INVOLVEMENT OF CYCLIC 3',5'-ADENOSINE MONOPHOSPHATE IN REPLICATION OF COLICINOGENIC FACTOR E_1 DNA

Atsushi Nakazawa and Terumi Tamada

Department of Biochemistry, Chiba University School of Medicine
Inohana, Chiba, Japan

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SUMMARY · The stimulatory site of cyclic 3',5'-adenosine monophosphate for the colicin E_1 synthesis was investigated in the mitomycin C-induced adenyl cyclase-defective mutant of Escherichia coli. Cyclic AMP increased the number of colicin E_1 producing cells which probably led to the increased synthesis of the colicin. The nucleotide failed to stimulate colicin E_1 production in the cells in which DNA synthesis had been arrested by nalidixic acid. Incorporation of [^{3}H]-thymine into colicinogenic factor E_1 DNA depended on the presence of cyclic AMP. Thus the site of cyclic AMP action appeared not to be after the transcription of DNA, but at the replication of DNA.

Colicinogenic factor E_1 (ColE_1) is an extrachromosomal DNA (1) that determines the production of an antibiotic protein colicin E_1 (2). ColE_1 is normally dormant with regard to active colicin production, but is induced to synthesize the colicin by treatment with mitomycin C (3) or ultraviolet (4). Although it has been already shown that DNA molecule of ColE_1 is multiplied after mitomycin C treatment (5), little has been known about the biochemical events of the regulatory process in the colicin induction.

We have recently found that cyclic 3',5'-adenosine monophosphate stimulates the synthesis of colicin E_1 in mitomycin C-induced Escherichia coli. The system seems unique among others that are regulated by cyclic AMP, since glucose is not inhibitory, but rather cooperative with the action of cyclic AMP (6). This paper describes the regulatory target of the nucleotide for colicin E_1 production. Available evidence indicates that cyclic AMP acts at the step of replication of ColE_1 DNA.

MATERIALS AND METHODS

Bacteria: E. coli K12 CA7902 cya$^-$ thi$^-$ (ColE_1, ColIb) and Y20 cya$^+$ thr$^-$

leu⁻ thi⁻ (ColE$_1$) have been described (6). Strain CA7902 cya⁻ thi⁻, originally isolated by Schwartz and Beckwith (7) and P678-54 thr⁻ leu⁻, a minicell producing strain of Adler et al. (8) were also used. Transfer of ColE$_1$ to these strains was made according to the method of Smith et al. (9) using E. coli K12 W3110 prototroph F$^+$ and Y20 (ColE$_1$). For the growth of bacteria the minimal salt media (M-9) supplemented with vitamin-free casamino acids (0.15%), thiamine-HCl (10 μg/ml) and glucose (0.4%), were used (6), which will be refered to as the basal media.

Colicin assays and lacunae counts: The conditions for the colicin induction and the colicin assay were previously described (6). For the colicin E$_1$ assay, E. coli K12 W3110 resistant to colicin Ib was used as the indicator strain. The number of individual cells producing colicin E$_1$ (lacunae counts) was measured according to the procedure of Ozeki et al. (4) using E. coli K12 20S0 str^r as the indicator strain.

Radioactive labeling of DNA: Cells were grown to log phase in the basal media containing deoxyadenosine (250 μg/ml). After washing by centrifugation, the cells (1.4×10^9 cells/ml) were incubated with mitomycin C (2 μg/ml), deoxyadenosine (250 μg/ml) and [^{3}H]-thymine (20 μCi/ml) in the presence or absence of cyclic AMP as in the colicin induction. The reaction was stopped by the addition of KCN (10 mM) at the given times. The lysis of the cells was performed by the lysozyme-Brij 58 procedure described by Clewell and Helinski (10). After removing the bulk of the host DNA by centrifugation at 30,000 × g for 20 min at 0°, aliquots of the cleared lysates (0.20 ml) were layered on a 15-50% neutral sucrose gradient and centrifuged in the RPS 65TA rotor at 50,000 rpm for 4.5 hours at 4° using a Hitachi 65P ultracentrifuge. Sucrose gradients were prepared in 0.05 M NaCl, 0.005 M EDTA and 0.03 M Tris, pH 8.0. Fractions (10 drops) were directly collected from the bottom of the tube on 25-mm square filter papers. The papers were washed successively with cold 5% trichloroacetic acid, ethanol, and ether, and the radioactivity was counted in a Beckman LS-100 liquid scintillation counter. Toluene containing 4.0 mg/ml PPO and 0.2 mg/ml POPOP served as the standard fluor.

TABLE I. Effect of cyclic AMP on number of colicin E_1 producing cells

Cyclic AMP	Incubation period (min)	Lacunae counts (per ml)	Viable cell counts (per ml)	Percent of cells as lacunae	Colicin E_1 activity
-	0	3.3×10^4	1.8×10^9	0.0017	0
-	30	4.4×10^4	4.4×10^8	0.0024	0
	60	4.6×10^6	7.0×10^8	0.65	8.8
	120	1.1×10^8	1.2×10^9	8.5	80
+	30	1.9×10^7	2.8×10^8	6.3	19
	60	6.7×10^8	2.2×10^8	75	200
	120	9.7×10^8	1.2×10^7	99	370

Strain CA7902 *cya*$^-$ (ColE_1) was aerobically grown to log phase in the basal medium. To the culture was added mitomycin C (2 μg/ml), and either cyclic AMP (1 mM) or distilled water. Incubation was performed without aeration at 37°. At the time indicated, a portion of cells was removed and lacunae counts and viable cells were measured as described in Materials and Methods. Percent of cells as lacunae was obtained as follows: Lacunae counts × 100/(Lacunae counts + Viable cell counts). Colicin E_1 activity was expressed as units per absorbancy at 660 nm of the incubation mixture.

RESULTS AND DISCUSSION

Cyclic AMP stimulated the increase of the number of colicin E_1 producing cells during mitomycin C induction of *E. coli* CA7902 (ColE_1) (Table I). Before induction, percent of the colicin producing cells was 0.0017 in a population. Upon addition of mitomycin C, however, the colicin producing cells markedly increased in the presence of cyclic AMP up to 99% of the population at 120 min. In contrast, in the absence of cyclic AMP the lacunae counts slowly increased to 8.5% at 120 min. The increase of the lacunae counts was paralleled by the accumulation of colicin E_1. Note that the stimulation of the increase of the lacunae counts by cyclic AMP was more remarkable in the early phase of incubation, which had been also observed in the colicin production.

It is known that the induced production of colicin E_1 is accompanied by the increase in the amount of ColE_1 DNA (5). The stimulatory site that cyclic

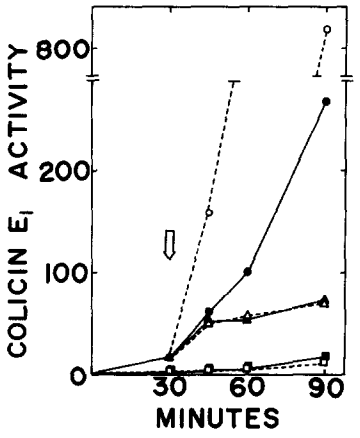

Fig. 1.

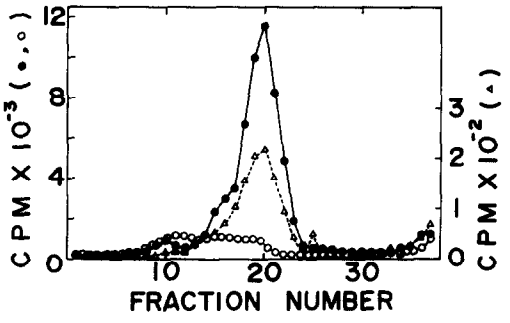

Fig. 2.

Fig. 1. Effect of nalidixic acid on colicin E_1 synthesis. Log phase cells of CA7902 cya$^-$ (ColE_1, ColIb) were incubated in the basal media containing mitomycin C (2 μg/ml). After 30 min (arrow) nalidixic acid was added at a final concentration of 200 μg/ml, together with either cyclic AMP at 1 mM (△) or distilled water (▲). As a control, distilled water was added at 30 min of incubation instead of nalidixic acid, together with either cyclic AMP (○) or distilled water (●). In the other set of experiments, nalidixic acid was added at the zero time together with mitomycin C and then incubated with (□) and without (■) cyclic AMP. Incubation was made at 37° without aeration. Colicin E_1 activity was expressed as units per absorbancy at 660 nm of the incubation mixture.

Fig. 2. Sucrose gradient analysis of the cleared lysates. Washed log phase cells of CA7902 cya$^-$ (ColE_1) (1.4×10^9 cells/ml) were incubated for 45 min at 37° in the 3-ml basal media containing mitomycin C (2 μg/ml), [^{3}H]-thymine (20 μCi/ml), and deoxyadenosine (250 μg/ml) in the presence and absence of cyclic AMP (1 mM). Colicin E_1 activities after incubation were 16 and 160 units per absorbancy at 660 nm in the absence and presence of cyclic AMP, respectively. Radioactivities incorporated into acid insoluble materials were 1.1×10^6 and 1.4×10^6 cpm in the absence and presence of cyclic AMP, respectively. The total counts in the incubation mixture was 6.8×10^6 cpm. For labeling minicells, a 20-ml culture of P678-54 (ColE_1) was grown for approximately two generations in the presence of [^{3}H]-thymine (5 μCi/ml) and deoxyadenosine (250 μg/ml) in the basal medium. Minicells were separated from the normal cells by low-speed centrifugation on sucrose gradients as described by Adler et al. (8). Conditions for lysis of the cells and the minicells and sucrose gradient analysis were described in Materials and Methods. The direction of sedimentation was from right to left: ●—●, CA7902 (ColE_1) incubated with cyclic AMP; ○—○, CA7902 (ColE_1) incubated without cyclic AMP; and △—△, minicells carrying ColE_1.

AMP affects in this system, therefore, may not be restricted to the transcription of DNA as in the case for the catabolic enzyme synthesis (11), but maybe the level of replication of ColE_1 DNA. In order to examine this possibility,

the following experiments were performed using nalidixic acid, an inhibitor of DNA synthesis (12). When the inhibitor was added to the cells of CA7902 (ColE_1, ColIb) at 200 μg/ml together with mitomycin C, essentially no synthesis of colicin E_1 was observed[1/] (Fig. 1), a confirmation that DNA synthesis is involved in the colicin induction. In the other sets of experiments, the cells were treated with mitomycin C and the colicin synthesis was allowed to proceed for 30 min. Nalidixic acid was then added to the reaction mixture at 200 μg/ml and the cells were incubated either with or without cyclic AMP. There was no effect of cyclic AMP on colicin E_1 accumulation after DNA replication was arrested by the inhibitor (Fig. 1). When the inhibitor was not included in the mixture, a marked stimulation by cyclic AMP was observed. Similar results were obtained in the experiments using 5-fluorouracil as an inhibitor. These results indicate that the synthesis of colicin E_1 after nalidixic acid addition is due to DNA and m-RNA corresponding to ColE_1 accumulated during the first 30 min of incubation, and consequently that the regulatory site of cyclic AMP is not a step after the transcription of ColE_1 DNA.

Direct evidence that cyclic AMP acts at the level of DNA replication was obtained from the experiments using [^{3}H]-thymine. Strain CA7902 (ColE_1) was incubated for 45 min with [^{3}H]-thymine under the conditions for colicin E_1 induction. The cleared lysates that were freed from host DNA were obtained as described and were analyzed by centrifugation on neutral sucrose gradients (Fig. 2). Three radioactive components were obtained in the presence of cyclic AMP; a sharp peak, a shoulder at the heavier side of the peak and a small peak near the bottom of the tube. In contrast, flat and broad bands which probably consisted of several components were observed in the absence of cyclic AMP[2/]. It has been shown that ColE_1 segregates into "minicells" (13), which are small, spherical, anucleated bodies produced during growth of a mutant, P678-54 (8). When [^{3}H]-

1/ At this concentration of nalidixic acid, host DNA synthesis was inhibited by over 95%, but host RNA synthesis still proceeded at a rate 45% that without the inhibitor.

2/ Under these conditions, [^{3}H]-thymine incorporation into the host DNA increased by 27% in the presence of cyclic AMP (see legend to Fig. 2).

labeled ColE_1 was isolated from the minicells carrying ColE_1 and examined by sucrose gradient centrifugation, the peak position[3/] exactly coincided with that of the above major peak in the presence of cyclic AMP (Fig. 2). This peak, however, was not present in the cleared lysates derived from CA7902, the non-colicinogenic parental cells. Furthermore, strain Y20 (ColE_1) gave the same radioactive peak even in the absence of cyclic AMP, and upon addition of cyclic AMP the radioactivity under the peak increased by about 25%. The radioactive peak was not observed when the cells of Y20 (ColE_1) were incubated in the presence of nalidixic acid (200 μg/ml). The above observations taken as a whole led us to the conclusion that the site of cyclic AMP action on colicin E_1 induction is the replication of the ColE_1 DNA.

Evidence has been accumulating that RNA synthesis is involved in the initiation of DNA replication in bacteria and phage (14, 15). Requirement of RNA synthesis for increased colicin E_1 production was shown by Kennedy (16) in chloramphenicol-treated cells. Recently Clewell *et al.* (17) demonstrated that rifampicin inhibited the ColE_1 replication after chloramphenicol induction. We also observed in mitomycin C-induced cells that rifampicin (50 μg/ml) inhibited the ColE_1 replication approximately by 50%, as judged by the sucrose gradient analysis. Protein synthesis, however, does not appear to be involved in mitomycin C-induced replication of ColE_1, since the presence of 100 μg/ml chloramphenicol did not affect [^{3}H]-thymine incorporation into ColE_1 DNA. From these findings it is attractive to assume that cyclic AMP participates in the synthesis of specific RNA species which are necessary for ColE_1 DNA replication.

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3/ The S value of ColE_1 isolated from minicells was reported to be 24.6 S (13), which corresponds to the value of either the supercoiled circular ColE_1 DNA (23 S) or the supercoiled circular DNA-protein complex (24 S) (10).

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