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Expression of the Adenylate Cyclase Gene during Cell Elongation in *Escherichia coli* K-12

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Expression of the adenylate cyclase gene (*cya*) in synchronized *Escherichia coli* cells was investigated by using the *cya-lacZ* protein and operon fusion plasmids. The regulation of *cya* expression during the cell cycle is characterized as follows: (i) *cya* is expressed during cell elongation; (ii) expression is repressed during cell division; (iii) regulation is exerted at the transcriptional level. To test *cya* expression during cell elongation, we constructed a plasmid (pLCR1) in which the *lacUV5* promoter operator was fused to the structural gene of *cya* and investigated the effect of *cya* expression by isopropyl-β-D-thiogalactopyranoside (IPTG) on the cell division of cells containing pLCR1. By the addition of IPTG, cell division was inhibited and filaments were formed. Such an inhibitory effect was antagonized by adding cyclic GMP to the culture medium and was not observed in the *crp* mutant.

Cyclic AMP (cAMP) has been implicated in a vast array of regulatory processes in mammalian cells. Both biochemical and genetic studies support the view that the primary mechanism of cAMP action is to affect the activity of cAMP-dependent protein kinase and, hence, to set the degree of phosphorylation of key proteins, the function of which is regulated by the reversible covalent modification (33). Recently, results of studies of *Saccharomyces cerevisiae* suggest that the cAMP-based regulatory cascade is required for cell cycle progression (21). Furthermore, in the lower organisms the changes of the endogenous cAMP levels have been shown to correlate with development or morphological change (8, 9, 27, 32).

On the other hand, a well-known example of cAMP-mediated regulation in *Escherichia coli* is catabolite repression (35). cAMP was shown to overcome the repression of the synthesis of catabolite enzymes by glucose when added to *E. coli*. In this case, cAMP and its receptor protein (CRP) act as a positive effector for transcription. In addition, the cAMP-CRP complex is also a transcriptional repressor in another set of genes (2, 3, 23, 24).

Thus, the mode of cAMP function in eucaryotes seems to be very different from that in *E. coli*. However, in both organisms cAMP may regulate cell growth. An inverse relationship was pointed out between the growth rate and cellular cAMP levels in bacteria and mammalian cells (20). Extensive studies in mammalian cells have led to the postulation that cellular cAMP levels play a central role in growth regulation and neoplastic transformation (25, 26). Recently, this postulation has been substantiated in yeast cells (34).

Bacterial rod shape is assumed to depend on the balance of two specific wall growth systems; one is responsible for cell elongation and one is responsible for septum formation (12, 30, 31). Kumar and co-workers (16, 17) reported that the synthesis of lateral wall- and minicell-producing cell division in *E. coli* are controlled by the cAMP-CRP complex. Recently, we found that cell elongation and inhibition of cell division are regulated by the cAMP-CRP complex in *E. coli* (37–39). These results indicate that the cAMP-CRP complex is closely concerned with the regulation of synthesis of lateral wall and septum formation.

Two promoters (p_1,p_2) of *cya* are known (3, 29), whereas our recent studies on the *cya* gene revealed that the transcription which depends on *cya* p_2 is negatively regulated by cAMP-CRP (3, 14). We believe that such autogenous regulation of *cya* is responsible for the normal growth of *E. coli*, because cell growth is inhibited by increased cAMP levels (10) and that the expression of *cya* could be closely related to regulation of cell cycle and growth.

Here we report the expression of *cya* during the cell cycle by using synchronized cells and *cya-lac* and *lac-cya* fusion plasmids. We demonstrate that expression of *cya* is closely coordinated with the cell cycle.

MATERIALS AND METHODS

Strains and plasmids. Tables 1 and 2 list the strains of *E. coli* and the plasmids used in this study, respectively.

Media. The rich medium was L broth (18) supplemented with 50 μg of thymine per ml. The minimal medium used was medium A (22), which was supplemented with 0.2% glucose–Casamino Acids (2.7 mg/ml; Difco Laboratories, Detroit, Mich.)–thymine (50 μg/ml)–thiamine (2 μg/ml)–ampicillin (50 μg/ml).

Preparation of plasmids and DNA fragments. The plasmids were purified essentially by the procedure of Birnboim and Doly (6) and Aiba et al. (4). Plasmid DNAs were digested with the appropriate restriction enzymes, and the resulting fragments were fractionated on polyacrylamide gels. The restriction fragments used in this study were prepared by the method described previously (4).

Enzyme assays. β-Galactosidase assays were performed essentially as described by Miller (22). Enzyme activity per milliliter was expressed as follows:

$$1{,}000 \times \frac{A_{420} - 1.75 \times A_{550}}{(\text{assay time, in min}) \times (\text{sample volume, in ml})}$$

Synchronization of cells. Cells were synchronized with *dnaA*(Ts) mutants by the method of Abe and Tomizawa (1). The overnight culture was diluted 30-fold with 60 ml of A medium and cultured at 30°C. When the A_{600} was 0.1 to 0.2

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TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source
LC343	F⁻ *thi leu thy sup-2* Δ(*pro-lac*) *dnaA*(Ts) *supE42*	J. Louarn 19
MC1061	*araD139* Δ(*ara leu*)*7699 galU galK* Δ*lac* (*IPOZY*)*X74 hsdR hsdM*⁺ *rpsL*	M. J. Casadaban 7
PA3092	F⁻ *thr-1 leuB6 thi-1 argH1 hisG1 trp-1 lacY1 gal-6 mtl-2 xyl-7 malA1 ara-13 rpsL9 thyA fhuAZ supE44 fic*[a]	Y. Hirota
AB1157	F⁻ *thr leu proA his arg thi lac gal ara xyl mtl thy str* T6ʳ	K. Imai
SK3891	AB1157 *pro*⁺ Tetʳ (Tn*10*)	P1 (CA8000::Tn*10*)[b] × AB1157
UR3359	AB1157 *pro* Tetʳ (Tn*10*)	P1 (SK3891) × AB1157
KU3041	PA3092 *pro* Tetʳ (Tn*10*)	P1 (UR3359) × PA3092
NO2404	Δ(*pro-lac*) *thi gyrA recA supE* ColE3ʳ/F′ *lacI*ᵠ*ZY*⁺ΔM15 *pro*⁺	K. Imai
NR7036	KU3041/F′ *lacI*ᵠ*Z Y*⁺ΔM15 *pro*⁺	NO2404 × KU3041
PP48	*cya*	I. Pastan

[a] *fic* was identified by Utsumi et al. (38).
[b] The culture of CA8000::Tn*10* was prepared by scraping off the tetracycline-resistant colonies transposed with Tn*10* (36).

after 4 to 5 h, the culture was changed to 42°C and incubated for 70 min. It was then immediately changed at 30°C, and synchronized cells were prepared.

RESULTS

Construction of fusion plasmids. Construction of *cya-lac* protein and operon fusion plasmids has been described by Kawamukai et al. (14). Briefly, the 529-base-pair (bp) *Bam*HI fragment carrying the major promoter (p_2) of *cya* and the first 88 codons for adenylate cyclase was inserted into the *Bam*HI site of pMC1403 (7) to form a *cya-lacZ′* protein fusion plasmid, pCL1. The plasmid pCL1 produces a *cya-lacZ′* hybrid protein that retains β-galactosidase activity but not adenylate cyclase activity. To obtain a *cya* p_2-*lac* operon fusion plasmid, pC2OL, the *Bam*HI fragment containing *cya* p_2 mentioned above, was inserted into the *Bam*HI site of pMS437C (14). Likewise, the 370-bp *Eco*RI-*Bam*HI fragment carrying *cya* p_1p_1' was placed between the *Eco*RI and *Bam*HI sites of pMS437C to obtain pC1OL (14).

Construction of *lacP-cya* fusion plasmids is shown in Fig. 1. The 95-bp *Alu*I fragment carrying the *lac* promoter-operator region from pKB252 (5) was ligated with both the 255-bp *Rsa*I-*Bam*HI fragment of pCA8 and the 4.5-kilobase-pair *Bam*HI-*Pvu*II fragment of pCA7. The ligation mixture was used to transform *cya* mutant PP48 to ampicillin resistance by using lactose-MacConkey agar indicator plates containing ampicillin. Plasmids were isolated from the transformants that formed red colonies. The structure of one of the *lacP-cya* fusion plasmids named pLCR1 is shown in Fig. 1. In pLCR1, an additional DNA fragment that corresponds to the *Rsa*I-*Rsa*I region in pCA8 was shown to be inserted between the *Alu*I-*Alu*I region (*lacP lacO*) and the *Rsa*I-*Bam*HI region. Perhaps, the *Rsa*I-*Rsa*I fragment derived from pCA8, the size of which is similar to the *Rsa*I-*Bam*HI fragment, was contaminated when the 255-bp *Rsa*I-*Bam*HI fragment of pCA8 was prepared. Although we did not determine the DNA sequence of pLCR1, the expression of *cya* in pLCR1 was confirmed to be induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG), because its addition increased cAMP levels (Fig. 2). During this experiment we found that the addition of both theophylline, which inhibits cAMP phosphodiesterase activity (15), and IPTG to the culture medium was effective in increasing the cAMP levels. Namely, it is obvious that the *cya* expression is under the control of the *lac* promoter-operator.

Characterized expression of *cya* in synchronized cultures. To study the expression of *cya* during the cell cycle, we synchronized the *dnaA* and Δ*lac* double mutant, LC343 harboring the *cya-lacZ′* protein fusion pCL1, and determined β-galactosidase activity in the cells. After a shift down from 42 to 30°C, the enzyme activity increased for the first 40 to 50 min and then stopped for several minutes and increased again (Fig. 3A). We also assayed for cell number in the same samples to determine the synchronized growth of cells. The data indicate that cell division occurred at 40 to 50 min after the temperature reduction (Fig. 3A). A periodic pattern of β-galactosidase activity was observed repeatedly in several independent experiments.

β-Galactosidase activity with the *cya-lacZ′* protein fusion reflects both transcriptional and translational regulation of *cya*. To clarify whether the observed repression of *cya* expression during cell division was at the level of transcription, we measured β-galactosidase activity in synchronized cells carrying the *cya-lac* operon fusion plasmids (Fig. 3B). The pattern of the β-galactosidase activity in LC343 harboring the *cya* p_2-*lac* operon fusion plasmid (pC2OL) during synchronized cultures was essentially the same as that in the case of the protein fusion (pCL1), although the inhibition of β-galactosidase synthesis was more evident (Fig. 3B). We also determined the synthesis of β-galactosidase in LC343 harboring the *cya* p_1p_1'-*lac* operon fusion (pC1OL) in syn-

TABLE 2. Plasmids used in this study

Plasmids	Characters	Source
pMC1403	For protein fusions	Casadaban et al. (7)
pCA3	*cya* cloned into pBR322	Aiba et al. (4)
pCL1	*cya-lacZ′* protein fusion carrying the p_2 promoter of *cya*	Kawamukai et al. (14)
pMS437C	For operon fusions	Shigesada et al. (14)
pC1OL	*cya-lac* operon fusion carrying p_1p_1' promoter of *cya*	Kawamukai et al. (14)
pC2OL	*cya-lac* operon fusion carrying p_2 promoter of *cya*	Kawamukai et al. (14)
pCA7	*Bam*HI-*Bam*HI fragment which corresponds to the N terminus of adenylate cyclase is deleted	Aiba et al. (4)
pCA8	Only *Bam*HI-*Bam*HI fragment of pCA3 which contains the *cya* promoter is cloned into PBR322	This study
pKB252	With *Alu*I-*Alu*I fragment carrying (*lacPUV5*) *lacO*	Backman et al. (5)
pLCR1	(*lacPUV5*) *lacO* was fused to the structural gene of *cya*	This study

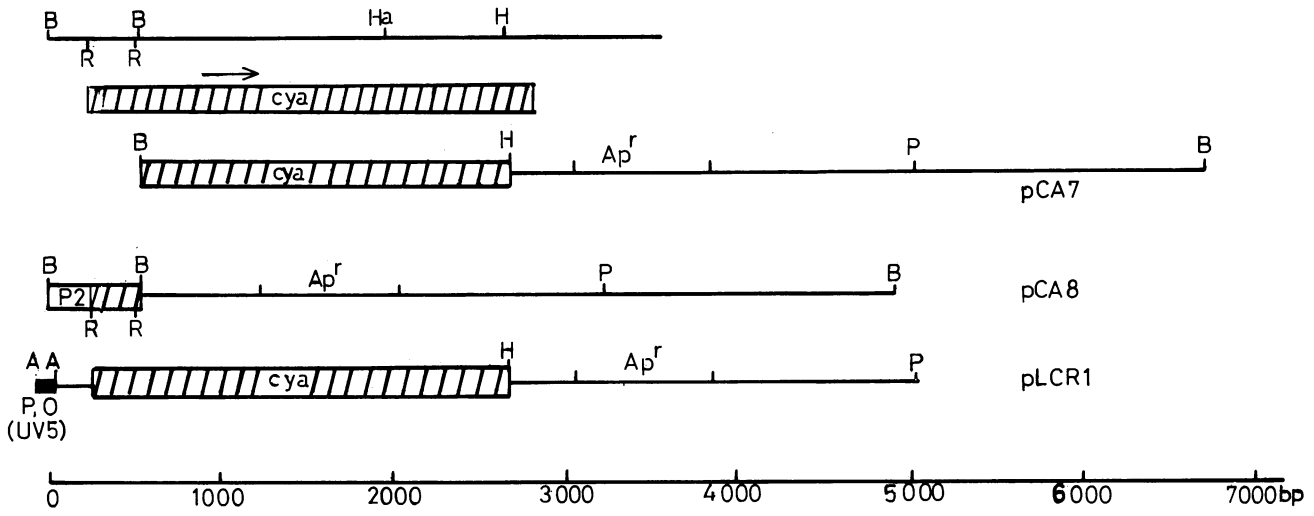

FIG. 1. Construction of pLCR1. The hatched bar represents the coding region for adenylate cyclase. The direction of transcription is from left to right. The restriction sites are designated as follows: A, *Alu*I; B, *Bam*HI; Ha, *Hpa*I; H, *Hin*dIII; P, *Pvu*II; R, RsaI.

chronized cultures. In this case, no inhibition of the synthesis of β-galactosidase was observed during cell division (Fig. 3C). These data indicate that the repression of *cya* expression during cell division is specific for p_2 and that it is exerted at the transcriptional level. At the same time, the data strongly suggest that the negative regulation is not due to the change of copy number of the plasmids during the cell cycle. However, the difference (Fig. 3A and B) between the change of β-galactosidase activity in the operon and the protein fusion might reflect the association of the translational regulation of *cya* expression. In fact, Reddy et al. (28) showed that the unique initiation codon of *cya* UUG is concerned with the translational regulation of *cya*, and Kawamukai et al. (14) also indicated the difference of β-galactosidase activity regulated by cAMP in pCL1 and pC2OL. Furthermore, to demonstrate that the regulation observed in pC2OL (Fig. 3B) is not due to the imposed temperature shifts, we performed the same experiment in the *dnaA*$^+$ strain. The characterized pattern was no longer observed (Fig. 3D).

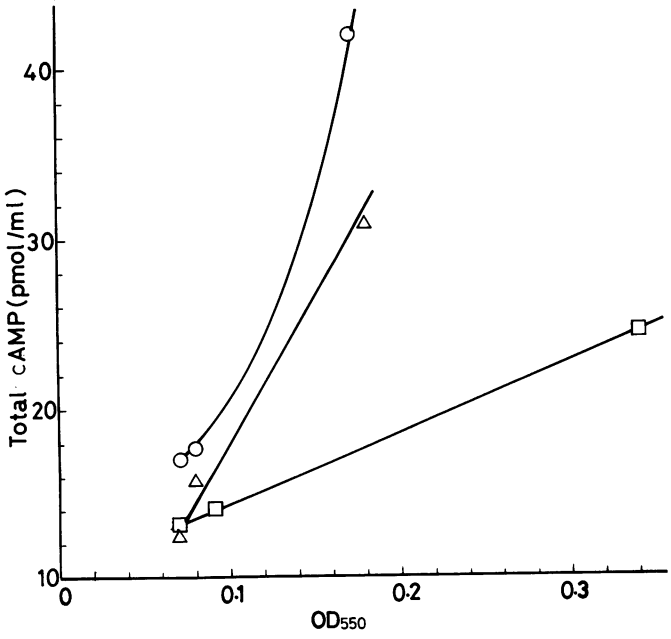

FIG. 2. Induction of cAMP by IPTG. Overnight culture in NR7036(pLCR1) was diluted 10-fold into L broth containing IPTG (0.5 mg/ml) (Δ), L broth containing both theophylline (1.8 mg/ml) and IPTG (0.5 mg/ml) (○), or L broth alone (□) and incubated at 42°C. Then, samples were taken at 0 (immediately), 10, and 120 min and boiled in 1 N HCl for 10 min (15). Total cAMP levels were assayed by the radioimmunological method described previously (15). OD_{550}, Optical density at 550 nm.

Effect of constitutive expression of *cya* on cell growth and division. By using synchronized cells containing *cya-lac* fusion plasmids, we showed the *cya* expression is dependent on the cell cycle. To study further the relationship between *cya* expression and the cell cycle, we constructed a *lacP-cya* fusion plasmid (pLCR1). In pLCR1 the *cya* promoter was replaced by the *lacUV5* promoter-operator region, and expression was under the control of *lac* promoter-operator (Fig. 2). We introduced the plasmid pLCR1 into *lacI*^{q} mutants and investigated the effect of *cya* expression caused by IPTG on cell division. The important feature of this system was that the *cya* gene is not under the control of *cya* promoter and that it is expressed simply by the addition of IPTG. By this addition, cell division of NR7036 containing pLCR1 was inhibited, and filaments were formed at 42°C (Fig. 4A and Table 3). Such an inhibitory effect at 30°C was not as remarkable as at 42°C. On the other hand, the addition of IPTG did not inhibit cell division of NR7036 without pLCR1 (data not shown). When IPTG was not added, cell growth of NR7036 containing pLCR1 was normal (Fig. 4B and Table 3). In addition, the inhibition of cell division induced by IPTG was antagonized by the addition of cyclic GMP (cGMP) to the culture medium (Fig. 4C) and was not observed in its *crp* mutant (Table 3). The filamentation induced by IPTG was also observed in another *lacI*^{q} mutant (NO2404) containing pLCR1.

These results obtained in NR7036 are consistent with results of our previous studies (38, 39), in which cell filamentation by the external addition of cAMP at 42°C was antagonized by the addition of cGMP and was not observed

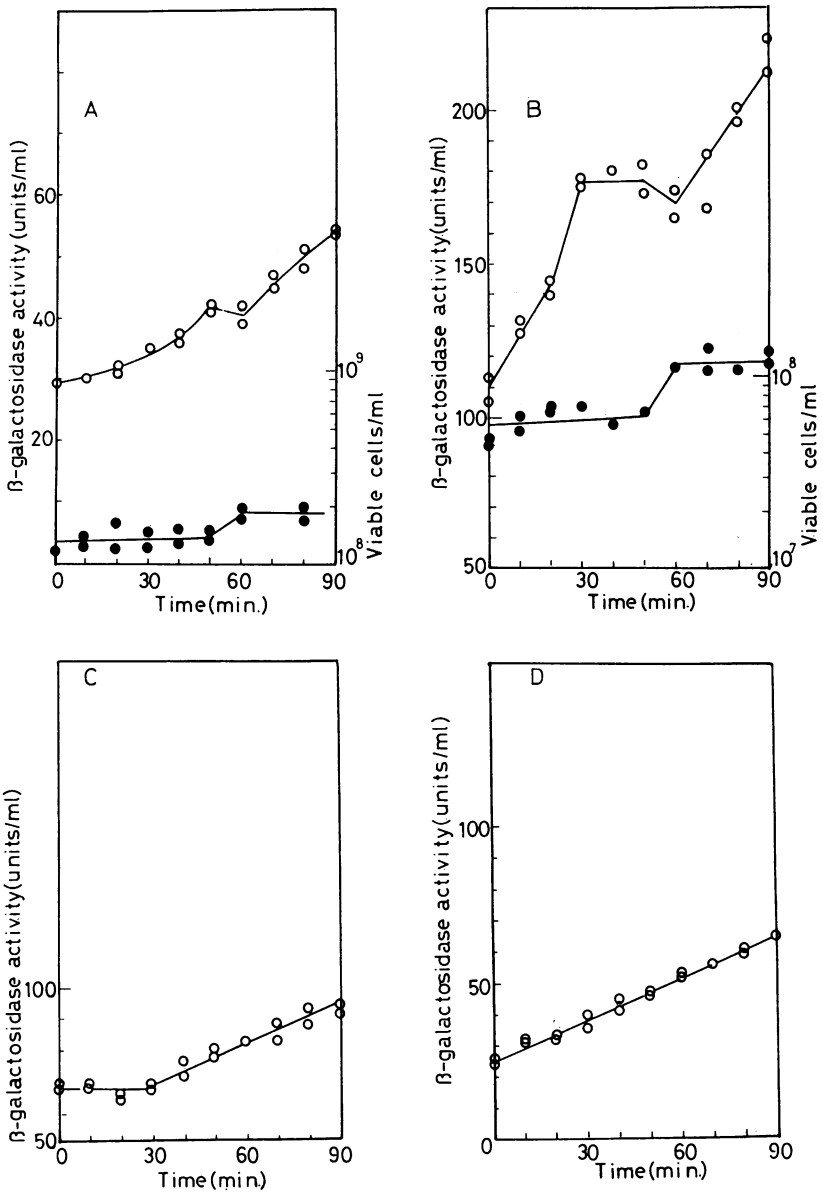

FIG. 3. Characterized expression of *cya* in synchronized cultures. (A) LC343(pCL1); (B) LC343(pC2OL); (C) LC343(pC1OL); (D) LC343 *dnaA*$^+$(pC20L). Synchronized cultures which were transferred from 42 to 30°C were immediately sampled and β-galactosidase activity (○) and viable cells (●) were assayed. LC343 (*dnaA*$^+$) was constructed by P1 transduction from W3110.

in its *crp* mutant. However, it should be noted that the *fic* mutation was necessary for cell filamentation induced by the addition of cAMP, while cell filamentation in I^q mutants containing pLCR1 induced by IPTG was not dependent on the *fic* mutation. This may reflect the difference of intracellular cAMP levels in both systems. The cAMP levels in cells containing pLCR1 after induction by IPTG may be significantly higher than that in cells supplemented with cAMP exogenously. In fact, during cell filamentation by IPTG, production of cAMP per 10^6 cells was about 20-fold that in the absence of IPTG. A concentration of 80 mM cGMP must be added to overcome the inhibition of cell division of I^q mutants containing pLCR1 in the presence of IPTG (Fig. 4C), while 5 mM cGMP is sufficient to antagonize the effect of exogenously added cAMP (39).

DISCUSSION

In this study we examined *cya* expression during the cell cycle by using synchronized Δ*lac* cells containing *cya-lac* fusion plasmids, because the activity of β-galactosidase should reflect the regulation exerted at the levels of transcription and translation of *cya* (3, 14); and the techniques used in this study might be suited for the study of gene expression during the cell cycle in synchronized cells, since the assay for β-galactosidase is simple and accurate.

The *cya* expression in synchronized cells is characterized as follows: (i) *cya* is expressed during cell elongation, (ii) its expression is repressed during cell division, (iii) the inhibition of *cya* expression is primarily exerted at the transcriptional level. We speculate that the expression of *cya* results

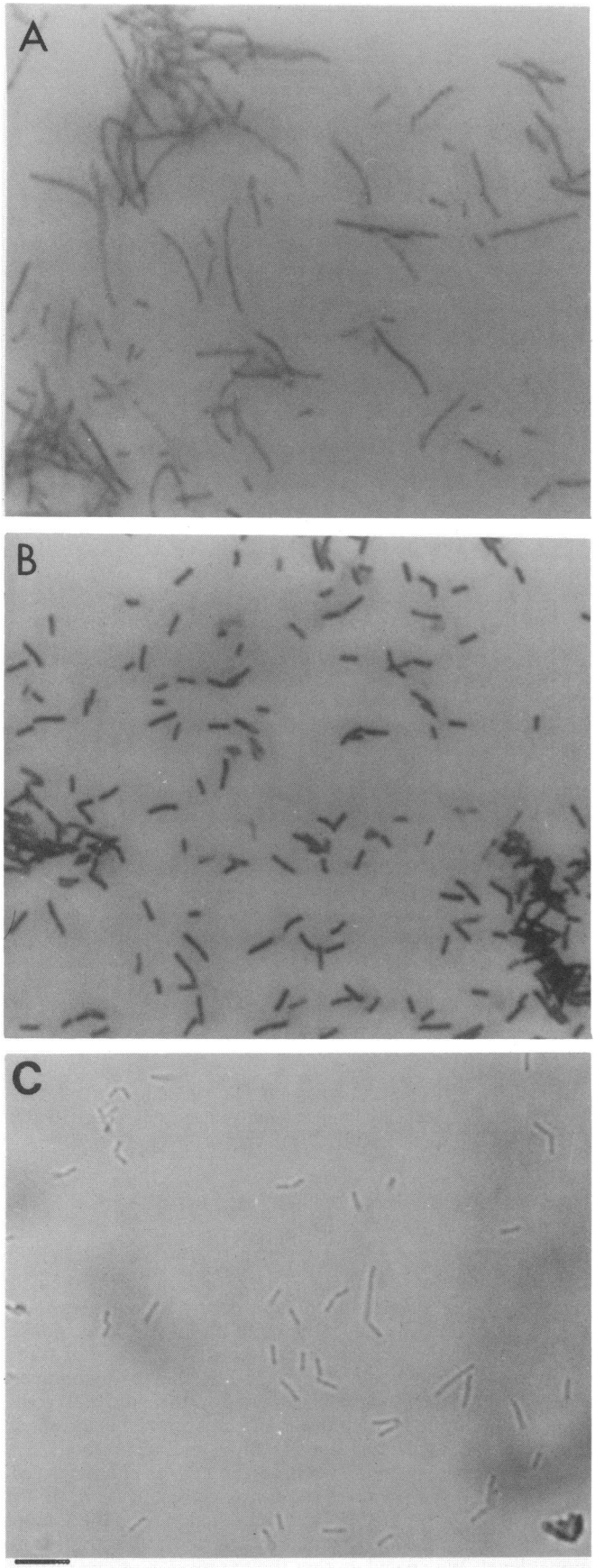

TABLE 3. Effect of constitutive expression of *cya* on cell growth[a]

Strain	IPTG (0.5 mg/ml)	Viable cells (10^{-7}/ml) at the following times:	
		0 h	2 h
NR7036(pLCR1)	+	57	57
	−	40	350
NR7036(pLCR1)(*crp*)[b]	+[c]	57	470
	−[c]	37	480

[a] Experimental procedure was the same as described in the legend to Fig. 4.
[b] NR7036 (*crp*) was derived as phosphomycin-resistant cells from NR7036.
[c] Under both conditions, filamentous cells were not observed.

in an increase in the intracellular cAMP concentration and causes cell elongation. When the concentration of cAMP reaches a certain level, its expression may be repressed by coordinating cell division. Recently, we found that the transcription of *cya* is negatively regulated by cAMP-CRP (3, 14). The repression of *cya* expression during cell division may be closely related to this autogenous regulation.

On the other hand, Höltje and Nanninga (13) reported that both intracellular and extracellular concentrations of cAMP increase logarithmically in synchronously growing cultures and concluded that cAMP by itself cannot regulate growth and division of the bacterium during the cell cycle. However, technically it is very difficult to detect severalfold differences in the cAMP concentration. Because of this the logarithmic increase in cAMP levels in synchronously growing cultures could not be concluded only from their data; it only proves the relationship between variation in the cAMP concentration and regulation of the cell cycle in *E. coli*. We cannot currently exclude the effect of a minor change in the cAMP concentration on cell growth. Furthermore, in addition to cAMP, cGMP, CRP, and other unknown factors also might be involved in control of the cell cycle. Results of Kawamukai et al. (15) and of this study (Fig. 2) show that the addition of theophylline, which inhibits cAMP-phosphodiesterase, caused increased cAMP levels; and filamentous cells appeared. This result suggests that cAMP metabolism is also important for the control of cell growth and division.

Cultures used in this experiment were synchronized by inhibiting the initiation of DNA synthesis with a *dnaA* mutant. This technique synchronizes DNA synthesis and cell division but it does not synchronize growth. Thus, it is interesting and valuable to apply the elutriation method used by Höltje et al. (13) to our system. Results obtained by use of this method should reflect characteristics of *cya* expression during synchronous growth.

Filamentation induced by IPTG in the cells containing pLCR1 was characterized as follows: (i) the addition of cGMP antagonized the inhibitory effect, (ii) filamentation was more remarkable at 42°C than at 30°C in NR7036 (*fic* mutant), (iii) filaments were not observed in a *crp* mutant. These results suggest that in the cells containing pLCR1, cAMP, the production of which was induced by IPTG, binds

FIG. 4. Cell filamentation induced by IPTG. Overnight culture in NR7036(pLCR1) was diluted 10-fold into L broth containing IPTG (0.5 mg/ml) (A), L broth alone (B), or L-broth containing both IPTG (0.5 mg/ml) and cGMP (30 mg/ml) (C) and incubated for 2 h at 42°C. Then, cells were observed. L broth contains thymine. Bar, 10 μm.

CRP competitively with cGMP (11), and its complex regulates the expression of genes involved with cell division. Temperature sensitivity of filamentation in the *fic* mutant cannot currently be explained. Recently, however, we succeeded in cloning *fic* with a product that was different from CRP. The *fic* gene product might regulate cell division with cAMP. Perhaps, increased cAMP sensitivity at 42°C in the *fic* mutant is due to temperature sensitivity of the *fic* gene product.

Kumar et al. (16) reported that cell elongation is inhibited in *cya* and *ftsA* double mutants at 42°C and that cAMP is required for lateral wall synthesis in *E. coli*. This is consistent with the finding of this study that *cya* is expressed during cell elongation. The interpretation of our results, however, involves the assumption that our results do not depend on gene copy numbers. If the construct in pC2OL or pCL1 were present as a single copy in the chromosome, we do not know whether the same results would be observed. In the future we think that a single-copy experiment should be performed.

To characterize *cya* expression during cell elongation, *cya* was expressed constitutively by using *lacP-cya* fusion systems. As expected, cell division was inhibited and filaments were formed. To eliminate the gene dosage effect of this system, we constructed the strain in which pLCR1 was inserted in chromosomal DNA by using the *polA*(Ts) strain and *cya* expression was induced by IPTG. In this strain, the addition of IPTG inhibited cell division and induced filaments (data not shown). These results are consistent with those shown in Fig. 4. Results of this study indicate that the cell cycle is dependent on *cya* expression and that its regulation is critical for the normal growth of *E. coli*.

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