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Cloning and promoter analysis of the *Escherichia coli* adenylate cyclase gene

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ABSTRACT

The gene for adenylate cyclase of E. coli has been cloned in the plasmid pBR322. The Cya^- strain transformed with the isolated plasmids produces significant amounts of adenylate cyclase and cAMP. Some of the Cya^+ plasmids were shown to direct the synthesis of a 85,000 dalton polypeptide in a cell-free system. The direction of transcription and the location of the cya promoter including the transcriptional start site were determined by an S1 digestion method. DNA sequence around the promoter region indicates that a putative coding region for adenylate cyclase begins at +233. The 233 bp leader region could encode a potential small polypeptide containing 30 amino acids. Two probable CRP binding sites were found in the leader region, suggesting a negative control at the transcriptional level by CRP-cAMP.

INTRODUCTION

Adenosine 3',5'-cyclic monophosphate (cAMP) is one of the most important regulatory elements in living cells. In bacteria, cAMP controls a variety of biological activities such as synthesis of many enzymes, envelope properties including cell division, and replication of certain plasmids (1-6). It is generally believed that in most of these processes the function of cAMP is mediated by its receptor protein (CRP). The cAMP-CRP complex regulates many E. coli genes positively and/or negatively at the transcriptional level.

The intracellular level of cAMP in E. coli varies depending on carbon sources in culture media (7-9). For example, it has been well known that cells growing on glucose contain particularly low levels of cAMP. However, it is not clear how glucose decreases the cAMP level, or more generally, how the concentration of cAMP in cells is regulated. It is apparent that the regulation

of the level and activity of adenylate cyclase, the enzyme responsible for the synthesis of cAMP, plays an important role in the control of cAMP concentration. Although some physiological studies related to the regulation of adenylate cyclase synthesis and its activity have been reported (10-13), little is known about the molecular mechanism of the regulation of cya gene coding for adenylate cyclase. To study directly the regulation of the adenylate cyclase gene, we have cloned the E. coli cya gene into plasmid pBR322, established the restriction map of the cya region, and analyzed the gene products in a cell-free system. We have also identified the promoter of the cya gene, and determined the nucleotide sequence around the regulatory region as well as the start site of the transcription. The analysis of the DNA sequence of the promoter-regulatory region suggests that the cya gene is negatively regulated by cAMP-CRP.

MATERIALS AND METHODS

Bacterial Strains

The following E. coli strains were used in this study: pp6 (cya^+) and pp48 (cya^-) from I. Pastan, SA 1039 (cya^-) from S. Adhya, and SG 20062 (cya^+) from S. Gottesman.

Enzymes and Chemicals

Sources of enzymes were as follows: restriction endonucleases, Bethesda Research Laboratories, New England BioLabs, and Takara Shuzo Co., Ltd.; calf intestine alkaline phosphatase, Boehringer Mannheim; T4 DNA ligase and T4 polynucleotide kinase, Takara Shuzo Co., Ltd.; nuclease S1, Bethesda Research Laboratories. All enzymes were used under the conditions specified by the suppliers. [γ-^{32}P]ATP (>5,000 Ci/mmol) and [^{14}C]leucine (>50 mCi/mmol) were purchased from New England Nuclear.

Isolation of DNA

Plasmid DNA was purified according to the procedure of Birnboim and Doly (14). DNA fragments were resolved in 5 to 8% acrylamide gels and extracted by the method of Maxam and Gilbert (15).

Construction of Recombinant Plasmids

E. coli gene libraries were constructed as described in

a previous paper (16). Briefly, chromosome DNA of E. coli SG 20062 was digested with restriction endonuclease Hind III or Pst I and the resulting fragments were ligated to the respective unique site on the plasmid pBR322. E. coli pp48, an adenylate cyclase deficient mutant, was transformed with the ligation mixture. The transformants were screened on a lactose MacConkey plate containing ampicillin (25 μg/ml) or tetracycline (10 μg/ml).

Synthesis of Plasmid Encoded Proteins

The proteins encoded by the plasmids were synthesized in vitro and analyzed by the procedure of Fukuda et al. (17).

Assay for Adenylate Cyclase and cAMP

Adenylate cyclase was assayed in vivo by the method of Botsford and Drexler (12). The cAMP level was determined by the radioimmunoassay procedure using the kit from Yamasa Shoyu Co., JAPAN.

Digestion with S1 Nuclease

Cellular RNA was extracted from early-logarithmic-phase cells of E. coli strain pp6 grown in L-broth medium by the SDS-hot phenol method (18). A DNA fragment ^{32}P-labeled at its 5' end was hybridized to cellular RNA, treated with S1 nuclease and analyzed according to the procedure described previously (18,19).

DNA Sequencing

DNA sequence was determined by the method of Maxam and Gilbert (15).

RESULTS

Cloning of the cya Gene

An adenylate cyclase-negative strain pp48 cannot metabolize lactose and forms a white colony on the lactose MacConkey plate. However, pp48 transformed with a hybrid plasmid carrying the cya gene should be Lac$^+$ to form a red colony on the indicator plate. On the basis of this strategy we have obtained two classes of recombinant plasmids which confer Lac$^+$ phenotype on pp48; one, designated as pCA1, is from the library of Hind III fragments and the other, designated as pCA2, is from the Pst I library. Plasmid pCA1 carries a 5.3 kb insert DNA at the Hind III

Table I Adenylate cyclase and cAMP levels in various E. coli strains

Strain	Relevant Genotype	cAMP[1]	Adenylate Cyclase[2]
pp6	*cya*$^+$	230.2	1.36
pp48	*cya*$^-$	6.5	0.00
pp48/pBR322	*cya*$^-$/*amp*$^+$	5.6	0.01
pp48/pCA1	*cya*$^-$/*amp*$^+$*cya*$^+$	158.2	0.96
pp48/pCA2	*cya*$^-$/*amp*$^+$*cya*$^+$	111.1	[3]

[1] Cells were grown in M9 medium containing 0.4% D-galactose and 0.1% casamino acids. 1 ml samples were withdrawn from the growing cultures at $A_{550} \doteqdot 0.5$, added to 0.1 ml of 11 M HCl, and boiled for 10 min. The cAMP in the samples was determined by the radioimmunoassay procedure. The level of cAMP is shown as pmol cAMP/ml/A_{550}.
[2] Cells were grown in 20 ml L-broth to $A_{550} \doteqdot 0.5$, harvested by centrifugation, and washed 3 times with 20 ml M9 medium containing 0.1% casamino acids and 100 µg/ml chloramphenicol. Cells were resuspended in the same buffer and shaked for 20 min at 37°C. 1 ml samples were taken at time 0, 10, 20 min. The cAMP level was determined as described above. Adenylate cyclase activity is given by the rate of cAMP synthesis (pmol cAMP/min/ml/A_{550}).
[3] Not determined.

site of pBR322, whereas pCA2 contains a 6.0 kb fragment at the Pst I site. To test further the ability of pCA1 and pCA2 to complement the Cya$^-$ phenotype, these plasmids were introduced in a different Cya$^-$ strain SA 1039. As expected, both pCA1 and pCA2 conferred a Lac$^+$ phenotype on SA 1039. Thus, based on the genetic complementation we concluded that the *cya* gene has been cloned in pBR322.

Adenylate Cyclase Activity and cAMP Level

To obtain further evidence that the *cya* gene had been cloned, we have determined adenylate cyclase activity of cells in the absence and the presence of plasmids by the aeration assay (12). The results are given in Table 1. The adenylate cyclase-negative strain pp48 shows essentially no detectable enzyme activity, whereas the wild type pp6 has a significant adenylate cyclase activity. When the pp48 is transformed by pCA1, the activity of adenylate cyclase is now comparable with that of the wild type strain. On the other hand, a control plasmid pBR322 does not affect the enzyme activity in pp48. We also determined the

level of cAMP in cells with or without plasmids. As expected, the cAMP concentration in the mutant pp48 is only 1/40 of that in the wild type strain. When the pp48 is transformed either by pCA1 or pCA2, the concentration of cAMP increases to the level of the pp6. Taken together these results show that pCA1 and pCA2 indeed contain the cya gene and the gene is expressed in host cells.

However, it is of interest to note that the levels of adenylate cyclase and cAMP in pp48 carrying the cya plasmid are almost the same as those of the wild type strain pp6 which does not contain the plasmid. The absence of gene dosage effect of the cya gene suggests that there exists a regulatory mechanism whereby the expression of cya gene is maintained at a constant level.

Mapping the cya Gene on the Cloned DNAs

As mentioned above, 5.3 kb and 6 kb DNA segments containing the cya gene have been isolated in pCA1 and pCA2, respectively. We could expect that the cya gene may be composed of about 3,000 bp, since the molecular weight of E. coli adenylate cyclase has been estimated to be about 95,000 daltons (20). To localize the cya gene on the cloned DNA, the restriction cleavege map of the insert DNAs was established (Figure 1), which indicates that the region from the Pst I site 1 to Hind III site 2 is included in both of the two cloned DNAs. Various DNA subfragments were then recloned into pBR322 and the resulting plasmids were tested for their ability to complement the Cya^- strain. So far we obtained four different subclones which still confer the Cya^+ phenotype; these are designated as pCA3, pCA4, pCA5 and pCA6 (see Figure 1). Among these pCA6 is the smallest, indicating that the 2.8 kb DNA region from the Bam HI site 1 to the Hind III site 2 contains the functional cya gene. When the 2.2 kb Bam HI-Hind III fragment was recloned into pBR322, the resulting plasmid pCA7 no longer conferred the Cya^+ phenotype. This implies that the region around the Bam HI site 2 essential for the cya gene.

Protein Encoded by the cya Gene

To identify the protein product of the cya gene, we have analyzed the proteins encoded by the cya plasmids in a cell-free

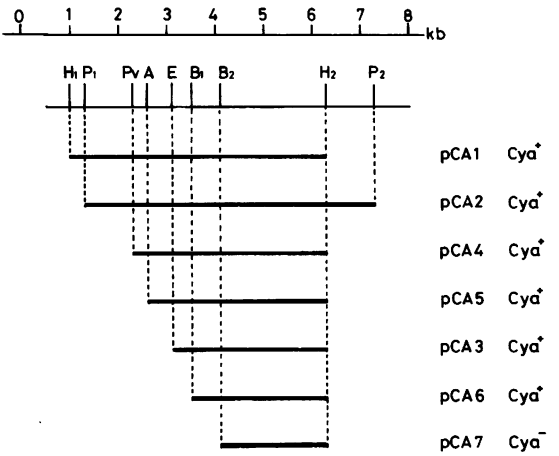

Figure 1. Restriction map of the cloned DNAs carrying the cya gene. The cloned DNA segments are indicated by solid bars. The restriction sites are designated as follows: Ava I, A; Bam HI, B; Eco RI, E; Hind III, H; Pst I, P; Pvu II , Pv. pCA1 and pCA2 are primary clones. pCA3 to pCA7 were constructed by deleting various fragments from pCA1.

S-30 system, followed by SDS-PAGE and autoradiography. As shown in Figure 2, the Cya^+ plasmid, pCA1 and pCA3, produced a protein of about 85,000 daltons (lanes 2-5) that was not obtained in the control plasmid pBR322. In a separate experiment, pCA6 also directed the synthesis of the 85,000 dalton protein while the Cya^- plasmid, pCA7, did not (data not shown). In addition, it has been shown by the analysis of the products of the cloned cya gene that the molecular weight of adenylate cyclase from Salmonella typhimurium is in the 80,000-100,000 range (23). Taken together, we concluded that the 85,000 dalton polypeptide represents adenylate cyclase of E. coli.

Direction of the cya Transcription

As an attempt to analyze the cya gene further, we determined the direction of the cya gene transcription. For this, the fine restriction cleavage map of the 2.8 kb DNA region was established with Dde I, Hinf I and Hpa II (Figure 3). Then, we have conducted an S1 nuclease protection experiment with the restriction fragments derived from the cya gene by cya mRNA. We prepared fragment 1 ^{32}P-labeled at its Hpa II 5' end (upper strand on Figure 3) and

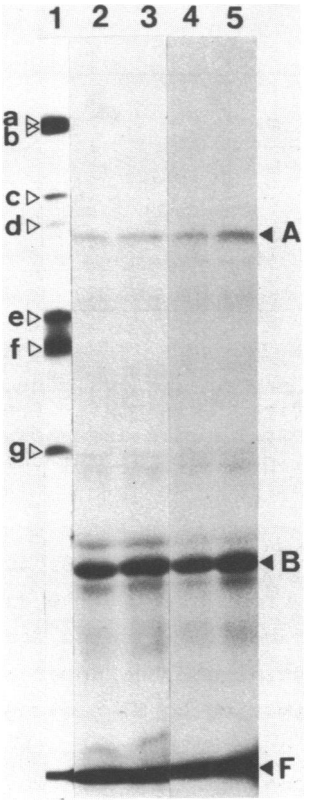

Figure 2. Proteins encoded by the cya plasmids. Protein synthesis in vitro directed by either pCA1 (2.1 µg for lane 2 and 4.2 µg for lane 3) or pCA3 (1.9 µg for lane 4 and 3.8 µg for lane 5) was performed essentially according to Fukuda et al. (17). Protein products labeled with [^{14}C]leucine were analyzed by electrophoresis on 9% polyacrylamide gel. The gel was treated for fluorography as described by Laskey and Mills (21). Bands A and B represent adenylate cyclase and β-lactamase, respectively, while band F shows the gel front. On the same gel, ^{3}H-labeled molecular weight marker proteins from E. coli (22) were run in parallel: a, RNA polymerase β' subunit (165K); b, RNA polymerase β subunit (155K); c, ^{T}m protein (100K); d, RNA polymerase σ subunit (90K); e, groE ATPase (64K); f, Protein A-binding protein (60K); g, RNA polymerase α subunit (38K).

fragment 2 ^{32}P-labeled at its Hpa II 5' end (lower strand on Figure 3). It is apparent from the location of these fragments that fragments 1 and 2 are parts of the cya structural gene (Figure 3). Each fragment was hybridized to cellular RNAs prepared

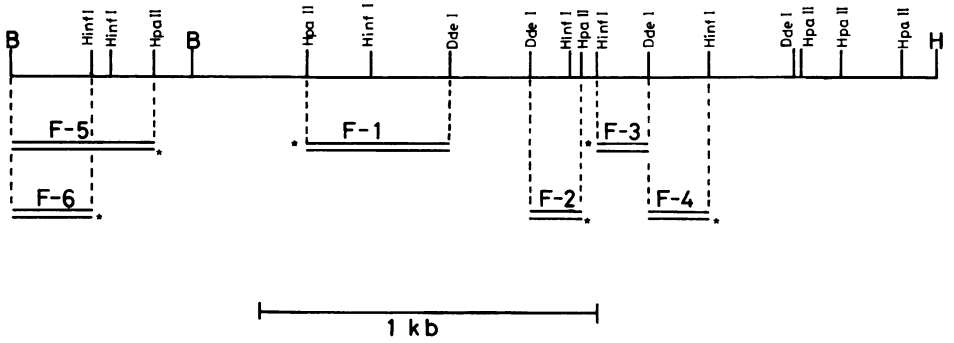

Figure 3. Restriction map of the cya gene. The 2.8 kb Bam HI-Hind III fragment is derived from pCA6. The restriction fragments used for S1 digestion experiments are shown under the map. Each fragment was labeled selectively at its unique 5' end with ^{32}P (shown by *).

from E. coli strain pp6, treated with S1 nuclease, and analyzed on a 7% acrylamide/8 M urea gel. If the direction is from left to right, the lower strand, ^{32}P-labeled fragment 2, should be protected from S1 digestion and the upper strand (^{32}P-labeled fragment 1) may be sensitive to S1 nuclease. If the direction is from right to left, one could expect the opposite situation (resistant fragment 1 and sensitive fragment 2). The results clearly show that fragment 2 is protected from S1 digestion, wheares fragment 1 is degraded by S1 nuclease (Figure 4). We carried out the same protection experiment using fragment 3 labeled at its Hinf I 5' end (upper strand) and fragment 4 labeled at its Hinf I 5' end (lower strand). The results again indicated that the lower strand was protected by the mRNA against S1 digestion (data not shown). The data strongly suggest that the cya gene is transcribed from left to right in the map given by Figure 3.

Identification of the cya Promoter

The above experiments together imply that the 540 bp segment between two Bam HI sites should contain at least part of the promoter of the cya gene. To test this possibility and, if so, to determine the precise location of the cya promoter, we performed an S1 nuclease mapping experiment using the 420 bp fragment 5 labeled at its Hpa II 5' end. The DNA probe hybridized to

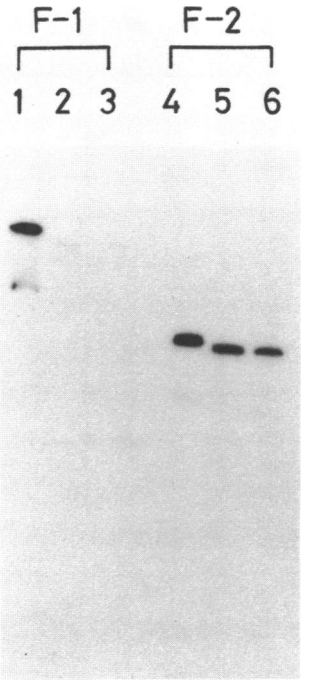

Figure 4. Determination of the direction of cya transcription. Fragments 1 (lanes 1-3) or 2(lanes 4-6) ^{32}P-labeled at their 5' Hpa II ends were mixed with 100 µg cellular RNAs from E. coli strain pp6 in 30 µl hybridization buffer (80% formamide, 20 mM Hepes [pH 6.5], 0.4 M NaCl). The mixture was incubated at 75°C for 10 min and cooled down to 37°C and the incubation was continued for 2 hr at 37°C. After adding 240 µl H_2O and 30 µl 10xS1 buffer (0.3 M sodium acetate [pH 4.6]. 0.5 M NaCl, 10 mM $ZnSO_4$, 50% glycerol), the mixture was treated with 100 U S1 nuclease (Bethesda Research Laboratories) for 2 min (lanes 2 and 5), and 5 min (lanes 3 and 6). The products were precipitated with ethanol and electrophoresed on a 7% polyacrylamide/8 M urea gel in TBE buffer. Lanes 1 and 4 represent the experiment without S1 treatment.

cellular RNAs was treated with S1 nuclease and the products were analyzed by electrophoresis on an acrylamide gel. As shown in Figure 5, a prominent protected DNA of about 300 bases long was obseved (lane 2). When we used 240 bp fragment 6 labeled at its Hinf I 5' end as a DNA probe, the size of the protected DNA was about 120 bases (lane 4). These results indicate that a promoter surely exists in the region 120 bp upstream from the

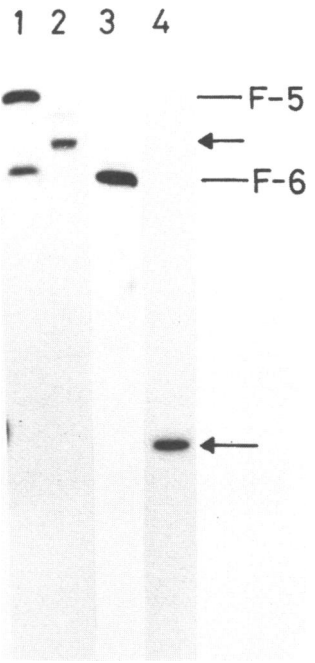

Figure 5. S1 mapping of cya RNA. Fragment 5 ^{32}P-labeled at its 5' Hpa II end or fragment 6 ^{32}P-labeled at its 5' Hinf I end was hybridized to 100 µg cellular RNAs from pp6, treated with S1 nuclease, and analyzed on a 7% polyacrylamide/8 M urea gel. The detail of the procudure is described in the legend of Figure 3. Lane 1, fragment 5 without S1 treatment; lane 2 fragment 5, treated with S1 for 5 min; lane 3, fragment 6 without S1 treatment; lane 4, fragment 6 treated with S1 for 5 min. Arrows indicate the protected DNA bands.

left most Hinf I site or 300 bp upstream from the Hpa II site to initiate the transcription from left to right. We believe that this is the promoter of the cya gene.

Nucleotide Sequence around the Promoter Region

The DNA sequence of the 540 bp Bam HI fragment was determined to verify the conclusion reached above and to uncover the features of the cya promoter region. The results are shown in Figure 6. We inspected the sequence and found a Pribnow box (24,25), -TAAATTG-, in the region about 130 bp upstream of the Hinf I site. The Pribnow sequence is preceded by a possible -35 region (25), -CTTTACG-. Since we have already detected an mRNA starting around

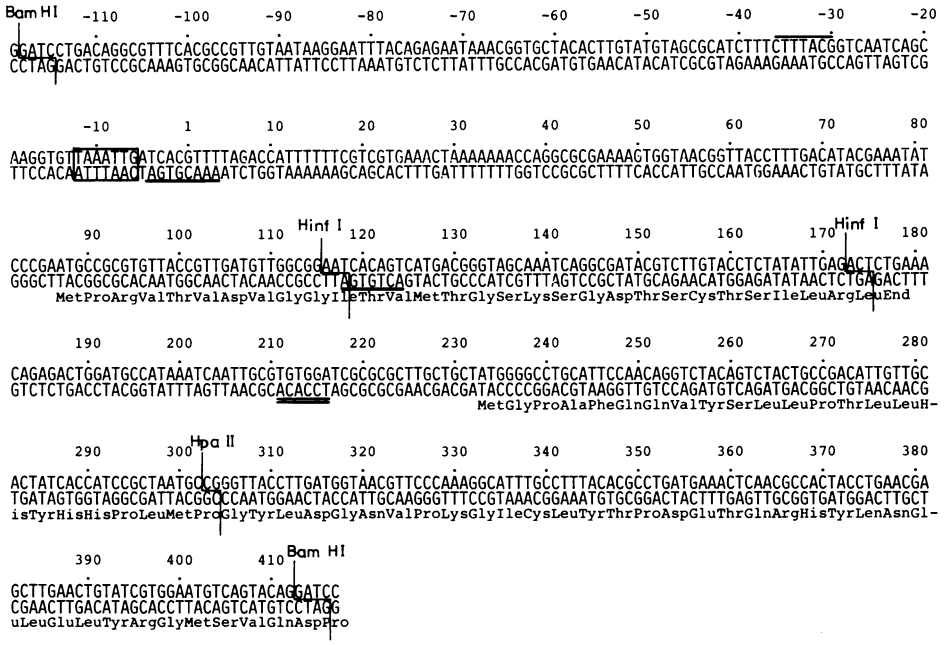

Figure 6. DNA sequence of the cya promoter and regulatory regions. The nucleotides are numbered from the site corresponding to the 5' end of cya mRNA. Pribnow sequence is boxed and -35 region is shown by a line over base pairs. The Shine-Dalgarno sequence for the proposed cya structural gene bebinning at position +233 is double-underlined. The nucleotides homologus to the consensus sequence for the CRP binding sites (31) are underlined. Relevant restriction sites are shown by broken lines.

this region by the S1 mapping experiment, it is reasonble to conclude that the Pribnow and -35 sequences presented here are elements of the promoter of the cya gene. Having known the DNA sequnece, it is now possible to determine precisely the start site of the transcription. We analyzed the products of S1 digestion on a sequencing gel (Figure 7). A predominantly protected DNA (shown by an arrow) can be observed at the position between thymine and guanine in the sequencing lanes. Because the sequencing reaction products are 1-1.5 nucleotides shorter than the products of S1 digestion (26), it is concluded that the protected band corresponds to cytocine and the transcription of the cya gene starts at the complementary guanine residue numbered as +1 in Figure 6. The analysis of the 5' end of the cya mRNA synthesized in vitro

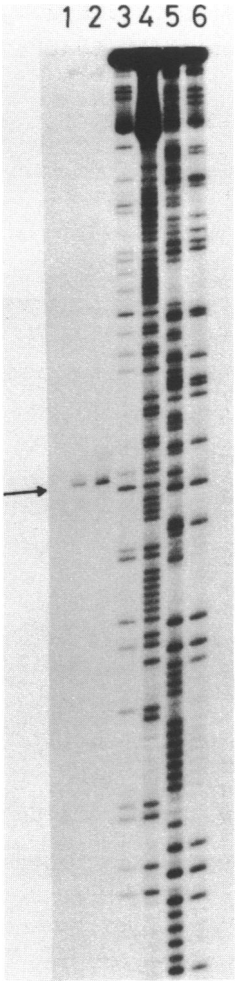

Figure 7. Mapping of the 5' end of cya RNA. Fragment 6 ^{32}P-labeled at its 5' Hinf I end was hybridized to cellular RNA, treated with S1 nuclease for 2 min (lane 2) or 5 min (lane 1) and analyzed on a 7% polyacrylamide/ 8 M urea gel. Arrow represents the protected DNA band. Lanes 3-6 indicate the products of the sequencing reactions of fragment 6: lane 3, G; lane 4, A+G; lane 5, C+T; lane 6, T.

showed that the start site of the in vitro transcription is the same as that of in vivo transcription (unpublished result).

DISCUSSION

From the E. coli DNA libraries we have isolated two classes of recombinant plasmids, pCA1 and pCA2, that are able to complement Cya$^-$ strains. The definite evidence that the cya gene has been cloned is that the Cya$^-$ strain harboring these plasmids produce significant amounts of adenylate cyclase and cAMP. By constructing various subclones the functional cya gene was localized in the

2.8 kb region between the Bam HI site 1 and the Hind III site 2. The 2.8 kb segment could code for a 85,000 dalton protein that may possess adenylate cyclase activity.

During this work, we learned that detaild analysis of the E. coli cya locus and its gene products had been reported by Roy and Danchin (27). The restriction map and the location of the cya gene presented here are consistent with those (27,28) of Roy and Danchin who isolated the cya gene from the E. coli DNA library of Clarke and Carbon (29). Using maxicells technique, they identified protein products encoded by the cloned cya gene and found that Cya^+ plasmids containing the region beyond the Hind III site 2 directed synthesis of a 95,000 dalton protein, while another Cya^+ plasmid missing the region to the right of the Hind III site 2 produced a 88,000 dalton protein that may be a truncated adenylate cyclase at its carboxy terminal end (27). It is apparent that the 88,000 dalton protein is identical with the 85,000 dalton protein obtained in our cell-free system.

The S1 mapping experiments allowed us to map the cya promoter between the two Bam HI sites. The existence of another promoter toward the cya structural gene is reported by Roy and Danchin (27). Although we also identified the second promoter between the Eco RI site and Bam HI site 1 by an S1 mapping experiment, the transcription from this promoter has been shown to be very weak (unpublished result).

The DNA sequence shown in Figure 6 reveals noteworthing features of the cya promoter-regulatory region. First, an open reading frame, which may code for the amino terminal region of adenylate cyclase, is found beginning at position +233. Preceding this initiation codon, there exists a putative Shine-Dalgarno sequence (30), -TGTGGA-, from +211 to +216. Second, in the 233 bp leader sequence, we found another reading frame starting at position +87 and ending at position +179. This region could code for a peptide composed of 30 amino acids. Third, there are two probable CRP binding sequences (31), -ACTGTGA-, from +124 to +118 and,-AAACGTGA-, from +4 to -4 on the lower strand.

Recently we have discovered that the cAMP-CRP complex acts as a repressor for the crp transcription, by binding to the unique site located in the crp leader region (19). Interestingly,

the location and the orientation of the putative CRP binding sequences in the cya gene are in good agreement with those of the crp gene. These structural features of the cya regulatory region suggest that there may exist some regulatory mechanism, including a negative control by cAMP-CRP complex, with the cya gene. The finding that the activity of adenylate cyclase in cells harboring the multicopy cya plasmids is almost the same as that of the wild type strain is well explained by assuming a negative control of the cya gene by cAMP-CRP.

It is known that mutants of E. coli lacking a functional CRP overproduce cAMP (8,12,32). Based on these observations, Botsford and Drexler proposed that the cya gene might be controled negatively by the cAMP-CRP complex at the transcriptional level (12). On the other hand, recent genetic and physiological studies suggest that the cAMP-CRP may not play a significant role in the regulation of the cya transcription or translation (33,34). Although our data support the model of Botsford and Drexler, the role of cAMP-CRP in the cya regulation still remains controversial. In vitro and in vivo studies with the cloned cya gene could give a clear answer to this important question and may unravel other aspects concerning the regulation of the cya expression.

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