The EMBO Journal Vol.2 No.5 pp.791 – 797, 1983

Regulation of adenylate cyclase synthesis in *Escherichia coli*: nucleotide sequence of the control region

A.Roy, C.Haziza[1] and A.Danchin*

Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, and [1]Institut de Microbiologie, Université de Paris-Sud, Centre d'Orsay, 91405 Orsay, France

Communicated by A.Danchin
Received on 25 February 1983

The regulatory region of the *cya* gene from *Escherichia coli* has been characterized by nucleotide sequence analysis and genetic approaches. Two promoters, P1 and P2, organized in that order with respect to the beginning of the *cya* open reading frame, were identified. Using *cya-lac* operon and protein fusions, it was possible to show that both promoters are active *in vivo*. P1 activity seemed sensitive to catabolite repression whereas activity of the stronger promoter, P2, did not respond to inhibition by glucose. No effect of cAMP or its receptor, catabolite activator protein (CAP), could be found although the DNA sequence reveals a consensus CAP site downstream of P2. The 548 nucleotides situated at the 3′ end of the sequence carry an open reading frame which can tentatively be assigned to the beginning of adenylate cyclase. Among noteworthy features of the corresponding sequence are an UUG codon as the putative start site of cyclase, and a long hydrophobic stretch of amino acids resembling leader peptides in secreted or membrane proteins.

Key words: bicephalic enzyme/*cya* gene/gene fusion/glucose effect/translation initiation codon UUG

Introduction

It has been known for several years that cholera toxin, as well as other toxins secreted by Enterobacteriaceae, permeate host cells and activate intracellular adenylate cyclase thereby creating a pathological situation. However, little attention had been paid to the possibility that soluble adenylate cyclases secreted by a number of pathogens could play a role in virulence until the recent work by Leppla (1982) on *Bacillus anthracis* and by Greenlee *et al.* (1982) on *Bordetella pertussis* revealed that bacterial adenylate cyclase is a main component of the bacterial toxins (Leppla, 1982; Confer and Eaton, 1982). It has been known for some time (Hewlett and Wolf, 1976) that *B. pertussis* secretes an adenylate cyclase, and a most remarkable feature of the enzyme has been discovered by Greenlee *et al.* (1982), namely that this bacterial cyclase is activated by an eukaryotic protein, calmodulin. These studies have suggested that the eukaryotic and prokaryotic enzymes might bear some resemblance to each other, and have given some impetus to understanding the regulation of bacterial adenylate cyclase activity and expression. Isolation of a regulatory mutant of *Escherichia coli* adenylate cyclase (Guidi-Rontani *et al.*, 1981) and study of the *cya* gene has suggested that the enzyme is comprised of two domains, catalytic and regulatory, which suggests an analogy with the cyclase of eukaryotic origin (Roy *et al.*, 1983). Study of the physical organization of the *cya* locus has revealed that transcription of the cyclase gene might display complex features. Indeed we could distinguish between two DNA regions which allowed transcription of the *cya* gene from different promoters (Roy and Danchin, 1982). To gain insight into the complex control, we analysed the DNA sequence of the *cya* promoter region (1200 bp) and constructed several *cya-lac* operon and protein fusions at known sites with respect to the putative promoters.

Here we describe the nucleotide sequence of the *cya* control region and present physiological data relevant to the expression of the genes. This allows us to propose localizations for the transcriptional as well as translational starts of cyclase mRNAs and protein. Amongst the various possible regulatory effectors of cyclase synthesis, neither cAMP nor its receptor, catabolite activator protein (CAP), seem to exert significant action. Glucose, however, appears to induce a negative control on *cya* transcription. The existence of two promoters and the structure of the putative start of the protein are discussed in the context of cyclase function.

Results

Nucleotide sequence of the cya gene control region

Figure 1 shows the sequencing strategy together with relevant restriction enzyme site, used to obtain the nucleotide sequence of the *cya* regulatory region. Figure 2 displays the DNA sequence encompassing the transcription and translation initiation regions of the *cya* gene. The corresponding 1200-bp fragment has been sequenced on both strands, and all restriction sites used for labelling have been checked by overlapping. Some of the noteworthy features of the sequence are given below.

(i) A long open reading frame (550 bp, Figures 2 and 3, register A1) which we identify as the beginning of the cyclase protein. An open reading frame of 300 bp in the region upstream of *cya* was found on the complementary strand. We tentatively identify it as the beginning of a polypeptide of 40 kd (P40) translated from a transcript diverging from the cyclase transcript (Roy and Danchin, 1982) (Figure 3, register B3).

(ii) Using the screening test of Stormo *et al.* (1982) for putative initiator codons, no AUG could be found in this open reading frame. The only possible initiator codon, preceded by the sequence AA--AGG--GAU, complementary to the 3′ end of 16S rRNA (Shine and Dalgarno, 1974) is

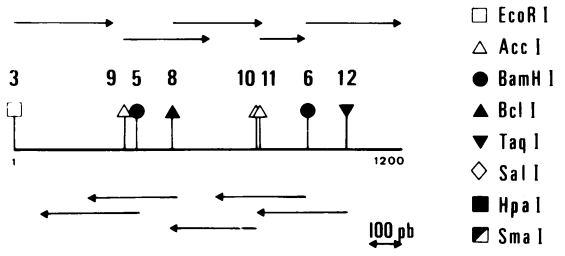

Fig. 1. Sequencing strategy for the *cya* gene regulatory region. The arrows indicate the sites used for 5′ labelling as well as the direction and extent of the sequences (only the sites used for labelling are shown).

*To whom reprint requests should be sent.

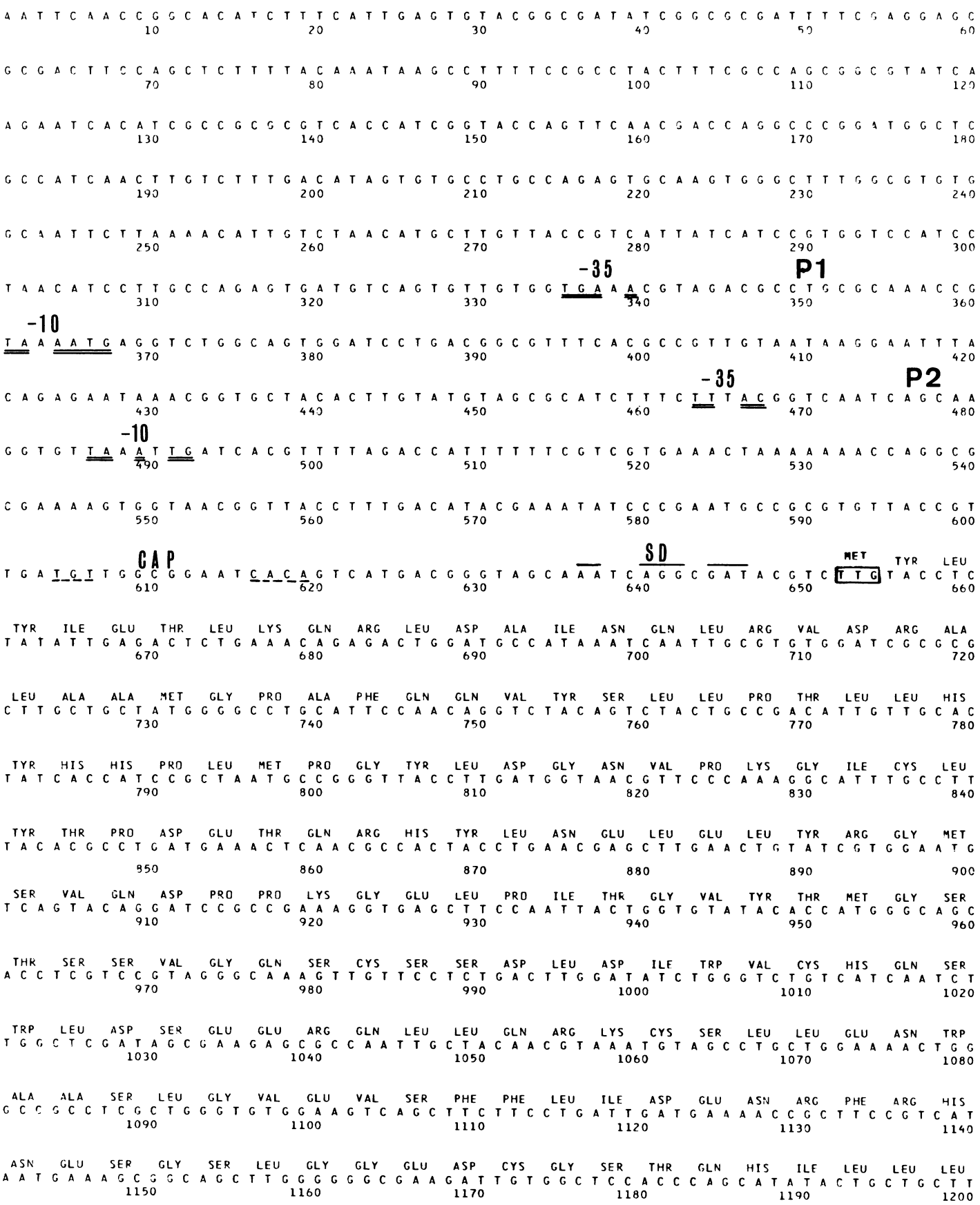

Fig. 2. Nucleotide sequence (1200 bp) of the *cya* gene control region. The amino acid sequence of the beginning of adenylate cyclase is deduced from the nucleotide sequence (see text). Promoter consensus bases are underlined with double lines and the CAP site with dashed lines. The UUG initiation codon is boxed and the Shine and Dalgarno consensus bases overlined.

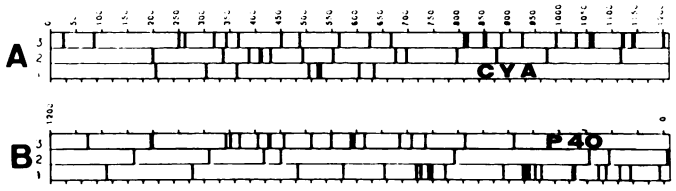

Fig. 3. Open-reading frames on each DNA strand. Vertical bars represent termination codons in all three registers labelled 1, 2, 3. 0 corresponds to the *Eco*RI site.

UUG. In addition to a good Shine and Dalgarno sequence, the putative start codon is preceded by a pyrimidine residue, known to promote more efficient formation of the initiation complex (Ganoza *et al.*, 1982).

(iii) Immediately following the putative UUG start site we can see a long stretch of hydrophobic amino acids which resembles the leader peptide of several secreted or membrane proteins in *E. coli* (Hall and Silhavy, 1981).

(iv) At least two promoter-like sequences can be localized upstream of the open reading frame. The first one, P1, is located in the restriction fragment *Eco*RI-*Bam*HI (3–5) (Figure 1), it possesses a −10 sequence (position 361) quite similar to the consensus Pribnow box, and around position 337 the sequence -TGA-A is not far from the consensus −35 TTGACA sequence (Figure 2). The second promoter, P2, located next to the site *Bcl*I (8), exhibits standard −10 and −35 sequences (position 486 and 463, respectively) (Figure 2) close to the consensus sequences G-TATAATG and TTGACA defined by Rosenberg and Court (1981). Transcription from this promoter would start at nucleotide G (498). Preliminary evidence using mRNA extraction and reverse transcriptase is consistent with this assignment. P2 is located 160 bp upstream from the translation start and this region is rich in palindromic sequence.

(v) A consensus CAP site (Queen and Rosenberg, 1981) is localized just before the UUG initiation codon (at position 604–619) exactly as is the *lac* operator site (Schmitz and Galas, 1979). Whether or not this CAP site is functional is discussed below.

Construction of cya-lac gene fusions

We have previously localized functionally two putative promoters for *cya* gene transcription (Roy and Danchin, 1982). It was therefore of interest to study their expression independently. Accordingly, we constructed operon and protein fusions placing the lactose operon under the control of either P1, P2 or both promoters, or placing the β-galactosidase gene under the cyclase translation start control. In strains harboring operon fusions, one can conveniently study transcriptional regulation exerted at *cya* promoter(s) by measuring synthesis of β-galactosidase [in fact a hybrid *trpA-lacZ* gene product in our constructions, (see Figure 4) showing normal β-galactosidase activity]. *cya-lacZ* protein fusions encode a hybrid protein in which the amino terminus of the β-galactosidase has been replaced by an amino-terminal portion of adenylate cyclase. Such hybrid proteins retained β-galactosidase activity and, in one case (plasmid pDIA 1861, see below), cyclase activity. Expression of hybrid proteins reflects the superimposition of transcriptional regulation exerted at *cya* promoters and additional regulations that might be exerted at the level of *cya* translation initiation.

Figure 4 displays the organization of plasmids carrying the

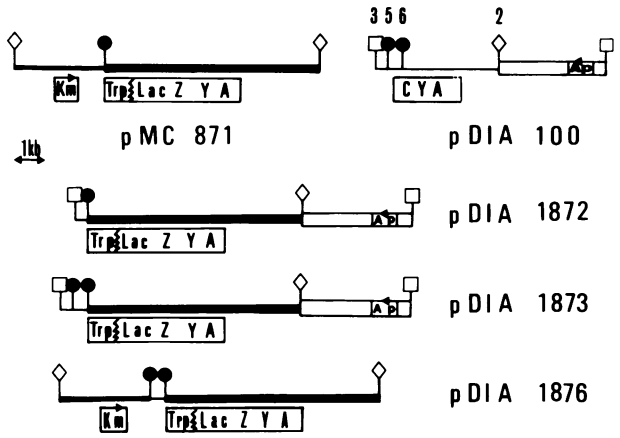

Fig. 4. Maps of the plasmids carrying *cya-lac* operon fusions. The double heavy line represents the *trp-lac* gene fusion, the single heavy line pMC871, the open line pBR322 and the thin line *E. coli* DNA. Gene products are indicated under the corresponding DNA segments The symbols are as given in Figure 1.

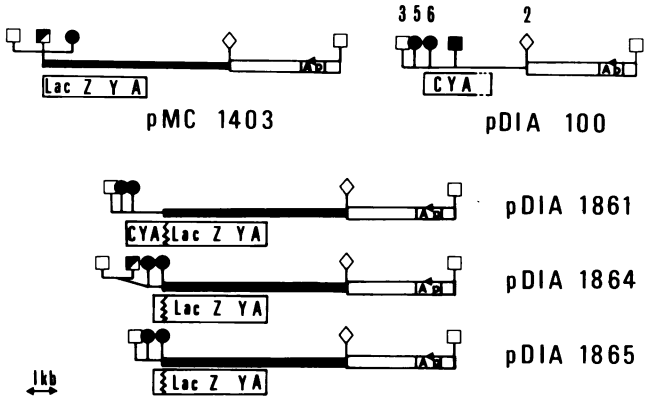

Fig. 5. Maps of the plasmids carrying *cya-lac* protein fusions. The heavy line represents the *lac* operon, the open line pBR322 and the thin line *E. coli* DNA. Gene products are indicated under the corresponding DNA segments. Symbols are as in Figure 1.

operon fusions that have been built in this study: (i) pDIA 1872 is an operon fusion that puts the *lacZYA* operon, from plasmid pMC871 constructed by Casadaban *et al.* (1980), under the control of the upstream *cya* promoter P1; (ii) pDIA 1873 allows expression of the *lac* operon through *cya* promoters P1 and P2; (iii) pDIA 1876 keeps only the second *cya* promoter P2 upstream of the *lacZYA* operon.

Plasmids carrying *cya-lacZ* protein fusions were of three different types. They are represented in Figure 5: (i) pDIA 1861 fuses the active N-terminal domain of the cyclase to the 8th amino acid of the β-galactosidase, from plasmid pMC1403 constructed by Casadaban *et al.* (1980). This plasmid exhibits both cyclase and β-galactosidase activities [strain TP 2006 (*cya*Δ *lac*Δ) carrying pDIA 1861 is Mal$^+$ Lac$^+$]; (ii) pDIA 1864 retains only the *cya* translation start site and the first 88 amino acids of cyclase which are fused to the 8th amino acid of β-galactosidase. In this case, transcription is controlled only by the second *cya* promoter, P2. In strain TP 2006 (*cya*Δ *lac*Δ) this plasmid expressed a Mal$^-$ Lac$^+$ phenotype; (iii) pDIA 1865 has an identical translation frame to pDIA 1864 but transcription is under the control of the two *cya* promoters P1 and P2.

All fusions were constructed *in vitro*, using appropriate

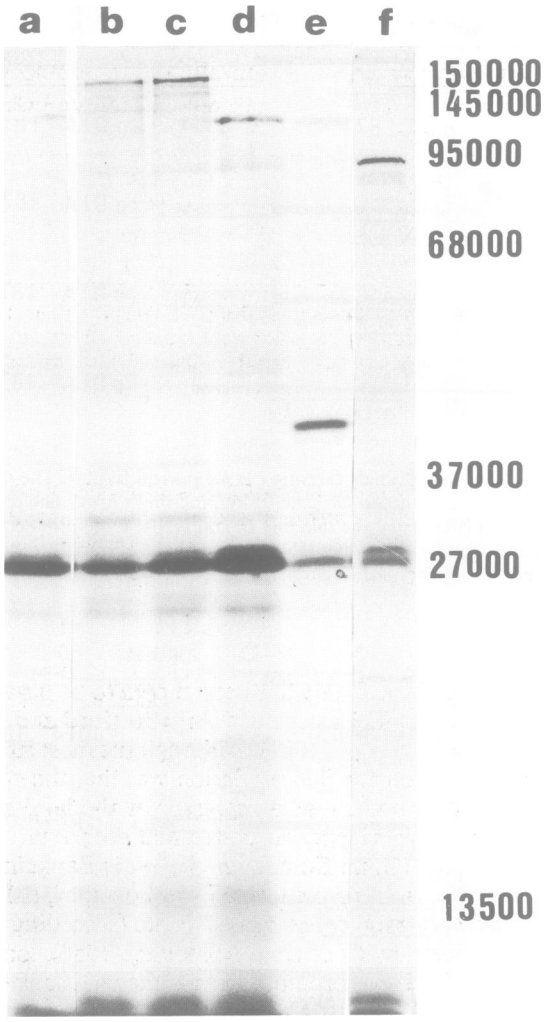

Fig. 6. Autoradiogram of SDS-polyacrylamide gel electrophoresis of proteins labelled in 'maxicells' system. **a**: pMC1403; **b**: pDIA 1861; **c**: pDIA 1861; **d**: pDIA 1872; **e**: pDIA 1873; **f**: pDIA 100. Radioactive bands are: **a**, β-lactamase (27 000) and precursor (31 000); **b** and **c**; β-lactamase and precursor, hybrid cyclase β-galactosidase (170 000); **d**, β-lactamase and precursor, β-galactosidase (125 000); **e**, β-lactamase and precursor, βD-galactoside permease (?) (45 000), B-galactosidase; **f**. β-lactamase and precursor, adenylate cyclase (95 000). Note that in **lane e**, the radioactive β-galactosidase band is very faint although it is extremely heavy in Coomassie blue staining (~2% of the total protein content of the cell). This is probably due to alteration of gene expression in irradiated CSR 603 cells.

restriction sites, then screened by complementation of a *lac*Δ strain. The nature of each fusion was controlled by restriction endonuclease digestion. The gene products specified by the fusions were identified in the 'maxicells' system (Figure 6). In the case of plasmid pDIA 1873, in addition to the β-lactamase and the β-galactosidase band on the autoradiogram, we could see a band migrating at 42 kd. We interpret this band as βD-galactoside permease, encoded by the *lacY* gene. Since the band is not visible in other *lacZYA* constructions, this suggests that pDIA 1873 is overproducing the *lacZYA* products, as compared with other plasmids. A confirmation of this interpretation comes from the observation (not shown) that staining the gel with Coomassie blue reveals a very intense band at the position of β-galactosidase in plasmid pDIA 1873 whereas the corresponding band is much weaker in other constructions.

Table I. Expression of β-galactosidase under control of *cya* promoter(s) in *crp*Δ or/and *cya*Δ backgrounds

Strain	Plasmid	Rate of β-galactosidase synthesis (units/mg)[a]	
		−cAMP	+ cAMP (1 mM)
TP 2100	pDIA 1872 (P1)	2590 ± 220	2320 ± 260
(*crp*$^+$ *cya*$^+$)	pDIA 1876 (P2)	10 480 ± 1570	10 270 ± 210
	pDIA 1873 (P1P2)	14 740 ± 1830	12 620 ± 520
TP 2006	pDIA 1872 (P1)	2600 ± 400	3340 ± 300
(*crp*$^+$ *cya*Δ)	pDIA 1876 (P2)	3900 ± 190	18 100 ± 900
	pDIA 1873 (P1P2)	5220 ± 800	17 300 ± 1400
TP 2139	pDIA 1872 (P1)	2320 ± 750	2600 ± 180
(*crp*Δ *cya*$^+$)	pDIA 1876 (P2)	4520 ± 700	4100 ± 260
	pDIA 1873 (P1P2)	10 060 ± 2830	11 430 ± 2210
	pDIA 1861	1650 ± 310	2120 ± 480
TP 2339	pDIA 1872 (P1)	2720 ± 140	2970 ± 80
(*crp*Δ *cya*Δ)	pDIA 1876 (P2)	4370 ± 140	4870 ± 810
	pDIA 1873 (P1P2)	6200 ± 480	6220 ± 150
	pDIA 1861	970 ± 40	850 ± 230

Measurements of β-galactosidase activity were performed on exponentially growing cultures and the carbon source was glucose. The plasmid copy number was measured as reflected by the amount of β-lactamase activity, it did not vary significantly except in the case of plasmid pDIA 1873 in strain TP 2006 (see Table II). Each figure represents the mean of several independent experiments. The large standard deviation in the highest activities reflects an important scatter due to instability of the plasmid in the corresponding background and growth conditions.
[a]Units of enzyme per mg (dry weight of bacteria).

Physiology of cya-lac fusions

The next step was to investigate the physiology of the fusions, especially the role of cAMP, its receptor CAP and carbon sources. To thoroughly evaluate a possible control mediated by the cAMP-CAP complex, the study was performed in isogenic *lac*Δ backgrounds, differing at genes *cya* or *crp*: *cya*$^+$ *crp*$^+$, *cya*Δ *crp*$^+$, *cya*$^+$ *crp*Δ, *cya*Δ *crp*Δ, using different growth media, with or without cAMP. The results obtained are represented in Table I.

(i) β-Galactosidase synthesis is 5-fold lower when expressed from P1 (pDIA 1872) as compared with P2 (pDIA 1876). When both promoters are present (pDIA 1873) the rate of β-galactosidase synthesis is the sum of the rates of synthesis coming from each individual promoter. This result holds true in each background tested and it cannot be ascribed to a variation in plasmid copy number (checked by measuring β-lactamase synthesis in each case).

(ii) cAMP synthesis is strongly enhanced in a *crp*$^-$ background. This has been ascribed to a repressor activity of the cAMP-CRP complex on adenylate cyclase synthesis (Bostford and Drexler, 1978). In our constructions, this phenomenon would have resulted in an inhibitory action of cAMP on the rate of β-galactosidase synthesis expressed from the *cya* promoters. However, we observe a stimulatory action of cAMP in the *cya*Δ *crp*$^+$ background. This effect is related to the nature of the carbon source on which the strains are growing [see below (v)].

(iii) Synthesis of β-galactosidase is unaffected by the absence of CAP either in operon or in protein fusions. It must be noted that plasmid pDIA 1861 carrying a *cya-lac* protein fusion exhibiting bicephalic activities (adenylate cyclase and β-galactosidase) is extremely unstable in a *crp*$^+$ background, presumably because it can synthesize high levels

Table II. Effect of carbon source on differential rates of β-galactosidase synthesis expressed from *cya* promoters

TP 2006 *cyaΔcrp⁺lacΔ*	cAMP (1 mM)	Rate of β-galactosidase synthesis[a] ΔZ/ΔB		Rate of β-lactamase synthesis[a] ΔAp/ΔB		$\frac{(\Delta Z/\Delta B)Glc}{(\Delta Z/\Delta B)Glp} \times \frac{(\Delta Ap/\Delta B)Glp}{(\Delta Ap/\Delta B/Glc}$
		Glycerol	Glucose	Glycerol	Glucose	
pDIA 1872	−	3120 ± 700	2600 ± 400	8600	13 300	0.54
	+	3840 ± 1200	3340 ± 300	8500	10 700	0.7
pDIA 1873	−	23 400 ± 5300	5220 ± 800	8700	1600	1.22
	+	27 000 ± 3200	17 300 ± 1400	13 800	3300	(2.7)

β-Galactosidase and β-lactamase rates of synthesis are measured in exponentially growing cultures. Note that β-lactamase activity, reflecting the plasmid copy number, fluctuates within a large range of values, especially when cells are grown on glucose. For this reason, we have shown the standardized ratio of β-galactosidase expression for cells grown on glucose (Glc) with respect to cells grown on glycerol (Glp) (third column). The values obtained in the case of pDIA 1873 in the presence of cAMP are only estimates, because of the toxic effect of the plasmid.
[a]Units of enzyme per mg (dry weight) of bacteria.

of cAMP incompatible with the presence of an active CAP (*E. coli* is sensitive to an excess of cAMP). This precludes the study of β-galactosidase synthesis from plasmid pDIA 1861 in the presence of a functional CAP.

(iv) In the *cya-lac* protein fusion (pDIA 1861) carrying the complete control region (P1 and P2), β-galactosidase synthesis is 6-fold lower than in the cognate operon fusion (pDIA 1873). This suggests that translation efficiency is lower when the *cya* start is in use.

(v) To study the influence of carbon source, β-galactosidase was determined when the strains were grown in minimal medium supplemented with glucose or glycerol in a *cyaΔ* background permitting growth on glycerol (Glp⁺). Since cAMP addition and the nature of carbon source might affect the plasmid content of the cell, the plasmid copy number was determined indirectly by monitoring β-lactamase as an internal standard (this is not possible with plasmid pDIA 1876 which does not harbour the gene for β-lactamase synthesis). As can be seen in Table II, the mean plasmid copy number per cell seems to be extremely sensitive to the carbon source when the plasmid carries the P2 promoter (pDIA 1873). That this must be due to a toxic effect of a high expression of the *lacZYA* operon (at least when bacteria are grown on glucose) is reflected by the fact that a large number of cells lose plasmid pDIA 1873 during their growth (data not shown), but do not lose plasmid pDIA 1872 in which the *lac* operon is expressed at a much lower level. Therefore, making the correction required by the variation in mean plasmid content of the cells allows us to propose the following tentative conclusions. Expression from promoter P1 seems to be sensitive to the carbon source. It is lowered by a factor of two when bacteria are grown on glucose as compared with glycerol, and this inhibitory effect is, at least in part, relieved by cAMP. On the other hand, no glucose effect is seen when β-galactosidase is expressed from P1 and P2 in tandem, whereas cAMP seems to exert a general stimlatory effect on the overall *lac* expression (one should note, however, that the corresponding level of *lac* expression is so high that this latter result must be considered with some caution).

The observation that cAMP appers to relieve the glucose-mediated repression of β-galactosidase synthesis expressed from P1 seems interesting. In this respect, synthesis of adenylate cyclase appears to be controlled, at least in part, in the same way as other systems sensitive to catabolite repression (i.e., inhibition mediated by glucose in the absence of cAMP relieved by addition of exogeneous cAMP, see Ullmann and Danchin, 1983). These results summarizing several experiments must however be considered with some caution because the plasmids are extremely unstable in minimal medium, but the variation they show is certainly significant qualitatively if not quantitatively.

Discussion

cAMP synthesis in *E. coli* has been shown to be negatively regulated by the cAMP receptor, CAP (Bostford and Drexler, 1978; Majerfeld *et al.*, 1981). Although the most straightforward explanation for this regulation was that the cAMP-CAP complex was acting as a repressor at the level of *cya* transcription, some evidence suggested that the picture might be more complex (Guidi-Rontani *et al.*, 1981; Bankaitis and Bassford, 1982). In the present work we show that, although there exists a very good consensus CAP site (according to the sequence proposed by Queen and Rosenberg, 1980), localized downstream of the *cya* promoters (as is the lactose operator in the *lac* operon), neither CAP nor cAMP have a negative action on *cya* gene expression. Since we use multicopy plasmids, one could argue that the CAP site located on the plasmid titrates the cAMP-CAP repressor complex. However, the number of CAP molecules in the cell is high [3000 molecules/cell (Guiso and Blazy, 1980)], this exceeds the copy number of the plasmid by at least 50-fold. It is, therefore, unlikely that a negative control mediated by CAP could have been hidden by the multicopy plasmids. Furthermore, we do see an effect of cAMP, but this effect, mediated by CAP (it disappears in *crp*⁻ strains, see Table I), is a stimulation of cyclase transcription when bacteria are grown on glucose. We have investigated the cAMP-CAP negative control of *cya* expression under a variety of growth conditions and found no such effect (Table I and unpublished data), but we cannot exclude a negative action of the cAMP-CAP complex under certain exceptional growth conditions which the bactria might meet in their natural environment. However, since CAP-dependent negative regulation of cAMP synthesis has been observed under growth conditions similar to those that we have used, it appears that CAP exerts its action at the level of cyclase activity (Joseph *et al.*, 1982). We cannot, at present, say whether such CAP-mediated regulation implies a direct action of the cAMP-CAP complex on cyclase or whether the complex controls the synthesis of a regulator of cyclase activity (such as the glucose:phosphoenol pyruvate-dependent phosphotransferase enzyme III^{Glc}, Postma *et al.*, 1981). The cAMP-positive effect on cyclase synthesis when bacteria are grown on glucose is worth noting.

In their study of cyclase expression, Bankaitis and Bassford (1982) failed to observe any action of the carbon source on cyclase synthesis, but they could only perform their investigation in a *cya*$^+$ background. Their results are indeed consistent with ours: we do not see an effect of cAMP on cells grown on glucose in a *cya*$^+$ background (strain TP 2100, Table I), whereas we do see such an effect in a *cya*$^-$ background (strain TP 2006, Table II). This strongly suggests that glucose exerts – in the absence of cAMP – a repressing effect on cyclase synthesis, and that this effect is at least in part relieved by cAMP (in the presence of a functional *crp* gene). In other words, adenylate cyclase synthesis seems to be sensitive to catabolite repression, mediated by an unknown factor, as described in other instances, and relieved by cAMP (for review, see Ullmann and Danchin, 1983). This is consistent with our previous results (Guidi-Rontani *et al.*, 1981) suggesting that the decrease in cAMP synthesis is a consequence rather than the cause of catabolite repression.

This glucose effect cannot yet be ascribed to specific molecular features of the *cya* control region, but it may be related to another noteworthy feature of *cya* expression, namely that it depends on the action of two promoters. We have localized the promoters using nucleotide sequence comparison with known sequences (Rosenberg and Court, 1979), knowing by *in vitro* constructions that P1 is located upstream of nucleotide 380 (Roy and Danchin, 1982, and this work) and that P2 lies between positions 380 and 910. Under the growth conditions investigated, both promoters seem to be regulated in the same way (except for the glucose control which seems to be specific for P1) and they appear to be additive (P1 + P2 used alone equal P1P2 in tandem), P2 is however five times stronger than P1. Their expression is modulated by the growth medium (enhanced in synthetic media as compared with rich medium; data not shown), and P1 is sensitive to a catabolite-like repression-negative control. The distance between the promoters could allow interaction with unknown regulatory molecules. In addition, P1 is located in an area where a strong divergent promoter is active (Roy and Danchin, 1982), its activity could, therefore, be stringently coupled to the activity of this divergent promoter.

Before discussing further the function of these promoters, we emphasize that we ensured that the actual *cya* locus organization was the one described here: indeed we isolated a cosmid complementing *cya* from an *E. coli* cosmid library and checked, by restriction enzyme analysis, that the map was identical with the map found in pDIA 100 (not shown). Several bacterial genes have been shown to be transcribed from two promoters [e.g., galactose operon, Musso *et al.*, (1977), rRNA genes, Nomura and Post (1979)] but the actual function for such an organization is still a matter of conjecture. Gene organization in *E. coli* is rather compact, but it could be that certain control regions exhibit some redundancy. We believe, however, that investigation of cyclase synthesis upon growth in stressing environments (such as shifts from anaerobiosis to aerobiosis, changes in pH, availability of nutrients, etc.) might reveal differential expression of the promoters. A more speculative view of such organization would be that the existence of two promoters reflects the remnants of an evolutionary gene transfer. The observation that a prokaryotic cyclase mimics properties of an eukaryotic cyclase (Greenlee *et al.*, 1982) is consistent with this view: bacterial cyclase could be phylogenetically related to the eukaryotic enzyme as the *E. coli* cAMP receptor seems to exhibit sequence homologies with an eukaryotic cAMP receptor (Weber *et al.*, 1982).

Our study also enables us to propose that translation of the cyclase protein starts at position 652: indeed we know the reading frame of the gene, because fusions at position 909 with the *Bam*HI site of pMC1403 (Casadaban *et al.*, 1980) yield a functional β-galactosidase (plasmids pDIA 1864 and pDIA 1865) and we obviously, also know that the start site is upstream of position 909. We looked, therefore, for initiation codons using the Stormo rule (Stormo *et al.*, 1982) and found no suitable candidate (the only AUG codons: 730, 796, 898 do not fit the rule, nor does GUG 708). We found, however, that the UUG codon located at position 652 might behave as a start codon. Indeed, if one changes the first U into an A the new AUG codon fits the Stormo rule well, and this is consistent with the fact that UUG (652) is preceded by a long sequence which is complementary to the 3′ end of 16S RNA (eight bases out of 11) (Shine and Dalgarno, 1974). It is also possible, from our study of protein fusion, to compare the translation efficiency of the *cya* gene with that of the *trpA* gene. Plasmid pDIA 1861 codes for a *cya-lac* protein fusion which can be compared with the *trpA-lac* protein fusion carried by plasmid pDIA 1873 (*trpA-lac* comes from pMC871, Casadaban *et al.*, 1980). The transcription control regions are identical in pDIA 1861 and pDIA 1873 (Figures 4 and 5), but, as shown in Table I, the amount of β-galactosidase synthesized in pDIA 1861 is six times lower than the amount synthesized in pDIA 1873. Since both are hybrid β-galactosidase they probably exhibit similar turnover numbers for the hydrolysis of the substrate (Welply *et al.*, 1980). Accordingly, it seems likely that translation initiation efficiency is lower in *cya* than in *trpA* by a factor of six. This is consistent with our proposal that a non-standard initiator codon is used in *cya*. It is known that UUG can be recognized as an initiation codon in the case of NADH dehydrogenase (Poulis *et al.*, 1981). In the absence of other data concerning UUG as an initiation codon one may wonder whether a coordinate control of translation initition may exist for certain proteins, based on UUG specific recognition. NADH dehydrogenase is a membrane-bound enzyme whereas there is only circumstantial evidence suggesting a similar localization in the case of cyclase (Janaček *et al.*, 1980). An interesting feature of the cyclase protein is that it begins with a long hydrophobic stretch of amino acids preceded by a basic amino acid (positions 718 – 744; note that the dipeptide Asp-Arg should probably be incorporated into the hydrophobic sequence because it can form an internal hydrogen bond, in such a case the stretch would be longer: 708 – 744). Adenylate cyclase is secreted in *B. pertussis* (Greenlee *et al.*, 1982) and *B. anthracis* (Leppla, 1982) and it would be interesting to see whether the 'leader peptide'-like sequence (Hall and Silhavy, 1981) found in the case of the *E. coli* enzyme could not have evolved towards, or from, a secreted form of cyclase.

Finally, using the figures given in Table I, we can compute that as many as 18 000 molecules of bicephalic cyclase-β-galactosidase protein are synthesized when plasmid pDIA 1861 is present. Assuming 50 copies of plasmid per cell, this suggests that as many as 400 molecules of cyclase are synthesized per *E. coli* cell. This is somewhat surprising in view of the low content of cAMP in the cell ($\sim 5\ \mu$M, Joseph *et al.*, 1982) and suggests either that excretion of cAMP must play a major role in the regulation of cAMP-dependent processes, or that cyclase activity is subject to stringent regulation.

Materials and methods

Strains and growth conditions

The *E. coli* strains used throughout this work were isogenic to strain TP 2100 (F⁻ *xyl ilvA argH1 lacΔX74*) (Roy and Danchin, 1982). They derived from strain TP 2111 *(aroB)* constructed as described in Guidi-Rontani *et al.* (1981) from TP 2100 and were TP 2139 *(aroB⁺ crpΔ39)*, TP 2339 *(aroB⁺ crpΔ39 ilv⁺ cyaΔ)*. Strain TP 2006 *(arg⁺ glp* cyaΔ)* was constructed by transducing strain TP 2000 (Roy and Danchin, 1982) with a P1 phage lysate grown on CAA 8306 (Guidi-Rontani *et al.*, 1981), selecting for growth in the absence of arginine and screening for growth on glycerol in the absence of cAMP. CSR603 (F⁻, *thr-1, leuB6, proA2, phr-1, recA, argE3, thi-1, uvrA6, ara-14, lacY1, galK2, xyl-5, mtl-1, rpsL31, tsx33,* λ⁻, *supE44*) was used in the 'maxicells' system. Growth media were either LB-rich medium or synthetic medium M63 supplemented with 0.4% glucose or glycerol, as the carbon source, thiamine (10 μg/ml) and the required amino acids (100 μg/ml) (Miller, 1972).

Enzymatic assays

β-Galactosidase was assayed according to Pardee *et al.* (1959) and 1 unit was defined as the amount of enzyme that converted 1 nmol substrate/min at 28°C. β-Lactamase-specific activity was measured as described by Chesnais *et al.* (1981) and 1 unit of β-lactamase is defined as the amount of enzyme which hydrolyses 1 nmol of ampicillin/min at 28°C. DNA digestion by restriction endonucleases and ligation of DNA fragments were performed according to the instructions of the suppliers. Analysis of restriction fragments was performed by standard gel electrophoresis and ethidium bromide staining (Davis *et al.*, 1980).

Nucleotide sequence determination

Plasmid DNA was obtained as previously described (Roy and Danchin, 1982) and further purified by centrifugation on a sucrose gradient (5–20% w/v). The DNA thus freed of contaminating RNA material was then submitted to hydrolysis by appropriate restriction enzymes and labelled in 5′ with [γ-^{32}P]ATP by T4 polynucleotide kinase using the exchange reaction described by Berkner and Folk (1977). Nucleotide sequences were determined according to the method of Maxam and Gilbert (1977).

Analysis of plasmid-coded translation products

Plasmid-coded proteins were labelled with [^{35}S]methionine (sp. act. ~1300 Ci/mmol) in the 'maxicells' system as described previously (Roy and Danchin, 1982).

Reagents and enzymes

DNA restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were purchased from Bethesda Research Laboratory (Bethesda, MD) or Boehringer (Mannheim, FRG). Agarose type II, ampicillin, kanamycin, *o*-nitrophenyl-β-D-galactopyranoside were from Sigma, [^{35}S]methionine and [γ-^{32}P]ATP from Amersham (Amersham, UK) and the other chemicals from Merck (Darmstadt, FRG).

Acknowledgements

This work benefited from competent advice from P.Stragier, for the sequencing method, and from E.Joseph and A.Ullmann. The nucleotide sequence was determined in the laboratory of J.C.Patte and we are indebted to him for his kind interest. This work was supported by grants from the C.N.R.S. (G.R. no. 18), D.G.R.S.T. (Convention 81.F.1202), I.N.S.E.R.M. (no. 82 1007) and Fondation pour la Recherche Médicale.

References

Bankaitis,U.A. and Bassford,P.J.,Jr. (1982) *J. Bacteriol.*, **151**, 1346-1357.
Berkner,K.L. and Folk,W.R. (1977 *J. Biol. Chem.*, **252**, 3176-3184.
Bostford,J.L. and Drexler,M. (1978) *Mol. Gen. Genet.*, **165**, 47-56.
Casadaban,M.J., Chou,J. and Cohen,S.N. (1980) *J. Bacteriol.*, **143**, 971-980.
Chesnais,J., Richaud,C., Ronceray,J., Cherest,H., Surdin-Kerjan,Y. and Patte,J.C. (1981) *Mol. Gen. Genet.*, **182**, 456-461.
Confer,D.L. and Eaton,J.W. (1982) *Science (Wash.)*, **217**, 948-950.
Davis,D.N., Botstein,D. and Roth,J.R. (1980) in *Advanced Bacterial Genetics*, Cold Spring Harbor Labortory Press, NY.
Ganoza,M.C., Sullivan,P., Cunningham,C., Hader,P., Kofoi,E.C. and Neilson,T. (1982) *J. Biol. Chem.*, **257**, 8228-8232.
Greenlee,D.V., Andreasen,T.J. and Storm,D.R. (1982) *Biochemistry (Wash.)*, **21**, 2759.
Guidi-Rontani,C., Danchin,A. and Ullmann,A. (1981) *J. Bacteriol.*, **148**, 753-761.
Guiso,N. and Blazy,B. (1980) *Biochem. Biophys. Res. Commun.*, **94**, 278-283.
Hall,M.N. and Silhavy,T.J. (1981) *Annu. Rev. Genet.*, **15**, 91-142.
Hewlett,E. and Wolf,J. (1976) *J. Bacteriol.*, **127**, 890-898.
Janaček,J., Nárprstek,J., Dobrová,Z., Jirešova,M. and Spižek,J. (1980) *Folia Microbiol.*, **25**, 361-368.
Joseph,E., Bernsley,C., Guiso,N. and Ullmann,A. (1982) *Mol. Gen. Genet.*, **185**, 262-268.
Leppla,S.H. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 3162-3166.
Majerfeld,I.H., Miller,D., Spitz,E. and Rickenberg,H.C. (1981) *Mol. Gen. Genet.*, **181**, 470-475.
Maxam,A. and Gilbert,W. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 560-564.
Miller,J.H. (1972) in *Experiments in Molecular Genetics*, published by Cold Spring Harbor Laboratory Press, NY.
Musso,R.E., Di Lauro,R., Adhya,S. and de Crombrugghe,B. (1977) *Cell*, **12**, 847-854.
Nomura,M. and Post,L.E. (1979) in Chambliss,G., Craven,G.R., Davies,J., Davis,K., Kahan,L. and Nomura,M. (eds.) *Ribosomes, Structure, Function and Genetics*, University Park Press, Baltimore, MD, pp. 671-691.
Pardee,A.B., Jacob,F. and Monod,J. (1959) *J. Mol. Biol.*, **1**, 165-178.
Postma,P.W., Schuitema,A. and Kwa,C. (1981) *Mol. Gen. Genet.*, **181**, 448-453.
Poulis,M.I., Shaw,D.C., Campbell,H.D. and Young,I.G. (1981) *Biochemistry (Wash.)*, **20**, 4178-4185.
Queen,C. and Rosenberg,M. (1981) *Nucleic Acids Res.*, **9**, 3365-3377.
Rosenberg,M. and Court,D. (1979) *Annu. Rev. Genet.*, **13**, 319-353.
Roy,A. and Danchin,A. (1982) *Mol. Gen. Genet.*, **188**, 465-471.
Roy,A., Danchin,A., Joseph,E. and Ullmann,A. (1983) *J. Mol. Biol.*, in press.
Schmitz,E. and Galas,D.J. (1979) *Nucleic Acids Res.*, **6**, 111-137.
Shine,J. and Dalgarno,L. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 1342-1346.
Stormo,G.D., Schneider,T.D., Gold,L. and Ehrenfeucht,A. (1982) *Nucleic Acids Res.*, **10**, 2997-3011.
Ullmann,A. and Danchin,A. (1983) *Adv. Cyclic Nucleotide Res.*, **15**, in press.
Weber,I.T., Takio,K., Titani,K. and Steitz,T.A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7679-7683.
Welply,J., Fowler,A., Beckwith,J. and Zabin,I. (1980) *J. Bacteriol.*, **142**, 732-734.