Identification of the cpdA Gene Encoding Cyclic 3',5'-Adenosine Monophosphate Phosphodiesterase in Escherichia coli*

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We have identified a gene, cpdA, located at 66.2 min of the chromosome of Escherichia coli that encodes cyclic 3',5'-adenosine monophosphate phosphodiesterase (cAMP phosphodiesterase, EC 3.1.4.17). The expression of β-galactosidase, which is a product of the lacZ gene, was repressed in cells that harbored multiple copies of the plasmid carrying the cpdA gene. Northern blotting showed that the transcription of the lacZ gene was inhibited in these cells. Multiple copies of the cpdA gene decreased the intracellular concentration of cAMP, which is a positive regulator for transcription of the lacZ gene. We found that the purified CpdA protein repressed in vitro transcription from the lacP1 promoter by decreasing CAMP. In addition, we showed that the CpdA protein hydrolyzed cAMP to 5'-adenosine monophosphate and that its activity was activated by iron. Our results suggested that regulation of intracellular concentration of CAMP is dependent not only on synthesis of CAMP but also on hydrolysis of CAMP by CAMP phosphodiesterase.

CAMP is an important cellular mediator in Escherichia coli. The role of CAMP in mediating glucose effects has been well investigated (1). The CAMP receptor protein (CRP) is a regulatory protein, which binds CAMP and mediates transcriptional regulation at several promoters (2). The CRP-cAMP complex is a positive transcriptional regulator of a number of catabolic operons, including the lac operon in E. coli, and as such, plays a role in catabolite repression, where secondary carbon sources are not catabolized in the presence of glucose (3, 4). This complex is involved not only in positive regulation of several catabolic functions but also in regulation of flagellum synthesis (5), toxin production (6), minicell production (7), coupling of DNA replication and cell division (8), and many other functions that are not directly related to catabolism. CRP forms an active conformation only when it binds CAMP. Therefore, the concentration of active CRP-CAMP complex and the biological responses mediated by active CRP-CAMP are influenced by the intracellular concentration of CAMP.

Another role of CAMP, which is independent of CRP transcription, has been reported. CAMP interacts directly with the DnaA protein and plays a role in the re-activation of DnaA, which is an essential element for initiation of DNA replication from the chromosome origin, oriC (9). Thus the cellular level of CAMP has some effects on controlling the initiation of DNA replication. Moreover, CAMP has a role in regulation of cell division in E. coli. Filamentation of growing cells is induced by elevated levels of CAMP (10). CAMP may affect cell division only indirectly through unidentified CAMP-dependent functions that are not obligatory (11).

In E. coli, the intracellular concentration of CAMP has been thought to be mainly controlled by its own synthesis. Synthesis of CAMP is catalyzed by adenylate cyclase, encoded by the cya gene, and its activity is regulated transcriptionally (12, 13) and post-translationally (14, 15). However, the existence of phosphodiesterase (EC 3.1.4.17), which hydrolyzes CAMP, had been reported in E. coli (16). Nielsen et al. (17) partially purified the enzyme. The gene encoding CAMP phosphodiesterase remained unidentified.

In the present study we have discovered the E. coli gene encoding CAMP phosphodiesterase, and we have purified this enzyme. We present here the nucleotide sequence of the gene (named cpdA) and properties of the enzyme.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media—We used derivatives of E. coli K-12, the prototroph strain W3110 (18), the Δlac pro rpsL thi ara strain CSH50 (19), the cpdA::kan strain SH8150 (this study), the uvrA recA strain CSR603 (20), the recD strain FS1576 (21), and the hsdS gal strain BL21(DE3), which is a lysogen of DE3, a lambda phage derivative that carries the gene for T7 RNA polymerase (22). A 2.8-kb BamHI-PstI chromosome segment located in the physical map 3229.50–3232.50-kb coordinate of the wild-type W3110 strain was cloned into pACYC184 vector plasmid (23), resulting in pAX903. This segment was treated with mung bean nuclease using a deletion kit for Kilo-Sequence (Takara Shuzo Co., Kyoto, Japan), and deleted segments were recloned into pACYC184. Resulting plasmids were named pAX904, pAX906, and pAX908. In addition, we constructed plasmid pAX923 carrying only the cpdA gene by digestion with VspI. The plasmid pX7477 carrying the lacZ gene was described previously (24). L medium (1% Bacto Tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.4) and peptone-agar (1% peptone, 0.5% NaCl, 1.4% agar, pH 7.4) were used. To test expression of β-galactosidase, peptone-agar plates containing 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were used. Isopropyl-β-D-thio-galactopyranoside (IPTG), 1 mM, was added to the medium for induction of β-galactosidase.

Construction of the cpdA-disrupted Mutant—The 2.8-kb BamHI-PstI chromosome segment of pAX903 was recloned into pUC119, resulting in plasmid pAX901. A HaeII-HaeII DNA fragment carrying a kanamycin-
resistant gene (kan), which derived from pACYC177 (23), was inserted into the BamHI site located downstream of the lacI gene, and the recombinant plasmid pAX910 was introduced into the chromosome of a recA mutant (FS1576). Kanamycin-resistant transformants were analyzed by Southern hybridization to confirm the disruption of the cpdA gene. The DNA fragment containing the disrupted cpdA gene was then sequenced, with the 205-base pair (bp) DNA fragment containing the disrupted cpdA gene being isolated from pAX910 by digestion with BamHI and PstI.

DNA Sequencing—We determined nucleotide sequence of the BamHI-PstI chromosome segment between the tolC gene and the parE gene, using the dideoxy method of Sanger et al. (25). The nucleotide sequences of the BamHI-PvuI and BglII-PstI chromosome segments already had been published (26, 27) and deposited in the EMBL/GenBank/DDBJ nucleotide data libraries under the accession numbers M37383 and X54409, respectively.

Assay for the β-Galactosidase Activity—Bacterial strains were grown in L medium with or without 1 mM IPTG. After the cell density reached 30 Klett units, IPTG was added at a final concentration of 35% (w/v) and stirred at 0°C for 30 min. After centrifugation of darkblue colonies (Fig. 1). We then assayed the activity of β-galactosidase in liquid culture of the wild-type strain E. coli (W3110) harboring plasmid pAX903, which has the BamHI-PstI segment (3229.50–3232.50 kb coordinate in the physical map), formed pale blue colonies on peptone-agar plates containing X-gal and the inducer IPTG of the lac operon. In contrast, the wild-type cells harboring the vector plasmid pACYC184 formed dark blue colonies. This suggested that when present at high doses, one product or a combination of products from three open reading frames in this segment inhibited the synthesis of β-galactosidase. We subcloned the segment and found that plasmids pAX908 and pAX923, both carrying ORF3, led to the formation of pale blue colonies, but pAX904, pAX906, and the vector pACYC184, none of which carry ORF3, led to the formation of dark blue colonies (Fig. 1). We then assayed the activity of β-galactosidase in liquid culture of the wild-type strain transformed with the pAX923 plasmid and the pACYC184 vector plasmid. Cells harboring pAX923 showed only about 20% of the activity of cells harboring pACYC184 (Fig. 2A, a). The results suggest that the ORF3 protein is an inhibitor of β-galactosidase synthesis.

To analyze the inhibitory mechanism of β-galactosidase synthesis, we measured the amount of lacZ mRNA in the presence and the absence of plasmid pAX923. The induced level of lacZ mRNA in the pAX923-harboring strain was 26% of that in the control strain (Fig. 2B). Thus we concluded that high levels of the ORF3 protein inhibited transcription of the lac operon. The inhibitory effect of multiple copies of the ORF3 protein on expression of β-galactosidase was also observed in a ΔlaciE (4044) deletion strain harboring the lacZ+ plasmid pX774 (Fig. 2A, b). This indicated that the inhibitory effect was independent of the LacI repressor.

Purification of the Inhibitor Protein—To identify the ORF3 protein responsible for the inhibitory action of β-galactosidase...

1. **Results**

Identification of the Novel Inhibitor of Expression of β-Galactosidase Independently of LacI Repressor—We sequenced the 66.2 min region of the E. coli chromosome located between the parE and the tolC (mukA) genes and found that there were four open reading frames (Fig. 1). The wild-type E. coli cells (W3110) harboring plasmid pAX903, which has the BamHI-PstI segment (3229.50–3232.50 kb coordinate in the physical map), formed pale blue colonies on peptone-agar plates containing X-gal and the inducer IPTG of the lac operon. In contrast, the wild-type cells harboring the vector plasmid pACYC184 formed dark blue colonies.

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CRP before the addition of template DNA and RNA polymerase, transcription from the \( \text{P2} \) promoter used as an internal control (compare Fig. 5, lane 3 and lane 4). When 100 pmol of the CpdA protein was preincubated with cAMP and CRP, transcription from the \( \text{lacP1} \) promoter was observed (Fig. 5, lane 5), and the level of the \( \text{lacP2} \) transcript was as high as that in the wild-type (Fig. 5, lane 6). These results suggest that the CpdA protein decreases the amount of CAMP in this assay solution. In fact, this inhibitory effect was not found when a 10-fold excess of CAMP was added (Fig. 5, lane 7).

We also examined the effect of the CpdA protein on transcription from the \( \text{gal} \) promoter, which consists of CRP-depend-
ent galP1 and CRP-independent galP2 promoter. Preincubation of the CpdA protein with cAMP and CRP caused repression of transcription from the galP1 promoter and produced instead a transcript from the galP2 promoter (data not shown).

Identification of the Inhibitor as cAMP Phosphodiesterase—We measured the concentration of cAMP after the incubation with the CpdA protein using the cAMP enzyme immunonoassay. This assay is based on competition between free cAMP and cAMP tracer linked to acetylcholinesterase molecules for a limited number of cAMP-specific rabbit antiserum binding sites. The amount of cAMP tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of free cAMP in the sample and will be measured by the activity of acetylcholinesterase.

The cAMP concentration was found to decrease markedly. As shown in Fig. 6A, the purified CpdA protein decreased cAMP concentration in a time-dependent manner. We found that FeCl₂ stimulates the enzymatic activity of CpdA protein. The addition of 100-fold cGMP did not inhibit the reaction of the CpdA with cAMP (Fig. 6A). Calculation of the data presented in Fig. 6B shows that the CpdA protein of E. coli has a $K_m$ of approximately 0.5 mM cAMP and a $V_{max}$ of 2.0 μmol/min/mg.

To identify the cAMP reaction products, cAMP (20 mM) was incubated with the purified CpdA protein, and the reaction product was separated by ascending chromatography on polyethyleneimine-cellulose (Fig. 7, lanes 1–6) or Whatmann 3MM paper (Fig. 7, lanes 7–9) with solvent (0.1 M LiCl/0.4 M formic acid). We observed the substrate, cAMP (Fig. 7, lane 1) and the product derived from the reaction of cAMP with the CpdA protein (Fig. 7, lane 2). cAMP was converted completely when CpdA was incubated with 1.5 μM of the CpdA protein (Fig. 7, lanes 3 and 8). On polyethyleneimine-cellulose, reaction products from cAMP (Fig. 7, lanes 2 and 3) had the same mobility as 5′-AMP (Fig. 7, lane 4) and adenosine (Fig. 7, lane 6) but not as 3′-AMP (Fig. 7, lane 5). On Whatmann 3MM paper, reaction products from cAMP (Fig. 7, lane 8) had the same mobility as 5′-AMP (Fig. 7, lane 7) but not as adenosine (Fig. 7, lane 10).

To confirm that the reaction product is 5′-AMP, 5′-nucleotidase was added to the reaction mixture after incubation with cAMP and the CpdA. 5′-Nucleotidase hydrolyzes 5′-AMP to adenosine. The mixture was further incubated for 2 h and was separated (Fig. 7, lane 9). The mobility of the product was not the same as that of 5′-AMP (Fig. 7, lane 7). It showed the same mobility as adenosine (Fig. 7, lane 10). Thus, 5′-nucleotidase hydrolyzed the reaction products to adenosine. We therefore concluded that the CpdA protein is a CAMP phosphodiesterase (EC 3.1.4.17) of E. coli that hydrolyzes CAMP to 5′-AMP, and we propose to designate this gene as cpdA (cyclic AMP phosphodiesterase).

Effect of cpdA Gene Dosage on the Intracellular Concentration of cAMP—Based on the results of in vitro analysis, we believed that the inhibitory effect by multiple copies of the cpdA gene on expression of β-galactosidase was caused by decreasing the intracellular concentration of cAMP. To test this possibility, we measured the intracellular concentration of cAMP in the wild-type strain harboring the pAX923 plasmid or the pACYC184 vector plasmid. The intracellular concentration of cAMP in the pAX923-harboring strain was only 11% of the level of that in the pACYC184-harboring control strain (Fig. 8). In addition, the concentration of cAMP in the cpdA-disrupted strain (SH8150), was 2-fold higher than the parental cpdA⁻ strain (W3110). These results suggest that the cpdA gene product also participates in decreasing the intracellular concentration of cAMP in vivo.

**DISCUSSION**

The results described in this paper from in vitro and in vivo experiments showed that the gene product that inhibited the expression of β-galactosidase was cyclic 3′,5′-adenosine monophosphate phosphodiesterase (EC 3.1.4.17), which hydrolyzes cAMP to 5′-AMP. We refer this gene as cpdA, which is located at 66.2 min of the E. coli chromosome. There are different types of cyclic 3′,5′-nucleotide phosphodiesterases; some enzymes hydrolyze cGMP as well as cAMP, whereas other enzymes prefer cGMP to cAMP as a substrate. This phosphodiesterase that we purified, CpdA, is specific for decomposition of cAMP, and its activity was not affected by the addition of cGMP. Its enzymatic parameters are as follows: $V_{max}$ is 2.0 μmol/min/mg, and $K_m$ is 0.5 mM.
The occurrence of cAMP phosphodiesterase in *E. coli* was first described by Brana and Chytil (16). Partial purification of cAMP phosphodiesterase was performed by Nielsen et al. (17), and they purified a cAMP phosphodiesterase from a crude extract of *E. coli* by about 100-fold. This enzyme has a molecular weight of about 30,000 and a Michaelis constant of 0.5 mM cAMP and is activated by iron. These properties of the cAMP phosphodiesterase, as purified by Nielsen et al. (17), are consistent with our present results with the purified CpdA protein. The cAMP phosphodiesterase activity detected by Nielsen et al. (17) is most likely the activity of the CpdA protein.

Current information relating to the phosphodiesterases in prokaryotic cells is not sufficient for domain analysis. However phosphodiesterases in eukaryotic cells are well characterized and have been divided into two classes, I and II (38). The amino acid sequences of representatives of the same class are homologous to one another. Phosphodiesterases in class I share a conserved domain of about 250–3270 residues. In this domain, there is a signature pattern of 12 residues that contains two conserved histidines: HD(L/I/V/M/F/Y)XH(A/G)XXN(L/I/V/M/F/Y) (39). The X is used for a position at which any amino acid is accepted. Ambiguities are indicated by listing between parentheses the acceptable amino acids for a given position. Repetition of an element of the pattern is indicated by following that element with an inferior number. On the other hand, class II enzymes have a highly conserved central region which contains three histidines in a signature sequence: HXXHLDH(L/I/V/M)X(G/S)(L/I/V/M/A)(L/I/V/M)XXS(A/P). The CpdA protein does not have the signature patterns of class I or II. Sequence similarities of CpdA with other proteins were not detected in the data base of GenBank using a Blast homology search, except for the predicted polypeptide, HI0399, in the genome of *H. influenzae* Rd. Hence, the CpdA protein belongs to a third class of cAMP phosphodiesterases.

Our first observation was that multicopy plasmids carrying the *cpdA* gene repressed expression of the *lacZ* gene in the wild-type strain in the presence of the inducer IPTG and also in the *lacI*-disrupted mutant, in which expression of β-galactosidase is constitutive. This indicates that the *cpdA* gene product inhibits *lacZ* expression independently of the LacI repressor.

Fig. 4. Nucleotide and deduced amino acid sequence of the *cpdA* gene. The nucleotide sequence of the *Pvu*I- *Bgl*II chromosome segment (see Fig. 1) is shown. The amino acid sequence derived from purified CpdA protein is underlined. The boundaries of cloning region of pXX904 and pXX906 are indicated by arrows above the nucleotide sequence. The nucleotide sequence data have been deposited in EMBL/GenBank/DDBJ sequence data libraries under the accession number D16557.

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We confirmed that the CpdA protein was cAMP phosphodiesterase, which hydrolyzed cAMP. Cells harboring the cpdA multicopy plasmid have a higher concentration of the CpdA protein by increased gene dosage, which increases the expression of cAMP phosphodiesterase. We measured the intracellular concentration of cAMP of the wild-type strain harboring the multiple copies of the plasmid carrying the cpdA gene and found that the presence of this plasmid markedly decreased intracellular cAMP. In turn, the intracellular concentration of cAMP was decreased, the formation of the CRP-cAMP complex was reduced, and expression of the \textit{lacZ} gene was repressed.

Decreasing intracellular concentration of cAMP by multiple copies of the \textit{cpdA} gene would inhibit expression of other CRP-dependent genes besides the \textit{lac} operon. We observed that in an \textit{in vitro} transcription system, expression of the \textit{gal} promoter, which was dependent on CRP-cAMP, was repressed by the CpdA protein. Using MacConkey plates, which indicate the utilization of a carbon source in the medium, we observed that cells harboring multiple copies of the \textit{cpdA} gene were partially inhibited for the utilization of galactose and maltose as well as lactose (data not shown). Besides expression of CRP-dependent genes, variation of intracellular concentration of cAMP by the \textit{cpdA} gene would be expected to influence cell division and the initiation of DNA replication.

It is known that adenylate cyclase plays an important regulatory role in the intracellular level of cAMP (1). The activity of adenylate cyclase, which synthesizes cAMP from ATP, is regulated transcriptionally and translationally. This enzyme is encoded by the \textit{cya} gene, and expression of the gene is negatively controlled by the CRP-cAMP complex (12, 13). Translation of \textit{E. coli} adenylate cyclase is initiated at a UUG codon,
and it was reported that the cya UUG codon limited cya expression at the level of translation (40). In addition to such gene regulation, the activity of adenylate cyclase is post-translationally regulated by phosphorylation via a phosphoenolpyruvate-dependent sugar phosphotransferase system (14, 15). Although the regulation of cAMP phosphodiesterase (CpdA) that hydrolyzes cAMP remains unclear, we found that translation of CAMP phosphodiesterase also is initiated at a UUG codon, as is adenylate cyclase. Thus, expression of the cpdA gene might be also regulated translationally.

The intracellular concentration of cAMP is very critical for regulation of various cellular systems; it influences expression of a great variety of genes by forming active CRP-cAMP complexes. Indeed, an excess of CRP-cAMP disturbed cell proliferation of a great variety of genes by forming active CRP-cAMP complexes. Moreover, under the condition that cells had an excess of CRP-cAMP, the cpdA-disrupted strain because the intracellular concentration of cAMP was twice as much as that of the isogenic cpdA+ strain (see Fig. 8). Taken together, these results indicate that the CAMP phosphodiesterase plays an important regulatory role in determining intracellular of cAMP content.

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