general stimulation of protein synthesis. Many studies have shown that polyamines stimulate protein synthesis *in vitro* and *in vivo*. Stimulation of protein synthesis is thought to be due, in part, to polyamines maintaining ribosome structure. In addition to their effect on ribosomes, polyamines have been shown to have a stimulatory effect on many reactions that are required for protein synthesis, e.g. renaturation of tRNA, binding of aminoacyl-tRNA and poly U to ribosomes, and the aminoacylation of tRNA (Tabor and Tabor, 1976). The

stimulation of ornithine decarboxylase relative to β-lactamase synthesis at higher concentrations of polyamines is consistent with previous reports that polyamines preferentially promoted the *in vitro* synthesis of higher molecular weight proteins (Atkins *et al.*, 1975; Watanabe *et al.*, 1981, 1983).

Repression of ornithine decarboxylase synthesis by putrescine and spermidine is in agreement with previous enzymic studies (Tabor and Tabor, 1969, Morris *et al.*, 1970). Tabor and Tabor (1969) reported that an ornithine auxotroph grown in an ornithine-limiting chemostat exhibited decreased ornithine decarboxylase activity following addition of putrescine and spermidine. The kinetics of inhibition suggested a combination of feed-back repression and inhibition. In another study, a mutant with a partial block in arginine decarboxylase activity was grown in the presence of arginine to cause a putrescine deficiency (Morris *et al.*, 1970). Under these conditions there was a four-fold derepression of ornithine decarboxylase activity. The repression of ornithine decarboxylase activity can be explained in light of the studies presented here as feedback repression. However, since polyamines have such a myriad of effects on protein synthesis, a study of the influence of polyamines on *speC* transcription may be best approached using an *in vitro* transcription system composed of highly purified components, or by gene fusion studies *in vivo*.

# Chapter 6

# GENE FUSION STUDIES

## 6.1. Introduction

In bacteria, gene expression is often controlled at the level of the initiation of transcription. Specific nucleotide sequences (promoters) in DNA determine the sites at which transcription starts. More than sixty promoter sites have been structurally defined with respect to nucleotide sequences. Two regions around -35 and -10 base pairs upstream from the initiation site appear to be conserved in promoters (Rosenberg and Court, 1979). Recent advances in recombinant DNA techniques have made it possible to place a known, easily assayable, gene function (e.g. β-galactosidase activity, tetracycline resistance, or galactokinase activity) under the transcriptional control of different regulatory elements. Gene fusions have proven extremely useful in the study of factors involved in regulation at the level of transcription initiation (McKenny et al., 1981).

In order to define more precisely the site at which cAMP-CRP negatively controls *speC* expression, the promoter region of the *speC* gene was fused to the structural gene for

tetracycline resistance (tet). The effect of cAMP, and other putative regulatory molecules, on tetracycline resistance of E. coli strains bearing a fused speC:tet gene was examined.

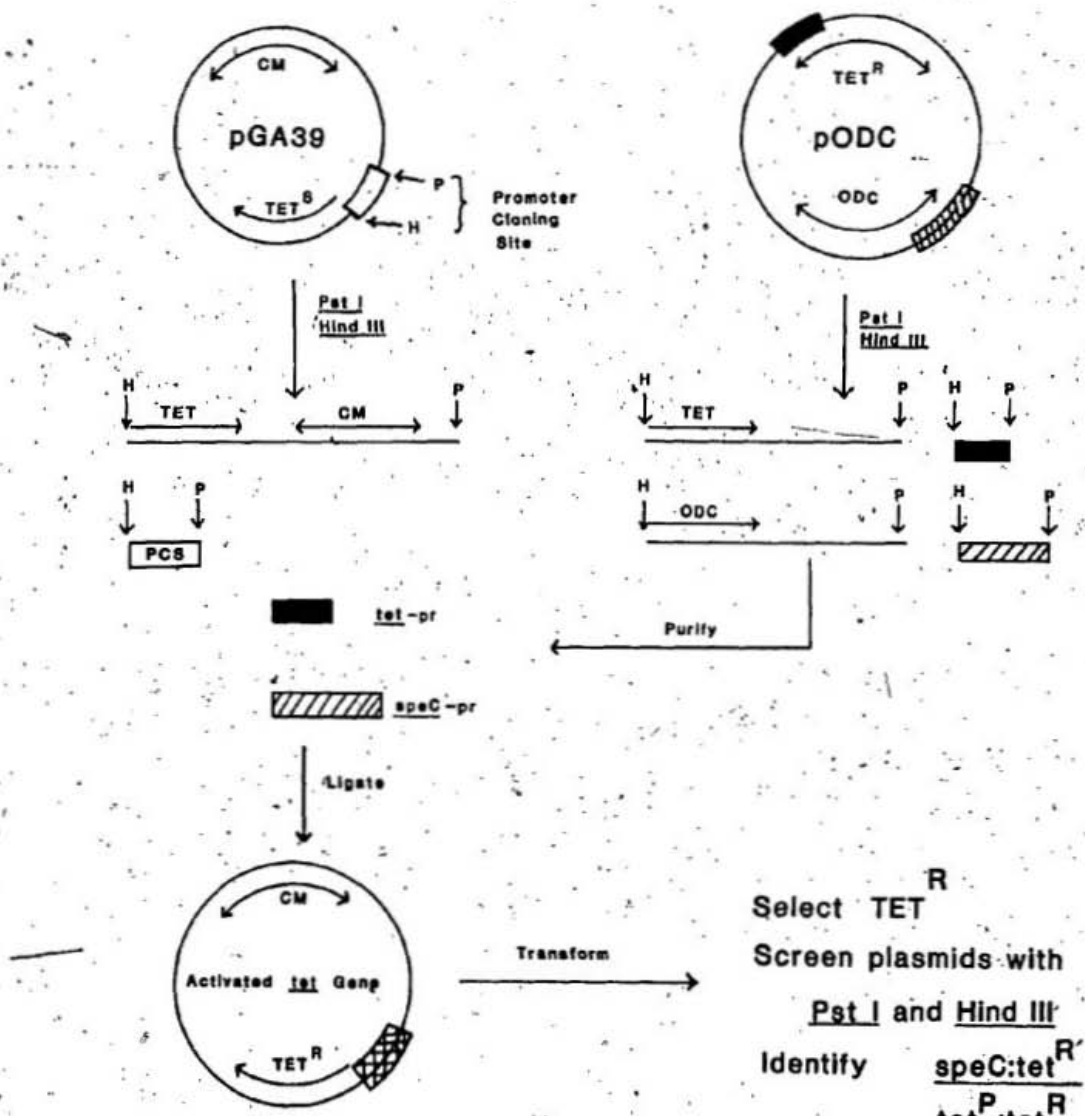
## 6.2 Results

### 6.2.1 Construction of a fused speC:tet gene

The construction of the fused speC:tet gene is shown in Figure 20. Plasmid pODC-1 DNA was digested with the restriction endonucleases, PstI and HindIII. The fragments generated by endonuclease digestion were fractionated by electrophoresis in low melt agarose gels as described in section 2.10. The 1.1 kb fragment bearing the speC promoter region was ligated into the promoter cloning site of plasmid pGA39 (An and Friesen, 1979). Plasmid pGA39 carries the tet structural gene that has had the promoter region deleted so that no tetracycline resistance is conferred on strains bearing this plasmid. The reconstructed plasmid, designated pCOD, conferred tetracycline resistance on host strains. Thus, the activation of the tet gene in plasmid pGA39 indicates that the 1.1 kb PstI-HindIII fragment carries the promoter for the speC gene. This result confirms the location of the speC promoter region on plasmid pODC-1 as deduced by cell-free transcription-translation experiments using pODC-1 restriction fragments as templates.

The 0.9 kb PstI-HindIII fragment of plasmid pODC-1, containing part of the tet promoter region was ligated into

Figure 20. Construction of a fused speC:tet gene. Plasmid pODC-1 DNA was digested with Pst 1 (P) and Hind III (H). The low molecular weight fragments containing the speC promoter (1.1 kilobase pairs) and the tet promoter (0.9 kilobase pairs) were resolved by electrophoresis in low-melting agarose. The purified fragments were separately ligated into the promoter cloning site of plasmid pGA39 in an orientation that would promote transcription of the tet gene. E. coli strain HB101 was transformed with the reconstructed plasmids and plated on Luria-agar plates containing 10 µg/ml tetracycline. The ligation of the promoter fragments into the pGA39 promoter cloning site was confirmed by Pst 1 - Hind III endonuclease digestion of plasmid DNA isolated from tetracycline resistant clones.


CM
pGA39
TET^S
P
H
Promoter Cloning Site
Pst I
Hind III
H
TET
CM
P
PCS
TET^R
pODC
ODC
tet-pr
speC-pr
Purify
Ligate
Activated tet Gene
Transform
Select TET^R
Screen plasmids with
Pst I and Hind III
Identify speC:tet^R
tet^P:tet^R

the promoter cloning site of pGA39, thereby reconstructing a normal *tet* gene. Strain HB101 transformed with this plasmid, designated pTET, exhibited no zone of growth inhibition around discs containing 75 µg of tetracyline.

### 6.2.2 Effect of cAMP on tetracycline resistance of strains bearing *speC:tet* gene fusion

The tetracycline resistance of *E. coli* strains transformed with plasmid pCOD, pTET or pGA39 was determined on MOPS-minimal plates with or without 5 mM cAMP. Figure 21 shows the plate assay for teteracycline resistance of *E. coli* strain HB101 transformed with plasmids pGA39, pCOD, and pTET. As shown in Figure 22 and Table 10, strain HB101 transformed with plasmid pCOD bearing the fused *speC:tet* gene was more sensitive to tetracycline when grown in the presence of 5 mM cAMP. This experiment was repeated four times with a similar extent of repression of tetracycline resistance by cAMP of wild-type and *cya* strains bearing plasmid pCOD. From one experiment to the other there was variability in the minimal inhibitory concentration. This was probably due to variability in preparation of fresh tetracycline discs prior to each experiment. The data shown are, therefore, from a representative experiment. Strain HB101 carrying plasmid pCOD, grown in the presence of cAMP, exhibited no zone of growth inhibition around discs containing up to 20 µg of tetracycline. In the absence of cAMP this strain exhibited no zone of growth inhibition up

Figure 21. Plate assay for tetracycline resistance. *E. coli* strain HB101 transformed with plasmid pGA39 (*tet*$^S$), pCOD (*speC*:*tet*), and pTET (*tet*$^R$) were streaked on MOPS-minimal plates with or without 5mM cAMP. Filter discs containing 1, 5, 10, 20, 30, 40, 50, 60, and 75 µg of tetracycline were placed on the surface of the agar plate (1 µg at top, with increasing amounts of tetracycline on discs placed counterclockwise, and 75 µg of tetracycline on the centre disc). The plates were incubated at 37°C for 18h. The clear zones around the filter discs indicate growth inhibition by tetracycline (see chapter 2, section 4).

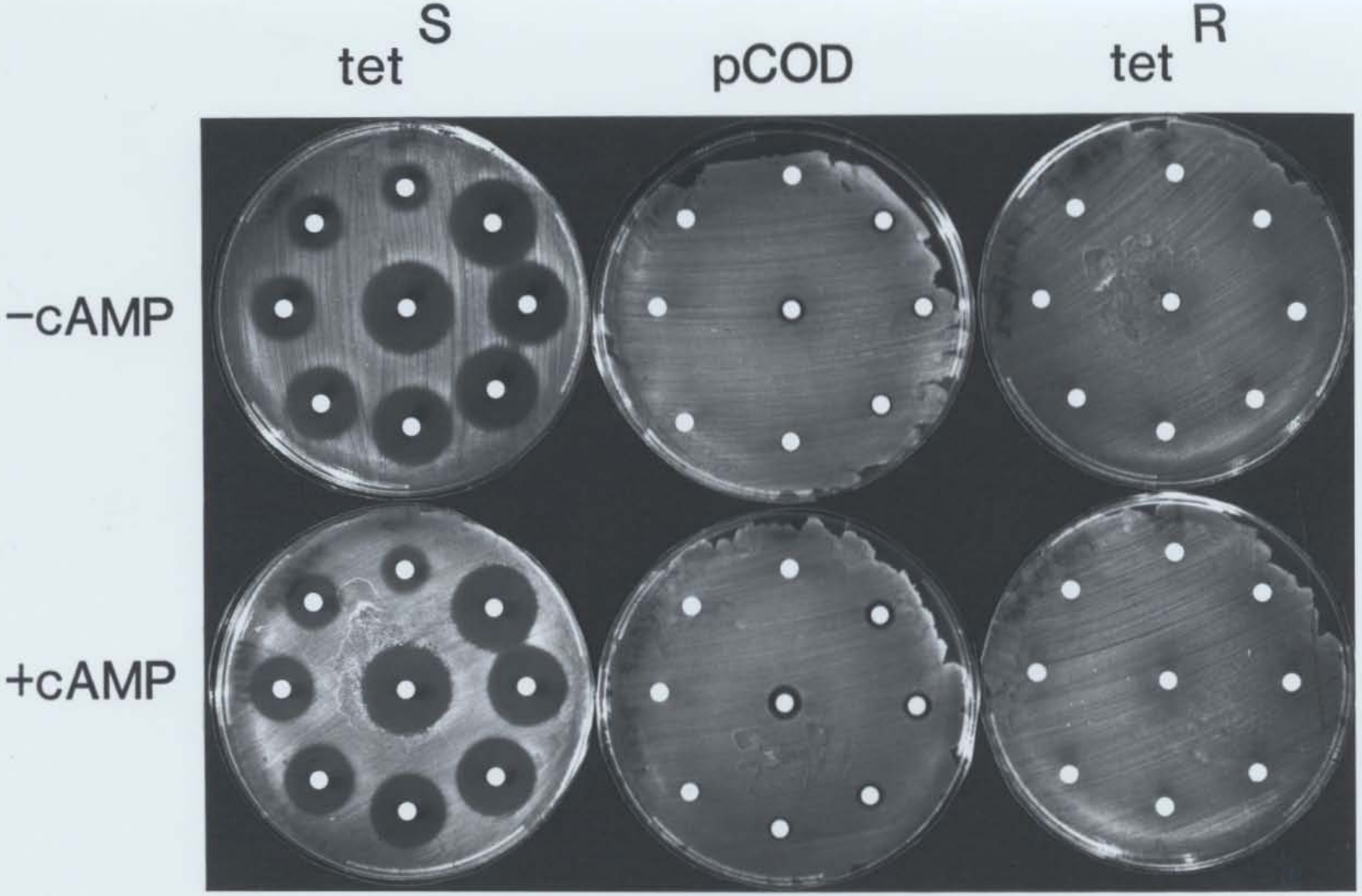

tet S
pCOD
tet R
−cAMP
+cAMP

Figure 22. Quantitation of the effect of cAMP on the tetracycline resistance of E. coli strains bearing a fused speC:tet gene. The diameter of the zone of growth inhibition around discs containing various amounts of tetracycline was determined for strains HB101, LS853 (cya), and LS854.1 (crp) transformed with plasmids pGA39 ($tet^S$, ▲), pCOD (speC:tet, ■) or pTET ($tet^R$, ●). Growth was in the presence (——) or absence (----) of 5mM cAMP.

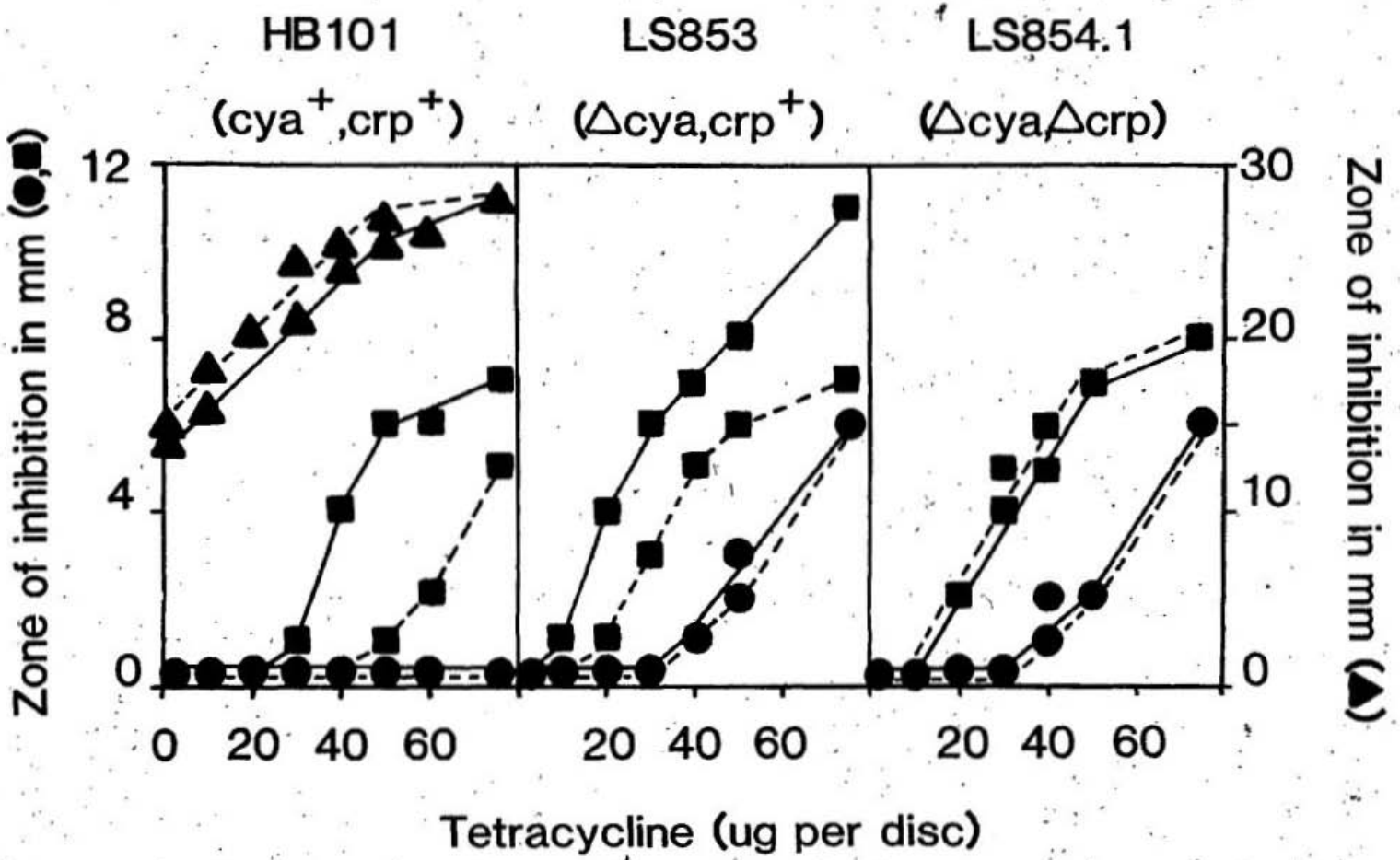

HB101
(cya+,crp+)
LS853
(Δcya,crp+)
LS854.1
(ΔcyaΔcrp)
Zone of inhibition in mm (●,■)
Zone of inhibition in mm (▲)
12
8
4
0
30
20
10
0
0 20 40 60
20 40 60
20 40 60
Tetracycline (ug per disc)

Table 10. Effect of cAMP on the tetracycline resistance of wild-type, cya, and crp strains transformed with plasmid pGA39, pCOD, or pTET.

| Strain | Plasmid | Resistance to tetracycline [a] | |
|---|---|---|---|
| | | Control | 5mM cAMP |
| HB101 | none | 1 | 1 |
| HB101 | pGA39 | 1 | 1 |
| HB101 | pCOD | 40 | 20 |
| HB101 | pTET | R | R |
| LS853 | none | 1 | 1 |
| LS853 | pGA39 | 1 | 1 |
| LS853 | pCOD | 20 | 10 |
| LS853 | pTET | 40 | 40 |
| LS854.1 | none | 1 | 1 |
| LS854.1 | pGA39 | 1 | 1 |
| LS854.1 | pCOD | 20 | 20 |
| LS854.1 | pTET | 40 | 40 |

Cultures of E. coli strains transformed with plasmids pCOD, pTET, and pGA39 were streaked over the surface of agar plates. Filter discs containing 1-75 µg of tetracycline were placed on the surface of the agar plate. The zones of growth inhibition were determined after 18h of incubation at 37°C.

a - The maximum amount of tetracycline (µg) on disc that did not produce a visible zone of growth inhibition. R - resistant to greater than 75 µg on the disc.

to 40 µg of tetracycline. In contrast, the tetracycline resistance of strain HB101 carrying the normal tet gene on plasmids pTET was unaffected by addition of cAMP. No zone of growth inhibition was seen around discs containing as much as 75 µg of tetracycline (Figure 22 and Table 10). It can be seen that cAMP had no effect on tetracycline resistance of the plasmid-free strain, or on the tetracycline resistance of those strains carrying a plasmid defective for tetracycline resistance (pGA39); both strains exhibited zone of growth inhibition around discs containing as little as 1 µg of tetracycline.

The decreased tetracycline resistance of strain HB101 transformed with plasmid pCOD grown in the presence of cAMP is consistent with reduced expression of the tet gene under control of the speC promoter. In order to ascertain whether CRP is involved in negative transcriptional control of the speC promoter, the tetracycline resistance of isogenic strains, LS853 (cya) and LS854.1 (cya, crp) carrying either plasmid pCOD or pTET was determined. In these experiments, strains were cultured on enriched rather than minimal plates as LS854.1 (cya, crp) grew very slowly on minimal plates. Although strain LS853 (cya) transformed with either plasmid pCOD or pTET was slightly more sensitive to tetracycline when grown on enriched rather than minimal medium, the effect of cAMP on tetracycline resistance of this strain was the same regardless of growth medium. Cyclic AMP decreased the tetracycline resistance of LS853 (cya) transformed with

pCOD but had no effect on the tetracycline resistance of this strain carrying plasmid pTET (Figure 22 and Table 10). Moreover, cAMP had no effect on tetracycline resistance of strain LS854.1 (*cya*, *crp*) transformed with either plasmid pCOD or pTET. These results indicate that CRP is required for cAMP to exert negative transcriptional control at the *speC* promoter.

### 6.2.3 Effect of other putative transcriptional regulatory molecules on tetracycline resistance of *speC:tet* bearing strains

The addition of ornithine or arginine at concentrations of $10^{-4}$ M to $10^{-2}$ M to the growth medium had no effect on the tetracycline resistance of strain HB101 transformed with either plasmid pCOD or pTET. Addition of putrescine to the growth medium at a concentration of $10^{-2}$ M reduced the tetracycline resistance of strain HB101 transformed with pCOD. Exogenous putrescine reduced the amount of tetracycline necessary to cause a visible zone of growth inhibition around the disc from 40 to 20 µg. The tetracycline resistance of HB101 transformed with plasmid pTET was not affected when grown in the presence of putrescine (Table 11). Spermidine had no effect on the tetracycline resistance of HB101 transformed with either plasmid pCOD or pTET at a concentration of $10^{-4}$ M. At concentrations greater than $10^{-4}$ M, spermidine caused a precipitate to form in the medium, such that no growth occurred on these plates.

Agmatine was also tested for its ability to regulate *speC* transcription. At a concentration of $10^{-2}$ M agmatine decreased the tetracycline resistance of strain HB101 carrying plasmid pCOD, but not strain HB101 carrying pTET (Data not shown). It was possible that agmatine did not have a direct effect on *speC* transcription but rather that the product of its hydrolysis by agmatine ureohydrolase (putrescine) was effecting repression of *speC* transcription. Therefore, an *E. coli* strain carrying a mutation in the *speB* gene (MA135), transformed with either plasmid pCOD or pTET was grown in the presence of agmatine. This strain is incapable of converting agmatine to putrescine due to a deficiency in agmatine ureohydrolase. The tetracycline resistance of strain MA135 transformed with plasmid pCOD was reduced when grown in the presence of $10^{-2}$ M agmatine. The tetracycline resistance of MA135 transformed with pTET was not changed by addition of agmatine to the growth medium (Table 11). These results indicate that agmatine, in addition to putrescine, represses transcription from the *speC* promoter.

## 6.3 Discussion

The activation of the *tet* gene on plasmid pGA39 by the 1.1 kb *Pst* I-*Hind* III fragment of plasmid pODC-1 confirms the location of, and the direction of transcription from, the *speC* promoter deduced from cell-free transcription-translation experiments described in section 5.2.4.

Table 11. Effect of amines on tetracycline resistance of strains HB101 and MA135 (speB) transformed with plasmid pGA39, pCOD, or pTET.

| Strain | Plasmid | Resistance to tetracycline[a] Control | $10^{-2}$M Putrescine |
|---|---|---|---|
| HB101 | none | 1 | 1 |
| HB101 | pGA39 | 1 | 1 |
| HB101 | pCOD | 40 | 20 |
| HB101 | pTET | R | R |

| Strain | Plasmid | Resistance to tetracycline Control | $10^{-2}$M Agmatine |
|---|---|---|---|
| MA135 | none | 1 | 1 |
| MA135 | pG39 | 1 | 1 |
| MA135 | pCOD | 50 | 30 |
| MA135 | pTET | R | R |

Cultures of E. coli strains transformed with pCOD, pTET, and pGA39 were streaked over the surface of agar plates. Filter disc containing 1-75 µg of tetracycline were placed on the surface of the agar plate. The zone of growth inhibition were determined after 18h of incubation at 37°C.

a - The maximum amount of tetracycline (µg) on disc that did not produce a visible zone of growth inhibition. R - resistant to greater than 75 µg of tetracycline on the disc.

It is unlikely that changes in the tetracycline resistance of *crp*$^+$ strains (HB101 and LS853) are due to cAMP influencing plasmid copy number. Unfortunately, it was not possible to determine plasmid copy number under the experimental conditions used here. However, since the plasmids pCOD and pTET are closely related in size and origin, the copy number of these two plasmids would be similarly affected (West *et al.*, 1979). Therefore, the influence of cAMP on plasmid copy number, if any, would be seen in similar changes in tetracycline resistance of *crp*$^+$ strains transformed with either pCOD and pTET. The differential effect by cAMP on the tetracycline resistance of these strains transformed with either pCOD or pTET would argue against changes in plasmid copy number being responsible for the observed effect of cAMP on tetracycline resistance of LS853 and HB101 transformed with pCOD. Also, it is known that cAMP does not influence uptake of tetracycline in *E. coli* (Botsford, 1981). The finding that the tetracycline resistance of *crp*$^+$ strains harbouring plasmid pTET was not changed by growth in the presence of cAMP corroborates the conclusions of Botsford (1981). Therefore, these data taken together suggest that repression of the tetracycline resistance of *crp*$^+$ strains bearing pCOD is the result of inhibition of transcription from the *speC* promoter. Moreover, the lack of repression by cAMP of the tetracycline resistnce of the CRP defective strain (LS854.1) transformed with pCOD indicates that CRP probably interacts

directly at the *speC* promoter. It is not possible to determine from the data presented here whether CRP binds to the RNA polymerase recognition site (as has been shown for the *ompA* and *galP2* promoters) or CRP binds to a leader sequence of the *speC* gene (as has been shown for the autogenous regulation of the *crp* gene). DNase footprinting studies of CRP protected *speC* promoter should clarify this point.

The repression of the tetracycline resistance of strains harbouring plasmid pCOD by putrescine is in agreement with the repression by putrescine of pODC-1 directed synthesis of ornithine decarboxylase *in vitro*. This result indicates that repression of ornithine decarboxylase synthesis (see preceeding chapter) and activity (Tabor and Tabor, 1969; Morris *et al.*, 1970) is mediated in part at the level of transcription. Since putrescine had no effect on the tetracycline resistance of strains transformed with pTET, repression of transcription by putrescine occurs as a function of the *speC* promoter.

Surprisingly, agmatine repressed the tetracycline resistance of strains bearing plasmid pCOD. It is tempting to speculate that agmatine may be involved in the switching from the putrescine biosynthetic pathway I (ornithine-putrescine) to pathway II (arginine-agmatine-putrescine) in cells grown in the presence of arginine (Morris *et al.*, 1970), i.e. increased agmatine due to decarboxylation of arginine causes cross-pathway feed-back repression.

However, earlier studies indicate that neither arginine nor agmatine influence the specific activity of ornithine decarboxylase in E. coli. At present the physiological role of agmatine in ornithine decarboxylase expression in E. coli remains obscure.

# Chapter 7

# QUICK BLOT ANALYSIS OF MESSENGER RNA IN E. COLI

## 7.1 Introduction

Our understanding of gene structure has been greatly enhanced by DNA blot techniques. In contrast, our understanding of gene expression has been hindered by the lack of corresponding RNA blot techniques. Recently, Bresser et al. (1983a,b) and Gillespie and Bresser (1983) have shown that eucaryotic mRNA in the presence of NaI and detergents can be selectively bound to nitrocellulose from whole cells. DNA can also be selectively bound to nitrocellulose by blotting the samples at elevated temperature and in the absence of detergent. The advantage of this quick blot method over previous methods is its selectivity, rapidity, and the fact that it can be easily quantitated. The mechanism of mRNA-nitrocellulose binding in the presence of NaI is not known. However, binding of mRNA to nitrocellulose is not dependent upon a 5' cap, 3' poly(A) tail, nor does it appear that secondary structure or G+C content are involved in selective binding of mRNA over rRNA and tRNA (Bresser et al., 1983a,b). Although little

protein is co-immobilized by this technique, incubation of the RNA-filter in $H_2O$, 70% ethanol and then acetic anhydride, reduces background in molecular hybridization experiments to a minimum.

Thus far, a dozen eucaryotic mRNAs have been shown to bind to nitrocellulose using the quick blot technique. At present, there are no reports that procaryotic mRNA can be selectively immobilized on nitrocellulose from whole cells. In this chapter, I describe experiments in which ornithine decarboxylase and β-galactosidase mRNAs are selectively immobilized on nitrocellulose from whole cells of E. coli. Furthermore, ornithine decarboxylase mRNA levels are repressed by cAMP-CRP, thereby confirming the negative transcriptional regulation of the speC gene of E. coli by cAMP.

## 7.2 Results

### 7.2.1 Selective immobilization of mRNA from whole cells of E. coli on nitrocellulose

The selective immobilization of mRNA and DNA from whole cells of E. coli strain LS853 (cya) on nitrocellulose is shown in Figure 23. Identical extracts of LS853 were treated with either ribonuclease A or deoxyribonuclease I for 30 min prior to immobilization of nucleic acids on nitrocellulose. The amount of radioactively-labelled pODC-1 that hybridized to nuclease treated samples was greatly reduced relative to untreated samples (Figure 23). This

indicates that there was very little non-specific binding of the pODC-1 probe to any co-immobilized protein. Similarly, there was no hybridization to samples of purified E. coli total RNA or chromosomal DNA treated under conditions that selectively immobilize DNA and RNA, respectively. Nor did immobilized mRNA and DNA from whole cells of Bacillis subtilis hybridize to the radioactively labelled pODC-1 probe. B. Subtilus DNA has no homology to the speC as determined by dot-blot analysis of purified chromosomal DNA (data not shown). These results taken together indicate that ornithine decarboxylase mRNA and DNA from whole cells of E. coli can be selectively immobilized on nitrocellulose by the quick blot method.

7.2.2 Estimation of the levels of ornithine decarboxylase and β-galactosidase mRNA in cya and crp strains of E. coli

Messenger RNA and DNA from whole cells of E. coli strain LS853 grown in the presence and absence of 0.5 mM IPTG and 2 mM cAMP were selectively immobilized on nitrocellulose and hybridized to either pODC-1 (speC) or pUC8 (lacZ) DNA (Figure 24). Relative levels of β-galactosidase mRNA in induced and uninduced cells were estimated by the densitometric scanning of autoradiograms. The hybridization signal to mRNA immobilized from a given number of cells divided by the hybridization signal to DNA immobilized from the same number of cells yielded a relative

Figure 23. Selective immobilization of mRNA and DNA from whole cells of E. coli on nitrocellulose. Samples were prepared for quick-blotting as described in Section 2.13. One half serial dilutions were made of samples a-f and i-l, and one fifth serial dilutions were made of samples g and h, prior to immobilization on nitrocellulose. (a) mRNA from whole cells of E. coli, (b) mRNA from whole cells of E. coli treated with RNase for 30 min. prior to immobilization, (c) purified E. coli total RNA (3.0 µg at lowest dilution), (d) purified E. coli total RNA treated under conditions for selective immobilization of DNA, (e) purified E. coli RNA treated with RNase for 30 min. prior to immobilization, (f) mRNA from whole cells of Bacillus subtilis. (g) DNA from whole cells of E. coli, (h) DNA from whole cells of E. coli treated with DNase for 30 min. prior to immobilization. (i) purified E. coli chromosomal DNA (1.5 µg at lowest dilution), (j) purified E. coli chromosomal DNA treated under conditions for selective immobilization of mRNA, (k) purified E. coli DNA treated with DNase for 30 min. prior to immobilization, (l) DNA from whole cells of Bacillus subtilis. The nitrocellulose filter was hybridized to pODC-1.

a b c d e f g h i j k l

mRNA level for a given gene (Gillespie and Bresser, 1983). No B-galactosidase mRNA was detected in LS853 (cya) grown in the absence of IPTG and cAMP (Table 12). The level of β-galactosidase mRNA in LS853 was induced by growth in the presence of IPTG.

The steady-state level of β-galactosidase in strain LS853 was further increased by growth in the presence of both IPTG and cAMP. This 2.3-fold increase in β-galactosidase mRNA levels is in agreement with the results of others (Contesse et al., 1970). The increase in β-galactosidase mRNA caused by growth in the presence ofcAMP and IPTG was reflected in a similar 1.5-fold increase in the specific activity of β-galactosidase (data not shown). There was no increase in β-galactosidase mRNA levels in the crp deletion strain LS854.1 grown in the presence of both cAMP andIPTG (Table 12). These results indicate that β-galactosidase mRNA from whole cells of E. coli can be selectively bound to nitrocellulose in the presence of NaI and detergents. Furthermore, the induction of the lac operon by cAMP demonstrates that cAMP-CRP facilitates normal positive regulation in this strain.

The levels of ornithine decarboxylase mRNA were found to be reduced by 84% when strain LS853 was grown in the presence of 2 mM cAMP (Table 13). The ornithine decarboxylase mRNA levels in strain LS854.1 (crp) were not, however, repressed by cAMP. Therefore, a functional CRP is required for negative transcriptional control of the speC gene

Figure 24. Autoradiogram showing ornithine decarboxylase and β-galactosidase mRNA levels in E. coli LS853. Cultures of LS853 (cya) were grown for 5-6 generations in MOPS-glucose medium, in the presence or absence of 0.5 mM IPTG and 2 mM cAMP. mRNA (R) and DNA (D) were prepared for quick-blotting as described in section 2.13. The nitrocellulose filters were hybridized to radioactive pODC-1 (speC) or pUC8 (lacZ).

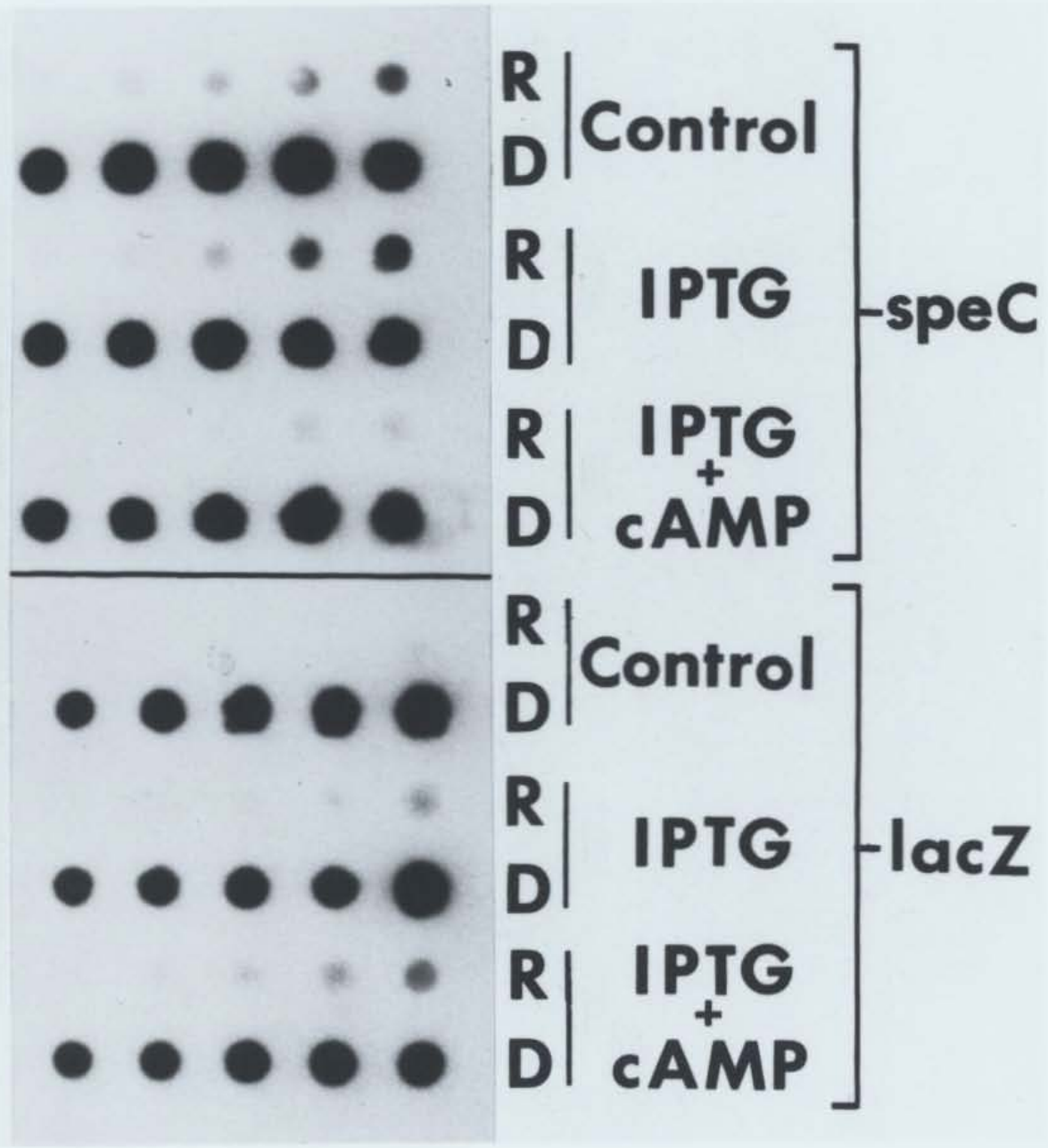

R
D
Control
R
D
IPTG
R
D
IPTG
+
cAMP
speC
R
D
Control
R
D
IPTG
R
D
IPTG
+
cAMP
lacZ

Table 12. Steady-state levels of β-galactosidase mRNA in *cya* and *crp* strains as a function of cAMP

| Growth conditions | mRNA level | |
|---|---|---|
| | LS853 | LS854.1 |
| Control | ND | ND |
| IPTG | 100 | 100 |
| IPTG + cAMP | 229 | 96 |

*E. coli* strains, LS853 (*cya*) and LS854.1 (*crp*) were grown in MOPS - glucose medium in the presence or absence of 0.5 mM IPTG and 2 mM cAMP for 5-6 generations. The mRNA levels were estimated by the quick blot method described in section 2.13. Values are expressed as a percentage of IPTG - induced levels. ND=not detectable.

Table 13. Effect of cAMP on the steady-state level of ornithine decarboxylase mRNA in *E. coli*

| Strain | Growth Conditions | Relative mRNA level | | | |
|---|---|---|---|---|---|
| | | Experiment | | | |
| | | 1 | 2 | 3 | Av. |
| LS853 | Control | 100 | 100 | 100 | 100 |
| | IPTG | 132 | 93 | 70 | 98 (±25) |
| | IPTG + cAMP | 11 | 24 | 12 | 16 (±6) |
| LS854.1 | Control | 100 | 100 | - | 100 |
| | IPTG | 91 | 89 | - | 90 |
| | IPTG + cAMP | 92 | 93 | - | 93 |

*E. coli* strains LS853 (*cya*) and LS854.1 (*crp*) were grown for 5-6 generations in MOPS - glucose medium in the presence or absence of 0.5 mM IPTG and 2mM cAMP. The mRNA levels were estimated by the quick-blot method described in section 2.13. Values in parentheses are standard deviations. All values are relative to the control.

## 7.3 Discussion

Ornithine decarboxylase mRNA, β-galactosidase mRNA and total DNA from whole cells of E. coli were selectively immobilized on nitrocellulose. This is the first report that mRNA from whole cells of E. coli can be immobilized on nitrocellulose in the presence of NaI.

The repression of steady-state levels of ornithine decarboxylase mRNA by cAMP in LS853 (cya) confirms that negative control by cAMP is exerted at the level of speC transcription. The lack of repression of ornithine decarboxylase mRNA levels in a crp deletion strain, LS854.1, indicates that negative transcriptional control by cAMP requires a functional CRP. Induction of β-galactosidase mRNA by cAMP in strain LS853 (cya) demonstrates that cAMP-CRP simultaneously exerts negative and positive transcriptional control in E. coli.

Steady-state levels of ornithine decarboxylase mRNA in cells grown in the presence of cAMP were approximately 20% of those grown in the absence of cAMP. The steady-state activity of ornithine decarboxylase in cells grown in the presence of cAMP was 40-50% of those grown in the absence of cAMP. It might be predicted that if mRNA levels are diminished by 80%, then there should be a corresponding 80% decrease in enzyme activity. These results suggest, therefore, that the difference in the extent of repression of enzyme activity and mRNA level caused by cAMP may be due to other factors, perhaps influencing translation of speC mRNA or enzyme activity.

# Chapter 8

# RELATEDNESS OF THE *SPEC* GENE IN ENTEROBACTERIACEAE

## 8.1 Introduction

As ornithine decarboxylase activity is so ubiquitous in living organisims (reviewed in Tabor and Tabor, 1976) it was of interest to learn if any homology exists between the *speC* gene of *E. coli* and DNA sequences in the chromosomes of other Gram negative and Gram positive bacteria. If homology does exist between the *speC* gene of *E. coli* and DNA sequences in the chromosomes of other genera, the intention was to use the *E. coli speC* gene as a probe to isolate the gene from other bacterial genera. Once cloned copies of the *speC* gene are available from several genera, a comparative study of the role of cAMP in *speC* transcription could be undertaken. The expectation that homology might exist was based on the assumption that extant bacterial genomes are derived from a common ancestor; i.e. the present day genomes of bacterial genera have diverged from an ancestral genome by base substitution and genome rearrangement (Riley and Anilionis, 1978). Once a function has been acquired by an organism, and the physiological requirements continue to

exist, there is strong selective pressure to conserve the DNA sequence of that gene (Riley and Anilionis, 1978). In addition, homology between sequences of taxonomically unrelated organisms may be the result of chromosomal transfer between such organsims after they diverged from a common ancestor (Filer and Furano, 1980).

## 8.2 Results

### 8.2.1 Characterization of the hybridization probe

It was necessary to characterize the pODC-1 hybridization probe because the 3.2 kb insert in the Pst 1 site of pBR322 originated from the ColE1 plasmid; pLC20-5, of the Clarke-Carbon bank (Clarke and Carbon, 1976) and may have contained sequences from ColE1. Hybridization of pODC-1 to Pst 1 fragments of E. coli chromosomal DNA showed that the 3.2 kb fragment of pODC-1 co-migrated with a Pst 1 fragment of E. coli chromosomal DNA (Figure 25A). This indicates that the entire 3.2 Kb fragment of pODC-1 is derived from E. coli chromosomal DNA. Another wild-type E. coli K-12 reference strain gave an identical result (data not shown).

It is important to note that the minimum amount of DNA required to code for an 80,000 dalton protein, the molecular weight of ornithine decarboxylase, is 2.0-2.2 Kb. Therefore, approximately 35% of the DNA of the 3.2 Kb fragment flanks the speC gene and may contain coding regions for other proteins. When radioactive polypeptides

Figure 25. Hybridization of [$^{32}$P] - labelled pODC-1 to restriction fragments of DNA from various sources. After electrophoresis in 1% (wt./vol) agarose the restriction fragments were transferred to nitrocellulose and hybridized to pODC-1 DNA. Numbers at left of figure refer to the size of molecular weight standards in kilobase pairs. Samples: (A) 0.025 µg of pODC-1, 0.025 µg of pBR322, and 2.5 µg of *E. coli* C600 DNA digested with 10 U of *Pst* 1. (B) 2.5 µg of *E. coli* C600, *E. coli* UW44 or *E. coli* DM22 (Δ *speC*) DNA digested with 24 U of *Pst* 1 or *Eco*R1 endonuclease. (C) 2.5 to 3.0 µg of *Citrobacter freundii*, *Salmonella typhimurium*, *Enterobacter aerogenes*, or *Klebsiella pneumoniae* DNA digested with 30 U of *Pst* 1 (P) or *Eco* R1 (E) endonuclease.

A
pODC-1
pBR322
E. coli C600
8.0
3.2
1.0
B
Pst1
E. coli C600
E. coli UW44
E. coli DM22
7.6
3.2
1.1
EcoR1
E. coli C600
E. coli UW44
E. coli DM22
7.6
3.2
1.1
C
P E
Salmonella
Citrobacter
Enterobacter
Klebsiella
7.6
3.2
1.1
0.9

Table 14

Biodegradative and biosynthetic ornithine decarboxylase activity and homology to speC among various bacterial genera

| Organism | Homology to speC | Specific activity[a] ODC[b] | ODI[c] |
|---|---|---|---|
| Escherichia coli C600 | + | 0.87 | 0.13 |
| Escherichia coli DM22 | + | < 0.001 | < 0.001 |
| Escherichia coli UW22 | + | 0.45 | 4.85 |
| Escherichia coli K-12 | + | 0.59 | 0.06 |
| Salmonella typhimurium | + | 0.28 | 420.41 |
| Citrobacter freundii | + | 0.51 | 0.09 |
| Enterobacter aerogenes | + | 0.31 | 445.33 |
| Klebsiella pneumoniae | + | 0.05 | 0.40 |
| Edwarsiella tarda | - | 0.93 | 203.34 |
| Morganella morgannii | - | 0.73 | 91.98 |
| Proteus mirabilis | - | < 0.001 | 24.30 |
| Serratia marcescens | - | 1.49 | 113.86 |
| Yersinia enterocolitica | - | 4.53 | 65.58 |
| Erwinia carotovora | - | < 0.001 | 0.16 |
| Pseudomonas putida | - | N.T. | N.T. |
| Coccochloris peniocystis | - | N.T. | N.T. |
| Halobacterium halobium | - | N.T. | N.T. |
| Halobacterium volcanii | - | N.T. | N.T. |
| Clostridium perfringens | - | N.T. | N.T. |
| Micrococcus lysodeiketicus | - | N.T. | N.T. |

[a] Specific Activity: μmoles $CO_2$ released/hr/mg protein.

[b] ODC-biosynthetic ornithine decarboxylase. Cells were grown in Luria broth. Preparation of extracts and assay conditions were as described in Section 2.3.

[c] ODI-biodegradative ornithine decarboxylase. Cells were grown in medium known to induce ODI in E. coli according to Applebaum et al (1975). Preparation of extracts and assay conditions were the same as for biosynthetic ornithine decarboxylase except that the breakage buffer and assay system was at pH 7.0.

N.T., = not tested.

synthesized in mincells containing pODC-1 are analyzed by sodium dodecylsulphate-polyacrylamide gel electrophoresis, however, only one protein of 80,000 daltons is observed in addition to those coded by pBR322 (see section 4.2.1).

8.2.2 Hybridization of pODC-1 to the gene for biodegradative ornithine decarboxylase

Hybridization of the pODC-1 probe to chromosomal DNA of E. coli strain UW44, a strain of E. coli that possesses both the biosynthetic and biodegradative ornithine decarboxylases (Table 14 and Applebaum et al., 1975), revealed a different pattern of radioactive bands than those detected in E. coli strain C600 (Figure 25B). Two fragments of 5.5 and 3.9 Kb were detected in Pst 1 endonuclease digests of E. coli UW44 DNA, compared to one fragment of 3.2 Kb in E. coli C600 DNA. Four fragments of 9.6, 7.4, 6.4, and 5.8 Kb were generated by Eco R1 endonuclease digestion of UW44 DNA that hybridized to speC, whereas three fragments of > 10.0, 6.4, and 5.8 Kb were detected in Eco R1 digests of C600 DNA. It is possible that the high molecular weight bands in the Eco R1 digests of C600 and UW44 DNA may represent undigested DNA.

The biodegradative and biosynthetic ornithine decarboxylases are remarkably similar in their subunit molecular weight, co-factor requirements and kinetic properties, but differ significantly in their pH optima and responses to activation by nucleotides (Applebaum et al., 1977). Although the genetic locus for biodegradative

ornithine decarboxylase has not been mapped, it is assumed that it is at a different position from *speC* (Applebaum *et al.*, 1977). Furthermore, it has been suggested that the biodegradative ornithine decarboxylase gene arose by gene duplication and subsequent divergence from the *speC* sequence (Applebaum *et al.*, 1977). Therefore, the additional radioactive bands detected in strain UW44 relative to *E. coli* K-12 strains which were found to lack biodegradative ornithine decarboxylase activity (Table 14), may represent portions of the biodegradative ornithine decarboxylase gene which are partially homologous to *speC*. This possibility is further supported by the hybridization pattern seen when pODC-1 was hybridized to *Pst* 1 and *Eco* R1 restriction fragments of a *speC* deletion strain of *E. coli*, DM22 (Figure 25B). The sizes of the restriction fragments that hybridize to the *speC* probe in DM22 chromosomal DNA are the same as the additional fragments seen in the hybridization pattern of *E. coli* UW44. Hybridization of [$^{32}$P]-labelled pBR322 to the chromosomal DNAs of *E. coli* strains detected no homologous sequences, indicating that the radioactive bands detected with the pODC-1 probe were homologous to the *speC* gene. No biodegradative ornithine decarboxylase activity was detected in strain DM22, however (Table 14). If the additional radioactive bands in strains UW44 and the *speC* deletion mutant represents sequences of the gene for biodegradative ornithine decarboxylase, these results suggest there are three classes of *E. coli* with respect to

the genes for the biosynthetic and biodegradative ornithine decarboxylases: One class that possesses an active speC gene (e.g. strain C600); a second class that possesses an active speC gene and an inactive gene coding for biodegradative ornithine decarboxylase (e.g. strain DM22 prior to the induced deletion of the speC gene); and a third class that possesses active genes for both biosynthetic and biodegradative ornithine dcarboxylases (e.g. strain UW44).

8.2.3 Intergeneric homology of speC among the Enterobacteriaceae

Of the bacteria listed in Table 14, Figure 25C shows only the four bacterial genomes that contain DNA sequences homologous to the speC region of E. coli genome. The sizes of the Pst 1 and Eco R1 restriction fragments that contained homologous sequences varied among the chromosomal DNAs, indicating that Pst 1 and Eco R1 recognition sites for the respective endonucleases occupied different positions within and outside the speC gene. When pBR322 was used as a hybridization probe, no homologous sequences were detected in the bacterial DNAs listed in Table 14. Therefore, the radioactive bands shown in Figure 25C correspond to sequences homologous to the speC region of the E. coli chromosome.

As can be seen from Table 14, the absence of homologous DNA sequences in a bacterial genome does not indicate that the organism lacks ornithine decarboxylase activity.

In addition to biosynthetic ornithine decarboxylase activity many of the Enterobacteriaceae possessed biodegradative ornithine decarboxylase activity. In E. coli strain UW44 (Applebaum, 1975), the biodegradative ornithine decarboxylase activity was ten fold higher than the biosynthetic ornithine decarboxylase activity. In several other Enterobacteriaceae (i.e. Salmonella, Enterobacter, and Proteus) the biodegradative ornithine decarboxylase activity was one thousand five hundred-fold greater than the corresponding biosynthetic ornithine decarboxylase activity.

In addition to screening bacterial DNA for homologous sequences to speC, radioactively-labelled pODC-1 DNA was hybridized to replicas of agarose gel electropherograms of restriction endonuclease digests of total DNA from Saccharomyces cerivisiae, Achlya ambisexualis, Chloroplast DNA from Zea mays, Trypanosoma gambiense, mouse liver, calf liver and HeLa cells. The amount of chromosomal DNA that was subjected to gel electropheresis was increased to 10 µg due to the increased complexity of the eucaryotic genome relative to the procaryotic genome. Also, the nucleic acid carrier in these hybridization reactions was E. coli tRNA instead of herring sperm DNA. This was done to preclude the possibility of herring sperm DNA sequences blocking potential hybridization sites for speC. None of these eucaryotic DNAs contained homologous sequences to pODC-1 as determined under the conditions used here (data not shown).

## 8.3 Discussion

The finding that only a limited number of Enterobacteriaceae (i.e. Citrobacter freundii, Salmonella typhimurium, Klebsiella pneumoniae, and Enterobacter aerogenes) contain homologous sequences to the spec region of the E. coli chromosome is in contrast to work done with other Enterobacteriaceae genes, using a similar experimental approach. The tuf gene, which encodes the elongation factor Tu, has diverged little, if at all, in different strains of E. coli and S. typhimurium, despite the significant sequence divergence between total genomes of these bacteria (Furano, 1978). Moreover, portions of tuf have been detected in the chromosomes of a diverse group of procaryotes (Filer and Furano, 1980) as well as the chloroplast DNA of Chlamydomonas reinhardtii (Watson and Surzycki, 1982). Portions of the lac, tna, trp and thy genes have been conserved in the genomes of many of the Enterobacteriaceae (Riley and Anilionis, 1980). The DNA sequence of the nif genes is also highly conserved in many nitrogen-fixing bacteria (Mazur et al., 1980: Ruvkun and Ansubel, 1980).

Although the genetic code is degenerate, that is, the DNA sequence need not be conserved to conserve protein sequence (see Efstratiadis et al., 1980; Jukes, 1980; Kimura, 1977; Kitamura et al., 1982; Nicols et al., 1980; for representative studies), the reports of DNA sequence conservation in various bacteria indicate that there is either strong selective pressure on particular DNA

sequences, or there has been intergeneric chromosomal transfer since divergence from a common ancestor. Filer and Furano (1980) concluded that the extensive intergeneric homology of tuf sequences is not the result of chromosomal transfer, but rather the result of strong selective pressure on tuf DNA sequences.

The limited intergeneric homology of speC sequences among the Enterobacteriaceae suggests that selective pressure on the DNA sequence of this gene has been less stringent than for other enterobacterial genes studied. Thus, the speC gene may be considered a rapidly evolving gene. This hypothesis is supported by the circumstantial evidence that only about one out of ten E. coli strains produce biodegradative ornithine decarboxylase, whereas all E. coli strains produce biosynthetic ornithine decarboxylase (Applebaum et al., 1977). The biosynthetic and biodegradative ornithine decarboxylases are considered to have only recently diverged in selected strains of E. coli (Applebaum et al., 1977) and suggests a tendency for degeneration of the speC DNA sequence. Alternatively, ornithine decarboxylase activity may be a recently acquired metabolic function in bacteria, and may have arisen independently in several genera, with each being capable of the same metabolic function. However, the ubiquity of ornithine decarboxylase activity in procaryotes and eucaryotes suggests a very ancient metabolic origin.

It is striking that some genera that are considered to

be closely related to the genus Escherichia, based on morphological, biochemical, and antigenic characteristics (Cowan, 1974), do not contain homologous sequences to the E. coli speC gene (e.g. Edwardsiella), whereas, more distantly related genera (e.g Enterobacter and Klebsiella) do contain homologous sequences to this region of the E. coli. It is not possible, at present, to speculate on the mechanism(s) which has conserved these sequences in genera less taxonomically related and not in genera more closely related.

# Chapter 9

## CONCLUDING REMARKS

The results of the experiments described in the preceeding chapters may be summarized as follows:

1) Cyclic AMP repressed the specific activites of biosynthetic ornithine decarboxylase, biosynthetic arginine decarboxylase, agmatine ureohydrolase, and glucose-6-phosphate dehydrogenase in wild-type and cya strains of E. coli. No repression of these enzyme activities was observed in a strain carrying a lesion in the crp structural gene, indicating that a functional CRP is required for repression. Nucleotides such as GTP and ppGpp and protein inhibitors reported to modulate the putrescine biosynthetic enzymes of E. coli were shown not to be involved in negative regulation of these enzymes by cAMP. Cyclic AMP exerted normal positive regulation of β-galactosidase, while simultaneously effecting negative regulation of ornithine decaboxylase and arginine decarboxylase. These results indicate that the negative control of ornithine decarboxylase and arginine decarboxylase by cAMP and CRP is exerted at the level of transcription.

2) Synthesis of ornithine decarboxylase directed by pODC-1 in minicells and in a cell-free protein synthesizing

system was repressed by cAMP. Repression required a functional CRP *in vitro*. These results indicate that repression of ornithine decarboxylase was direct rather than mediated by a cAMP-induced repressor protein of *speC* transcription.

3) The tetracycline resistance of wild-type and *cya* strains of *E. coli* harbouring the *tet* gene under the control of the *speC* promoter region was repressed by cAMP. Cyclic AMP had no effect on the tetracycline resistance of *crp* strains carrying the *speC-tet* gene fusion, nor wild-type strains bearing a normal *tet* gene. This indicates that cAMP-CRP interact at the *speC* promoter region to facilitate negative control of the *speC* gene.

4) Steady-state levels of ornithine decarboxylase mRNA in a *cya* strain, but not in a *crp* strain, were repressed by exogenous cAMP. This result confirms that negative control of the *speC* by cAMP and CRP is exerted at the level of transcription.

Until recently, it was believed that cAMP-CRP acts solely as an activator of catabolite-sensitive operons. The finding that cAMP-CRP exerts negative transcriptional control of the *speC* gene as well as negative transcriptional control of *galP2* (Musso *et al.*, 1977), spot 42 RNA (Sahagan and Dahlberg, 1979), possibly *ompA* (Movva *et al.*, 1981), and autogenous regulation of the *crp* gene (Aiba, 1983) extends the role of cAMP-CRP complex in procaryotic gene regulation.

The specific activities of two other putrescine

biosynthetic enzymes, arginine decarboxylase and agmatine ureohydrolase, were found to be repressed by cAMP and CRP. It is conceivable that negative control of these enzymes by cAMP and CRP is also exerted at the level of transcription. It therefore appears probable that cAMP co-ordinately controls the biosynthesis of putrescine, and hence spermidine in E. coli.

It is of particular interest that the level of this nucleotide regulates the production of polyamines, molecules that are thought to be intimately involved in the control of macromolecular synthesis. The level of cAMP varies inversely with the growth rate of E. coli cells. Cells cultured on glycerol exhibit high intracellular levels of cAMP, whereas cells cultured on glucose, which allows a faster growth rate have reduced intracellular levels of cAMP (Buettner et al., 1973; Epstein et al., 1975; Pastan and Adhya, 1976). The rates of synthesis of DNA, RNA and protein are markedly increased in rapidly growing cells (Nierlich, 1978). If polyamines are required to facilitate macromolecular synthesis, it seems that the levels of these compounds would be elevated in rapidly growing cells.

Cyclic AMP coordinates the mobilization of potential reserves of carbon and energy when readily available sources become limiting. As a result, cAMP has been called an "alarmone" or "hunger signal", sensing the metabolic state of the cell. Similarly, in liver and muscle cells of mammals, cAMP acts as a hunger signal; cAMP transmits the

signal in these cells via a protein kinase rather than by enhancing the transcription of catabolite-sensitive operons. The role of cAMP may be more extensive in procaryotic physiology. Several years ago, Sanwal (1970) reported that spermidine can replace magnesium as an activator of E. coli glucose-6-phosphate dehydrogense and may well be the natural activator of this enzyme. The ribose of RNA in E. coli is derived mainly from the pentose phosphate pathway, of which glucose-6-phosphate dehydrogenase is a key enzyme. Cohen (1971) has suggested that spermidine, a compound thought to facilitate RNA synthesis at the level of transcription, may also stimulate the supply of RNA precursors. The finding that cAMP negatively controls the production of spermidine via the putrescine biosynthetic enzymes as well as glucose-6-phosphate dehydrogenase (section 3.2.6) would be consistent with this scheme.

Insel and Fennel (1978) reported that cAMP repressed the activity of ornithine decarboxylase in mammalian cells. In S49 mouse lymphoma cells, cAMP-mediated repression of ornithine dearboxylase and S-adenosylmethionine decarboxylase activities requires a cAMP-dependent protein kinase. Although it is tempting to speculate on the possibility of a general regulation of polyamine biosynthesis by cAMP in both procaryotes and eucaryotes, there are many reports of cAMP stimulating ornithine decarboxylase activites in eucaryotic cells (reviewed in Jungman and Russell, 1977; Tabor and Tabor, 1976).

The repression of the tetracycline resistance of E. coli crp+ strains bearing a fused speC:tet gene by polyamines suggests that these cations negatively regulate speC transcription. This idea is supported by the finding that low concentrations of polyamines repress the synthesis of ornithine decarboxylase in a cell-free transcription-translation system. There are several possible mechanisms for negative control of speC transcription by polyamines. 1) These cations may intercalate directly at the speC promoter to reduce initiation of transcription. 2) They may interact with RNA polymerase to specifically reduce initiation or elongation of transcription of the speC gene. 3) Polyamines may be ligands for a speC repressor. Since evidence presented here indicates that CRP interacts with the speC promter, this regulatory molecule is a candidate for such a role. This hypothesis could be tested experimentally by examing the effect of polyamines on speC mRNA levels in a crp deletion strain. 4) Several reports indicate that the expression of certain genes in E. coli are regulated by the extent of DNA supercoiling (Gomez-Eichelman, 1981; Kano et al., 1981; Menzel and Gellert, 1983). The polyamines stimulate DNA gyrase activity, and inhibit the activity of topoisomerase I in vitro (Lipetz et al., 1980; Liu and Wang, 1978). Thus, polyamines have the potential to increase DNA supercoiling in vivo. An increase in negative superhelicity of the chromosome may cause local changes in the region of the speC promoter leading to

decreased initiation of transcription. Examining the effect of polyamines on ornithine decarboxylase mRNA levels in E. coli strains with defective topoiosmerase activities would test this hypothesis. Also, the effect of polyamines on speC transcription in a cell-free system directed by linearized pODC-1 could be examined.

Four members of the Enterobacteriaceae were shown to possess homologous sequences to the speC gene. Assuming that these sequences code for ornithine decarboxylase in these bacteria, this finding offers an opportunity to clone the speC gene from other bacterial genera. Once cloned copies of the gene from other genera are available, a comparative study of cAMP-CRP control of speC transcription can be undertaken. This type of study may lead to a clearer understanding of the relationship between cAMP and the polyamines, and their role in bacterial physiology.

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