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Stimulation by Cyclic Adenosine Monophosphate of Plasmid Deoxyribonucleic Acid Replication and Catabolite Repression of the Plasmid Deoxyribonucleic Acid-Protein Relaxation Complex

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Colicinogenic factors ColE1 and ColE2 are bacterial plasmids that exist in *Escherichia coli* as supercoiled deoxyribonucleic acid (DNA) and as strand-specific, relaxation complexes of supercoiled DNA and protein. Newly replicated ColE1 DNA becomes complexed with protein after the replication event. This association of DNA and protein can take place under conditions in which DNA or protein synthesis is arrested. The addition of cyclic adenosine monophosphate (c-AMP) to normal cells growing in glucose medium results in a six- to tenfold stimulation in the rate of synthesis of the protein component(s) of the complex and a three- to fivefold stimulation in the rate of ColE1 DNA replication. Employing mutants deficient in catabolite gene activator protein or adenylate cyclase, it was shown that synthesis of both the plasmid-determined protein colicin E1 and the protein component(s) of the ColE1 relaxation complex is mediated through the c-AMP-catabolite gene activator protein system. Addition of c-AMP to ColE2-containing cells results in the stimulation of synthesis of ColE2 DNA and relaxation protein(s) as well as in the production of a protein component of the ColE2 relaxation complex that renders it sensitive to induced relaxation by heat treatment. In the case of ColE2, synthesis of the relaxation protein(s) is not dependent upon catabolite gene activator protein.

The colicinogenic factors E1 (ColE1) and E2 (ColE2) are bacterial plasmids which determine the production of antibiotic proteins (colicins). From gently lysed spheroplasts of *Escherichia coli*, they can be isolated both as noncomplexed, covalently closed, (supercoiled) circular deoxyribonucleic acid (DNA) molecules and as relaxation complexes of supercoiled DNA and protein (3-6). The complexed, supercoiled DNA is converted to an open, circular form upon treatment with alkali, heat, proteases, or certain ionic detergents (4, 5). When such a conversion occurs, a strand-specific nick or gap appears in the circular DNA (4, 6).

The percentage of ColE1 DNA that can be isolated as relaxation complex is dependent upon the presence of glucose in the growth medium (7). When extracted from cells grown in a glucose-containing medium, approximately one-third to one-half of the isolated ColE1 DNA is in the form of relaxation complex. In contrast, greater than two-thirds of the isolated plasmid DNA is complexed when extracted from cells grown in the absence of glucose.

This apparent "glucose effect" suggested that control over synthesis of either the relaxation complex or its protein component(s) may be exerted through the process termed "catabolite repression." The presumed effect of glucose or its catabolites is to depress the intracellular concentration of cyclic adenosine 3',5'-monophosphate (c-AMP) (16). It has been demonstrated that operons sensitive to catabolite repression require c-AMP for their expression (17). It has been shown recently that transcrip-

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tion of the *lac* and *gal* operons results from the interaction between a cellular protein, termed catabolite gene activator protein (CGA), and DNA (2, 9, 16, 18, 21). The effect of c-AMP is the activation of the CGA protein which must take place prior to its binding to DNA (1, 18).

In this paper we shall be concerned with the factors which regulate the synthesis of ColE1 and ColE2 DNA and their respective relaxation protein(s). We will show that replication of ColE1 and ColE2 DNA is enhanced by c-AMP and that synthesis of the proteins of the ColE1 relaxation complex and colicin E1 is mediated through the c-AMP-CGA system. In addition, evidence is presented for two types of ColE2 relaxation complexes, the synthesis of one being c-AMP dependent.

MATERIALS AND METHODS

Materials. Reagents and enzymes and their sources are as follows: Triton X-100, Bass Chemical Co.; sodium dodecyl sulfate (SDS), Fischer Scientific; Sarkosyl NL30 (sodium dodecyl sarcosinate), Geigy Chemical Co.; CsCl (technical grade), Penn Rare Metals division of Kawecki Chemical Co.; ethidium bromide, a gift from Boots Pure Drug Co., Ltd., Nottingham, England; mitomycin C, Nutritional Biochemicals; chloramphenicol (CAP), Calbiochem Co., c-AMP, Calbiochem and Sigma Chemicals; [*methyl*-^3H]thymine (50 Ci/mmol), [2-^{14}C]thymine (55.2 mCi/mmol), and [*methyl*-^3H]thymidine (50 Ci/mmol), New England Nuclear Corp.; lysozyme (egg white, crystallized three times), trypsin (pancreatic, crystalline, A grade) and Pronase (B grade), Calbiochem. Colicins E1 and E2 were purified to homogeneity by S. Schwartz (20).

Bacterial strains. *E. coli* K-12 strains JC411 Thy$^-$ and CR34 Thy$^-$, colicinogenic for the plasmid ColE1 or ColE2, have been described previously (4, 5). Derivatives of JC411 Thy$^-$ (ColE1) and JC411 Thy$^-$ (ColE2) which carry the R64 plasmid were constructed by conjugal mating with *E. coli* strain J5-3 (R64), obtained from E. Meynell. Strain 5336, a mutant of HfrH 1100, was obtained from I. Pastan. In the absence of added c-AMP, this strain cannot utilize carbohydrates such as lactose, maltose, or L-arabinose due to a deficiency in adenylate cyclase activity (17). In minimal medium employing glucose, this strain grows poorly and tends to revert. Strain CA-7900, a mutant of HfrH was obtained from J. Beckwith. This strain cannot utilize lactose, maltose, or arabinose in the presence or absence of added c-AMP due to a deficiency in the c-AMP binding protein, CGA (18, 21). YS40 ColV^r Az-r and C600 Thy$^-$ Az-r are sensitive to the killing action of purified colicins E1- and E2 or ColE1- and ColE2-containing strains.

Media. M9 medium, described by Roberts et al. (19), contained 0.2% glucose and 0.01% required amino acid. AB-3, L broth, and tryptone broth have been described previously (1). Tryptone broth was used with 0.5% glucose. When c-AMP was employed, it was added in final concentration of 2.5×10^{-3} M to M9 medium or 4×10^{-3} M to complex medium. For thymine-requiring strains, thymine was used at a concentration of 1.5 μg/ml.

Growth of bacteria and preparation of cleared lysates. Unless otherwise indicated, cells were grown at 37 C in batch culture employing either [^{3}H]thymine (10 μCi/ml) or [^{14}C]thymine (1 μCi/ml), Deoxyadenosine (300 μg/ml) was used in cases where the strain did not require exogenous thymine. For pulse experiments [^{3}H]thymidine (10 μCi/ml) with or without deoxyadenosine was used. Turbidity was recorded on a Klett-Summerson colorimeter (green filter). In experiments where the medium or label was changed during growth, the cells were harvested by centrifugation at 25 C, washed once in unlabeled medium lacking an essential component for growth, and suspended in new medium.

Cleared lysates were made by the procedure of Clewell and Helinski (6) with some modifications. Log-phase were harvested by centrifugation at 4 C. The cell pellet was suspended in a 25% sucrose, 0.05 M tris(hydroxymethyl)aminomethane (Tris, pH 8.0) solution, and spheroplasts were made by the addition of lysozyme and ethylenediaminetetraacetic acid (EDTA). Lysis was performed by the addition of a lytic mixture containing 10% Triton X-100, 0.05 M Tris (pH 8.0), and 0.0625 M EDTA to an equal volume of spheroplasts. This crude lysate was centrifuged at 46,000 × *g* for 30 min to remove the bulk chromosomal DNA, and the supernatant fluid (cleared lysate) was saved.

Purification of DNA-protein relaxation complex. Cleared lysates were centrifuged through 30-ml 15 to 50% sucrose gradients containing 0.05 M Tris (pH 8.0), 0.005 M EDTA, and 0.05 M NaCl for 16 h at 4 C at 22,500 rpm in a Beckman SW25.1 rotor. The fractions containing Col factor were pooled and dialyzed against TES (0.05 M Tris [pH 8.0], 0.05 M EDTA, 0.05 M NaCl). The resulting dialysate is termed "purified complex."

Extraction of Col factor DNA from lysate pellet. The pellets from the centrifugation of lysed spheroplasts were suspended in a 0.5-ml solution containing 0.05 M Tris (pH 8.0), 0.2 M EDTA, and 0.5 M NaCl, and were stored at 23 C for 16 h. Pronase (1 mg) and Sarkosyl (3% final concentration) were added, and the mixture was incubated with occasional stirring at 37 C for 4 h. At the end of this period a homogeneous, viscous solution was observed. All of the radioactivity estimated to be present in the pellet was removed by this procedure.

Sucrose density gradient centrifugation and counting of radioisotope. Cleared lysates or purified complex, either untreated, treated with 0.2% SDS at 23 C for 10 min, or heated to 65 C for 15 min, were centrifuged in 5-ml 5 to 20% sucrose gradients containing TES and 0.5 M NaCl by the methods described elsewhere (5). Dropwise fractionation of the gradient onto Whatman no. 1 filter paper and counting of radioisotope have been described previously (5). In some instances, gradients were fractionated directly into scintillation fluid containing 57% toluene, 34% Triton X-100, and 0.2% 2,5-diphenyloxazole. The percentage of relaxation for any given treatment of the plasmid DNA was determined

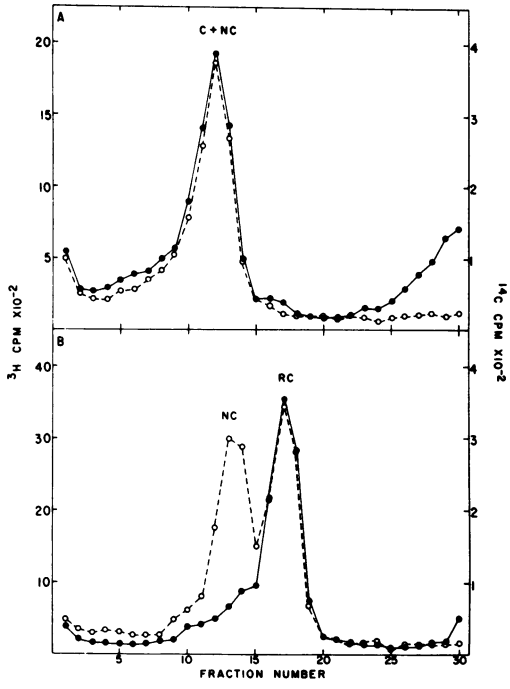

FIG. 1. *Effect of c-AMP on the stimulation of synthesis of ColE1 relaxation complex. Neutral sucrose density gradient profile of ColE1 relaxation complex in cleared lysates of CR34 (ColE1). Symbols:* ●, [^{3}H]*-thymidine-labeled ColE1 DNA from cells grown in M9-glucose and* 2×10^{-3} *M c-AMP;* ○, [^{14}C]*thymine-labeled ColE1 DNA from cells grown in M9-glucose without c-AMP. A, Lysate layered directly onto gradient; B, lysate layered onto gradient after incubation with 0.2% SDS for 10 min at 23 C. C indicates complexed ColE$_1$ DNA; NC indicates noncomplexed, supercoiled ColE1 DNA; RC indicates the relaxed form of the complexed ColE1 DNA.*

by comparing the total counts observed in the 17*s* peak (open, circular DNA) to the total counts observed in both the 17 and the 23*s* (supercoiled DNA) peaks (see Fig. 1).

Dye-bouyant density-equilibrium centrifugation. Equilibrium centrifugation in cesium chloride-ethidium bromide has been described in detail elsewhere (3).

Immunity and colicin determination. Immunity to the killing action of a colicin upon a strain was determined as described previously (10). The colicin titer was determined as the highest dilution of purified colicin which exhibited a clear zone of growth inhibition. Colicinogenicity of a particular strain was determined by overlaying the colonies with a strain sensitive to the killing action of the presumptive colicin. After 24 h of incubation, if the test strain were colicinogenic, the sensitive overlaid strain exhibited a clear area of no growth.

RESULTS

Effect of c-AMP on levels of relaxation complexes of ColE1 and ColE2. JC411 and CR34 strains carrying either the ColE1 or the ColE2 factor were grown in M9-glucose- and M9-glycerol-containing media to a density of 3×10^8 cells/ml. Each culture was then divided into two equal portions with c-AMP and [^{3}H]thymine added to one and [^{14}C]thymine added to the other. After being allowed to shake for an additional hour, the respective portions were mixed together and chilled, and the cells were harvested. Lysozyme-EDTA spheroplasts were made and lysed subsequently with Triton X-100. Cleared lysates, prepared from these cultures, were subjected to various treatments and analyzed by centrifugation in neutral sucrose gradients for both the amount of Col factor DNA and the percentage of plasmid DNA in the complexed state. In the case of CR34 (ColE1) (Table 1), a slight glucose effect upon the relative amount of ColE1 DNA in the form of relaxation complex had occurred. The effect was more striking in the JC411 host in which approximately a fourfold reduction in the level of relaxation complex was found when glucose

TABLE 1. *Effect of c-AMP on the level of ColE1 relaxation complex*

Strain	Growth conditions[a]	SDS-induced relaxation[b] (%)
CR34 (ColE1)	Glycerol	64.2
CR34 (ColE1)	Glycerol + c-AMP	88.8
CR34 (ColE1)	Glucose	48.0
CR34 (ColE1)	Glucose + c-AMP	78.7
CR34 (ColE1)	Glucose + c-AMP + CAP	32.3
JC411 (ColE1)	Glycerol	65.1
JC411 (ColE1)	Glycerol + c-AMP	71.5
JC411 (ColE1)	Glucose	17.2
JC411 (ColE1)	Glucose + c-AMP	66.5

[a] Cells were grown in M9 basal salts medium containing 50 μg/ml of essential amino acids and 0.2% of the carbon source specified. Cultures containing c-AMP were labeled with [^{3}H]thymine (100 μCi/ml). c-AMP was employed in a final concentration of 2.5×10^{-3} M and was present during the last hour of growth prior to harvesting. Cultures without c-AMP were labeled with [^{14}C]thymine (1 μCi/ml). When CAP was employed, it was added in final concentration of 100 μg/ml at the same time as the addition of c-AMP, and the culture was allowed to incubate for 60 min in its presence. At a cell density of 5×10^8 cells/ml, cultures employing the same strain and carbon source (± c-AMP) were mixed together and harvested, and cleared lysates were made as described in Materials and Methods. Cleared lysates were left untreated or treated with 0.2% SDS for 10 min at 23 C and centrifuged on 5 to 20% sucrose gradients.

[b] Determined as described in Materials and Methods.

was substituted for glycerol as the carbon source. In the case where c-AMP was added to the growth medium, an increase in the relative amount of total ColE1 DNA extracted in the complexed state was observed in both strains and in both glucose- and glycerol-containing media (Table 1 and Fig. 1). Similarly, in the case of the ColE2 factor in CR34,c-AMP was found to stimulate an increase in the relative amount of SDS-relaxable plasmid DNA isolated from glucose- or glvcerol-grown cells (Table 2). Little or no stimulation was found in the JC411 host which contained very high levels of relaxation complex. Although the increase in the relative amount of relaxation complex stimulated by c-AMP in CR34 (ColE2) appeared to be low (10–15%), the fact that c-AMP also stimulated an increase in the amount of total ColE2 DNA produced (see Table 9) indicates that the synthesis of the ColE2 relaxation complex was also stimulated substantially by the nucleotide.

Addition of CAP at the same time as the addition of c-AMP blocked the stimulation in the amount of ColE1 relaxation complex induced by the nucleotide (Table 1). The reduction in the amount of relaxation complex observed after CAP treatment is the result of the increase in total plasmid DNA since ColE1 DNA is synthesized in the presence of CAP. These data indicate that the effect of c-AMP is on the synthesis of the protein components of the relaxation complex.

Heat treatment of ColE2 DNA-protein complex results in the relaxation of a portion of complexed ColE2 DNA and an inactivation of the remainder (4). In addition to stimulating an increase in the total amount of complexed ColE2 DNA in CR34, c-AMP also stimulated an increase in the sensitivity of this complex to heat treatment (Table 2).

TABLE 2. *Effect of c-AMP on level of ColE2 relaxation complex*

Strain	Growth conditions[a]	SDS-induced relaxation[b] (%)	Heat-induced relaxation[c] (%)
CR34 (ColE2)	Glycerol	69.5	26.5
CR34 (ColE2)	Glycerol + c-AMP	80.3	45.2
CR34 (ColE2)	Glucose	62.5	17.5
CR34 (ColE2)	Glucose + c-AMP	83.5	53.0
CR34 (ColE2)	Glucose + c-AMP + CAP	41.5	—[d]
JC411 (ColE2)	Glycerol	79.7	—
JC411 (ColE2)	Glycerol + c-AMP	86.1	<2
JC411 (ColE2)	Glucose	87.4	<2

[a] Growth conditions are as described in Table 1.
[b] Determined as described in Materials and Methods.
[c] Cleared lysate of the culture was heated to 65 C for 15 min prior to analysis on sucrose gradient.
[d] Determination not made.

Synthesis of protein components of ColE1 and ColE2 relaxation complexes. The glucose effects observed upon the level of the ColE1 and ColE2 relaxation complexes suggest that the synthesis of the proteins of these complexes is under the control of the process termed catabolite repression. Since catabolite repressible operons require both c-AMP and CGA protein for their expression (17, 21), the production of relaxation complex was tested in the strains 5336, an adenylate cyclase-deficient mutant, and CA-7900, a CGA protein-deficient mutant.

Cultures of HfrH and CA7900 strains carrying either ColE1 or ColE2 were grown in M9-glucose-[^{3}H]thymine or [^{3}H]thymidine with or without c-AMP. In the case of the 5336 (ColE1) and (ColE2) strains, portions of each culture which had been growing in a tryptone-glucose-c-AMP-[^{3}H]thymidine medium were transferred to an homologous medium lacking c-AMP and allowed to grow for eight more generations. The percentages of the total Col factor DNA isolated as SDS-inducible relaxation complex were determined. It can be seen (Table 3) that c-AMP stimulated a 1.8-fold increase in the percentage of ColE1 relaxation complex in the HfrH (ColE1) strain, the parent of both CA7900 and 5336. In CA7900 (ColE1) relaxation complex was reduced to approximately 16% of the total ColE1 DNA molecules whether or not c-AMP was present during growth. Similarly, in 5336 (ColE1), eight generations after the removal of c-AMP from the growth medium, the level of relaxation complex dropped from 75% to less than 5%. Under these conditions there was no noticable change in the rate of synthesis of ColE1 DNA. The residual level of ColE1 relaxation complex in CA7900 (ColE1) and 5336 (ColE1) may be at least partially due to the observed low numbers of revertants (5–15%) in the cultures.

Stimulation of ColE2 relaxation complex by c-AMP was also found in the HfrH (ColE2) strain. In both the CA7900 (ColE2) and 5336 (ColE2) strains, 65% or greater of the ColE2 DNA molecules were in the form of the relaxation complex, and this level was not dependent upon c-AMP. These results indicate that c-AMP may stimulate, but is not required for, the synthesis of the protein components of ColE2 relaxation complex; nor is this synthesis mediated through a system involving the CGA protein.

A more thorough analysis of the effect of the

removal of c-AMP from 5336 (ColE1) on the synthesis of relaxation complex was carried out. This strain was grown in a tryptone-glucose-c-AMP medium for many generations. A portion of the culture was pulsed with [^{3}H]thymidine for 20 min (one-half doubling time). The balance of the culture was washed free of the medium and suspended in tryptone-glucose without c-AMP. Samples were removed after each succeeding generation and pulsed for 20 min with [^{3}H]thymidine. The culture was diluted after each doubling to ensure that all samples were pulsed at the same cell density. After the pulse, the samples were immediately chilled and harvested. Lysis was performed after all the samples were taken. Assays for the percentage of relaxation complex were performed on cleared lysates. It was observed (Fig. 2) that the relative amount of relaxation complex decreased as the cells were incubated for increasing periods of time without c-AMP. No significant differences in the rate of ColE1 DNA synthesis were observed in any of these samples. For the first 3.5 succeeding generations after removal of c-AMP, the percentages of relaxation complex observed is indicative of the total cessation of synthesis of the protein components of the complex and the dilution of the preexisting complex formed prior to removal of c-AMP.

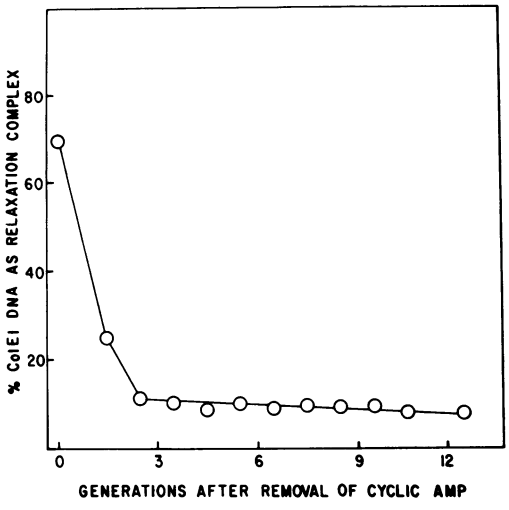

FIG. 2. *Effect of removal of c-AMP on synthesis of relaxation complex in 5336 (ColE1) cells were grown in a tryptone-glucose-c-AMP medium for 10 generations, transferred to fresh medium without c-AMP, and allowed to grow with dilution after each doubling for 12 doublings. Samples were taken every generation after removal of the c-AMP and pulsed for 30 min with [^{3}H]thymidine. Sucrose gradient analysis for the percentage of SDS-induced relaxation was performed on cleared lysates as described in Materials and Methods.*

These findings, along with those described in Table 3, indicate that synthesis of the protein components of ColE1 DNA-protein relaxation complex is mediated through the c-AMP-CGA system.

Effect of c-AMP on immunity, colicin production, and transferability of Col factors. Purified colicins E1 and E2 were tested for their killing effect upon the strains HfrH (ColE1), 5336 (ColE1), and CA7900 (ColE1). All these strains are immune to colicin E1 but sensitive to colicin E2 (Table 4). 5336 ColE1 is immune to

TABLE 3. *Level of complexed plasmid DNA in CA7900 and 5336*

Strain	c-AMP in growth medium[a]	SDS-induced relaxation[b] (%)
HfrH (ColE1)	−	29.3
HfrH (ColE1)	+	52.3
CA7900 (ColE1)	−	17.3
CA7900 (ColE1)	+	16.3
5336 (ColE1)	−	4.5
5336 (ColE1)	+	75.5
HfrH (ColE1)	−	59.1
HfrH (ColE1)	+	80.0
CA7900 (ColE2)	−	89.5
CA7900 (ColE2)	+	84.5
5336 (ColE2)	−	72.4
5336 (ColE2)	+	67.7

[a] HfrH and CA7900 strains were grown in M9-glucose plus c-AMP as described in Table 1. Strain 5336 was grown in tryptone-glucose-c-AMP (4×10^{-3} M) for 10 generations, transferred to tryptone-glucose, and allowed to grow for 8 generations with dilution.

[b] Determined by sucrose gradient analysis as described in Materials and Methods.

TABLE 4. *Immunity of CA7900 (ColE1) and 5336 (ColE1) to colicin E1*

Strain	Additions[a]	Titer[b]	
		Colicin E1	Colicin E2
HfrH	None	>1,600	>1,600
HfrH (ColE1)	None	<2	>1,600
CA7900	None	>1,600	>1,600
CA7900 (ColE1, R64)	None	<2	>1,600
5336	None	>1,600	>1,600
5336	c-AMP	>1,600	>1,600
5336 (ColE1, R64)	None	<2	>1,600
5336 (ColE1, R64)	c-AMP	<2	>1,600

[a] Strains were allowed to grow as a lawn on AB-3 plus c-AMP (4×10^{-3} M) where noted. Purified colicin E1 and E2 were diluted serially from stocks (1 mg/ml) and spotted onto the bacterial lawns.

[b] Highest dilution of colicin that cleared the bacterial lawn after 36 h of incubation at 37 C.

colicin E1 in the absence of c-AMP. These data suggest that the c-AMP-CGA system does not exert control over the immunity characteristic of ColE1-containing strains.

The method of constructing CA7900 (ColE1) or (ColE2) and 5336 (ColE1) or (ColE2) made use of the mechanism of R64-promoted transfer of the ColE1 and ColE2 factor. Exconjugants from the cross of the appropriate donor and recipient strains were screened for colicinogenicity by overlaying with a colicin-sensitive strain. Under these conditions, a colicinogenic strain synthesized a sufficient amount to produce a detectable clearing in the lawn of the overlaid test strain. Not a single exconjugant of the crosses JC411(ColE1, R64) × CA7900 or 5336, plated without c-AMP, could be demonstrated to be colicinogenic for ColE1 by the overlay assay (Table 5). Yet 10 independent CA7900 (R64) exconjugants were found to transfer the ColE1 factor in a subsequent cross. In the case of the exconjugants of JC411(ColE1, R64) × 5336 cross, colicinogenicity, by use of the colicin production assay, could be detected upon addition of c-AMP to the medium. Mitomycin C-induced synthesis of colicin E1 was also tested in both 5336 (ColE1) and CA7900 (ColE1) strain. It was found that the latter did not produce colicin, whereas the former required c-AMP for induced synthesis. These findings indicate that the c-AMP-CGA system is required for the spontaneous and induced production of colicin E1.

In the case of ColE2 (Table 5), it was found that the 5336 (ColE2) strain produced a detectable amount of colicin in the absence of c-AMP. Similarly CA7900 (ColE2) also produced detectable amounts of colicin upon prolonged incubation. This finding suggests that the c-AMP-CGA system does not control the spontaneous production of colicin E2. c-AMP-induced stimulation of ColE2 synthesis was not examined.

TABLE 5. *Transfer of Col factors to CA7900 and 5336*[a]

Recipient	Additions	Donor JC411 (ColE1, R64)		Donor JC411 (ColE2, R64)	
		Exconjugant ColE1$^+$ (%)	Exconjugant R64$^+$ (%)	Exconjugant ColE2$^+$ (%)	Exconjugant R64$^+$ (%)
HfrH	None	32	94	70	96
CA7900	None	0	16	24[b]	12
5336	None	0	10	52	45
5336	c-AMP	8	46	72	40

[a] Donors and recipients were crossed as described in Materials and Methods and plated on M9-glucose. Where c-AMP is noted, it was used in 3×10^{-3} M concentration during the mating and all subsequent platings. Colicinogenicity and the R64$^+$ characteristic of exconjugants were tested as described in Materials and Methods.

[b] Small zones of inhibition of overlaid strain were observed after 48 h of incubation.

To test whether c-AMP affects the conjugal transfer of ColE1, cultures of 5336 (ColE1, R64) grown with or without c-AMP, were crossed with C600 Thy$^-$, Az-r, and the isolated exconjugants were tested for colicinogenicity by the overlay technique. It was found that approximately 40% of the exconjugants of the crosses performed in the presence or absence of the nucleotide were colicinogenic for ColE1, indicating that c-AMP neither stimulates nor is required for R64 promoted transfer of ColE1 DNA.

Level of protein components of ColE1 relaxation complex synthesized in the presence of c-AMP. Since a direct assay for the protein components of the ColE1 relaxation complex is not as yet available, a direct examination of the cell for the amount of these proteins cannot be made. However, since ColE1 DNA can replicate in the presence of CAP, and this agent has been shown to block the synthesis of the relaxation complex (Table 1), it was possible to determine whether an amount of protein of the complex in excess of that required to fully saturate the ColE1 DNA molecules could be stimulated by c-AMP. CR34 (ColE1) was grown in M9-glucose medium employing [^{14}C]thymine for many generations. The cells were then washed free of the label and suspended into two portions of homologous media. Only one portion received c-AMP. After 75 min, CAP and [^{3}H]thymine were added to both portions. Samples of each culture were taken every hour for 5 h after the addition of CAP, and the percentage ^{3}H-labeled ColE1 DNA in the form of relaxation complex (ColE1 DNA synthesized in the presence of CAP) was determined as described previously. It was observed (Fig. 3), in the culture grown without c-AMP prior to CAP treatment, that 1 h after the addition of CAP, 25% of the ColE1 DNA synthesized was complexed. In succeeding samples the percentage of complex was rapidly diluted by the production of noncomplexed ColE1 DNA. In addition, after 1 h, the percentage of relaxation complex in the preexisting ColE1 DNA (^{14}C-labeled ColE1 DNA) also decreased by a factor of two, presumably due to the exchange in vivo of relaxation proteins between newly synthesized and unreplicated DNA. Since after the 1 h of growth in the

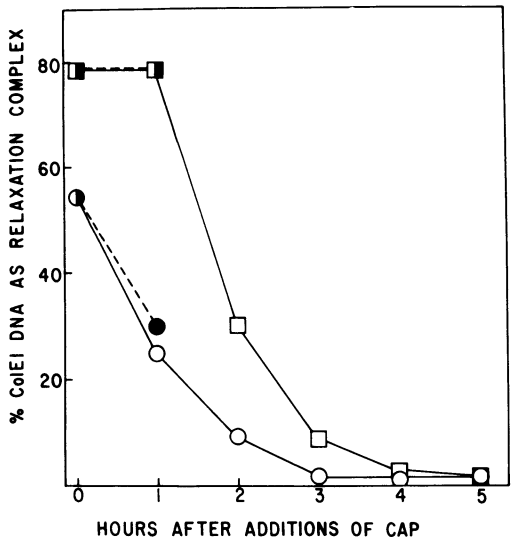

FIG. 3. *Overproduction of relaxation proteins in the presence of c-AMP. A culture of CR34 (ColE1) was uniformly labeled in M9-glucose with [^{14}C]thymine for 12 generations to a density of 3 × 10^8 cells/ml. The cells were washed free of the label and suspended into two portions of homologous medium, one containing 2 × 10^{-3} M c-AMP and the other containing no c-AMP. After 25 min, CAP (150 μg/ml) and [^3H]thymine were added to both cultures, and CAP (150 μg/ml), [^3H]thymine, and c-AMP were added to the one which did not receive CAP previously. Percentages of ColE1 DNA as relaxation complex were determined from cleared lysates taken at times indicated as described in Materials and Methods. Symbols: (●) ^{14}C-labeled ColE1 DNA and (○) ^3H-labeled ColE1 DNA from cells incubated in the absence of c-AMP prior to the addition of [^3H]thymine; (□) ^3H-labeled and (■) ^{14}C-labeled ColE1 DNA from cells incubated in presence of c-AMP prior to addition of [^3H]thymine.*

presence of CAP, approximately a 2.5-fold increase in the number of ColE1 DNA molecules per cell had occurred, these data indicate that cells grown in M9-glucose in the absence of c-AMP possess a level of relaxation proteins that is sufficient to bind to only one-half of the ColE1 DNA molecules. On the other hand, when the cells were grown in the presence of c-AMP prior to CAP treatment, approximately 80% of the ColE1 DNA synthesized in the first hour in the presence of CAP was found to be in the complexed state (Fig. 3). No loss of relaxation complex among preexisting molecules had occurred. After the first hour, the proportion of ColE1 DNA in the form of relaxation complex was diluted by noncomplexed ColE1 DNA being synthesized in the presence of CAP. These results indicate that when the cells are grown in the presence of c-AMP, relaxation proteins are synthesized in an amount sufficient to saturate 80% of the ColE1 DNA present in the cell as well as a threefold excess of noncomplexed ColE1 DNA produced in the presence of CAP. Since the addition of c-AMP also resulted in a 2.5-fold stimulation of ColE1 DNA synthesis during the pretreatment period, approximately a 6- to 10-fold increase in the amount of relaxation proteins alone had resulted from a 75-min incubation period with the nucleotide.

Independent synthesis of ColE1 DNA and relaxation proteins. The findings of Clewell and Helinski (7), as well as those reported in the preceeding experiment, indicated that ColE1 DNA can be synthesized in the absence of synthesis of the ColE1 relaxation proteins. The following experiment indicates that the relaxation protein can be synthesized in the absence of ColE1 DNA synthesis. CR34 (ColE1) was grown to a density of 5 × 10^8 cells/ml at 23 C in M9-glycerol containing [^3H]thymine. The cells were then washed free of thymine, suspended in M9-glycerol, and incubated under the following conditions: (i) 23 C, [^3H]thymine; (ii) 43 C, no thymine; (iii) 43 C, no thymine; (iv) 43 C, no thymine, CAP. After 90 min of incubation, the cells were harvested and the level of relaxation complex was determined. It can be seen (Table 6) that at 23 C the cells contained very little ColE1 DNA in the form of relaxation complex. When the cells were shifted to 43 C in the presence or the absence of thymine, approximately 50% of the DNA was found to be complexed. In the latter case, complex protein had bound to preexisting nonreplicating DNA. The finding that CAP blocks the conversion of nonreplicating ColE1 DNA into the complexed state (Table 6) indicates that the conversion which took place under conditions where the ColE1 DNA could not replicate was dependent upon protein synthesis and suggests that the relaxation proteins

TABLE 6. *Effect of thymine starvation on relaxability of ColE1 DNA*

Incubation conditions[a]	ColE1 DNA as relaxation complex[b] (%)
(i) 23 C, thymine	5.4
(ii) 43 C, thymine	49.3
(iii) 43 C, no thymine	50.0
(iv) 43 C, no thymine, CAP	<2.0

[a] CR34 (ColE1) cells were pregrown in M9-glycerol at 23 C and transferred to M9-glycerol with or without thymine at the temperature noted and incubated for 90 min.

[b] Determinations made on cleared lysates as described in Materials and Methods.

can be synthesized in the absence of replication of ColE1 DNA.

Rate of complex formation of newly synthesized ColE1 DNA. Although it has been demonstrated that the DNA and protein components of the ColE1 relaxation complex can be synthesized independently of each other, it has not been established whether, in the case of normal ColE1 replication, the relaxation complex is synthesized as a unit. A culture of CR34 (ColE1) was therefore uniformly labeled with [^{14}C]thymine, washed free of the label, and suspended in medium containing either thymine or thymine plus c-AMP. The cultures were allowed to incubate for 60 min, after which time each received a pulse of [^{3}H]thymidine for 1 min followed by a chase with a 1,000-fold molar excess of unlabeled thymidine for a period of 10 min. The cultures incubated in the absence of c-AMP received the nucleotide at the time of the pulse. Samples were taken 1 min before the pulse, at the end of the pulse, and after 2, 5, and 10 min of the chase. The samples were assayed for the level of relaxation complex. It can be seen (Fig. 4A) that, in the case of the culture preincubated in the absence of c-AMP, the percentage of complex among the prelabeled molecules was approximately 50% prior to the pulse and through the pulse and the chase. Of the ColE1 DNA molecules synthesized in the pulse, very few were complexed (less than 5%). During the periods of the chase an increasing proportion of ColE1 DNA molecules synthesized in the pulse became complexed. After 10 min approximately 30% of the molecules synthesized in the pulse were found complexed. Since there is not a sufficient amount of relaxation proteins to saturate the ColE1 DNA under these conditions and since new, unlabeled ColE1 DNA molecules were synthesized during the chase period, it is perhaps not surprising that the level of complex among the pulsed molecules did not approach that for the preexisting DNA in a period of 10 min.

In the case in which the cells were preincubated in the presence of c-AMP, it was found (Fig. 4B) that approximately 80% of the prelabeled ColE1 DNA molecules were complexed. Despite the fact that enough relaxation proteins were present to saturate at least twice as much ColE1 DNA than the cells carried, only 15% of the ColE1 DNA molecules synthesized in the 1-min pulse period were found to be complexed. After 10 min of the chase, this proportion increased to approximately 50%. In the case in which the cells were preincubated in the presence of c-AMP and in the absence of thymine (not shown), a condition which results in the

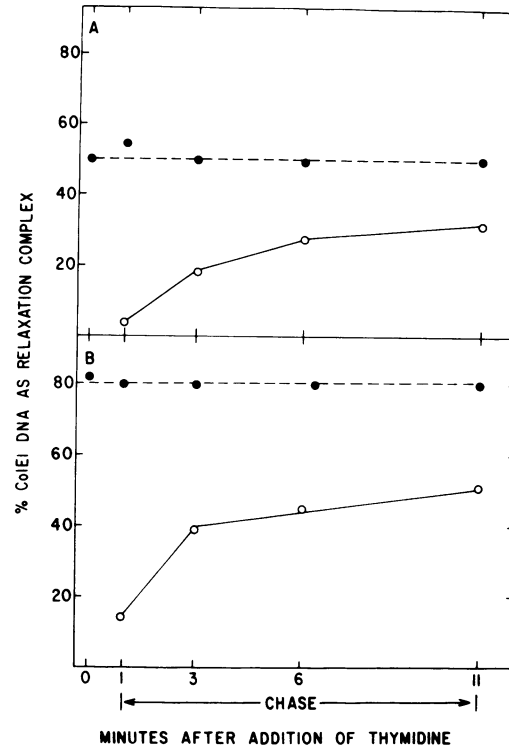

FIG. 4. *Kinetics of production of ColE1 relaxation complex. CR34 (ColE1) was uniformly labeled with [^{14}C]thymine for 13 generations to 5 × 10^{8} cells/ml, washed free of the label, and suspended into homologous medium containing unlabeled thymine and either no c-AMP or 2 × 10^{3} M c-AMP. After 60 min, [^{3}H]thymidine was added to both cultures, followed 1 min later by a 1,000-fold molar excess of cold thymidine. Percentage of ColE1 DNA as relaxation complex was determined in samples taken prior to the pulse, after the pulse, and during the chase period as described in Materials and Methods for both* (○) *^{14}C-labeled ColE1 DNA and* (●) *^{3}H-labeled ColE1 DNA. A, Cells grown without c-AMP; B, cells grown in presence of c-AMP.*

synthesis of greater than eightfold more relaxation protein than a corresponding amount of ColE1 DNA, only 15% of the molecules appearing in the pulse were found to be complexed but became fully complexed (80%) after 10 min of the chase. These findings suggest that the ColE1 relaxation complex is not synthesized as a unit but is formed shortly after synthesis of supercoiled ColE1 DNA.

Heat-induced relaxation of ColE2 complex. Blair et al. (4) have demonstrated that of the ColE2 molecules extracted from cells grown in the absence of c-AMP, approximately 25 to 33% of the molecules which can undergo relaxation in the presence of SDS are capable of undergo-

ing relaxation upon heat treatment. Furthermore, the balance of those molecules not induced to relax upon heat treatment are no longer capable of undergoing SDS-induced relaxation. These findings led Blair et al. (4) to suggest that the heat treatment of CR34 (ColE2) complex results in the inactivation of the relaxation proteins. We have shown (Table 2) that the addition of c-AMP to CR34 (ColE2) cells results in a substantial increase in the sensitivitiy of relaxation complex of ColE2 DNA and protein to heat-induced relaxation. To demonstrate the heat-relaxable ColE2 DNA-protein complex, the ColE2 DNA was extracted from CR34 (ColE2) and JC411 (ColE2) cells grown in either the presence or absence of c-AMP and purified on neutral sucrose gradients (see Materials and Methods). The purified complex was analyzed subsequently for the degree of relaxation in response to heat or SDS treatment. It was found (Table 7) that purified ColE2 complex extracted from CR34 (ColE2) grown without c-AMP was less than 10% as relaxable by heat treatment as by SDS treatment. Purified complex from the c-AMP culture was 72% as heat relaxable as SDS relaxable. ColE2 complex from JC411 (ColE2) was not found to be heat relaxable even when extracted from cells grown in the presence of c-AMP. These findings indicate that c-AMP stimulates the production of a heat-inducible ColE2 relaxation complex in CR34 (ColE2).

Heat-relaxable complex and ColE2 DNA replication. For examination of production of heat-relaxable ColE2 complex during plasmid replication, a culture of CR34 (ColE2) was uniformly labeled with [^{14}C]thymine in M9-glucose medium to a density of 3×10^8 cells/ml. The cells were washed free of the medium and suspended into two portions of homologous medium, each containing [^{3}H]thymine but only one containing c-AMP. The cultures were allowed to grow for 1 h in [^{3}H]thymine. Samples were taken prior to the label change and 5 min and 60 min after the label change. SDS-induced and heat-induced relaxation of ColE2 DNA were examined in the cleared lysates prepared from these samples. It was found (Table 8) in the case in which just the label was changed, in all the samples taken, that the amount of heat-relaxable complex never was greater than 17% of the total ColE2 molecules extracted. As was found in the case of ColE1 DNA, newly synthesized ColE2 DNA (5-min ^{3}H-labeled sample) was observed to be less SDS relaxable than preexisting unreplicated molecules. After 60 min the SDS-induced relaxability was more evenly distributed among the ^{3}H- and the ^{14}C-labeled molecules. In the case where c-AMP was added at the label change, in the 5-min sample newly replicated ColE2 DNA was found to be approximately 72% as heat-relaxable as SDS-relaxable. The ColE2 ^{14}C-DNA, representing both unreplicated molecules and newly replicated molecules was only 33% as heat relaxable as SDS relaxable. In the 60-min sample, the distribution of heat relaxability is still greatly in favor of the newly replicated (^{3}H) molecules. These data suggest that preexisting, non-heat-relaxable ColE2 complex cannot become heat relaxable in

TABLE 7. *Heat relaxability of complexed ColE2*

Strain	Growth medium[a]	Induced relaxation[b] (%)	
		SDS	Heat
CR34 (ColE2)	Glucose	50.0	4.4
CR34 (ColE2)	Glucose + c-AMP	72.7	51.7
JC411 (ColE2)	Glucose	75.5	7.8
JC411 (ColE2)	Glucose + c-AMP	73.9	8.7

[a] Cells were grown as described in Table 1. ColE2 DNA was extracted and purified as described in Materials and Methods.

[b] Relaxation tested as described in Tables 1 and 2.

TABLE 8. *Synthesis of relaxation complex in CR34 (ColE2)*

Sample[a] time (min)	c-AMP added	Treatment[b]	Induced relaxation[c] (%)	
			^{14}C	^{3}H
0	No	SDS	55.7	
		Heat	17.2	
15	No	SDS	66.6	33.6
		Heat	6.2	4.8
60	No	SDS	65.8	56.0
		Heat	15.7	9.7
15	Yes	SDS	77.1	67.7
		Heat	25.9	50.3
60	Yes	SDS	83.4	78.3
		Heat	29.4	53.8

[a] Cells were grown in M9-glucose plus [^{14}C]thymine and transferred to M9-glucose plus [^{3}H]thymine with and without c-AMP. Samples were taken at 0, 15, and 60 min after the label shift.

[b] Treatments of cleared lysates were carried out as described in Tables 1 and 2.

[c] Determinations made as described in Tables 1 and 2.

the presence of c-AMP by a simple addition of a c-AMP-stimulated component. Rather, it appears, as in the case of ColE1 relaxation complex, that the DNA is synthesized as a supercoiled molecule to which is added either SDS-inducible or heat-inducible relaxation proteins, or both, synthesized in the presence of the nucleotide.

Effect of c-AMP upon the synthesis of ColE1 and ColE2 DNA. It has been indicated above that c-AMP, in addition to inducing a stimulation of the production of relaxation proteins, also caused an increase in the relative amount of ColE1 DNA and (in one case) ColE2 DNA extracted from the cell. These results are summarized in Table 9. Increases of 1.8- and 2.5-fold were found in the proportions of ColE1 DNA extracted from CR34 (ColE1) and JC411 (ColE1) strains, respectively, grown in the M9-glucose medium. Slight stimulations by c-AMP in these strains were also found to occur in the glycerol medium. The HfrH (ColE1) strain, and its derivatives, CA-7900 and 5336, did not exhibit a stimulation in the relative amount of ColE1 DNA upon the addition of c-AMP. In the case of ColE2 DNA synthesis, the only instance in which a stimulation by c-AMP took place was in the CR34 (ColE2) strain.

To demonstrate that the effect of c-AMP in the CR34 (ColE1) strain is a stimulation of synthesis of Col factor and not an enhancement of release of plasmid into the cytoplasm from sites in the cell to which the DNA molecules are bound, the following experiment was carried out. A CR34 (ColE1) culture, growing in M9-glucose, was divided into two portions. [^{3}H]Thymine and c-AMP were added to one portion and [^{14}C]thymine to the other portion. After 20 min of incubation, the cultures were chilled, mixed together, and harvested. Lysozyme-EDTA spheroplasts of the mixture were lysed with Triton X-100. The lysate was centrifuged at 20,000 rpm for 20 min, the supernatant fluid (cleared lysate) and the pellet were saved. The pellet was subsequently subjected to extensive treatment with SDS and Pronase (see Materials and Methods), and the solubilized pellet material was centrifuged to equilibrium in a cesium chloride-ethidium bromide density gradient (Fig. 5). A single peak, consisting mainly of chromosomal DNA was found for both the ^{3}H (c-AMP) and ^{14}C-(no c-AMP) labels. Various fractions of the gradient were pooled as described in Fig. 5. The pools were dialyzed and subjected to sucrose gradient analysis for the appearance of either 23*s* (supercoiled) or 17*s* (open, circular) ColE1 DNA. The cleared lysates were also analyzed in a similar manner (Table 10). Of the total Col factor DNA isolated in the case of c-AMP-grown cells, approximately 70% of the DNA was found in the cleared lysate. In the case of the culture grown without the nucleotide, an even greater proportion, 85%, of the ColE1 DNA was recovered in the cleared lysate. Summing the amount of radioactivity of Col factor and chromosomal DNA, a twofold enrichment was found to have occurred in the case in which c-AMP was added. These data indicate that the effect of c-AMP is not a facilitation of release of ColE1 DNA during the extraction process from sites in the cell to which the plasmid is bound.

TABLE 9. *Percentage of total DNA as Col factor DNA*[a]

Strain	Glycerol	Glycerol + c-AMP	Glucose	Glucose + c-AMP
CR34 (ColE1)	1.04	1.20	0.98	2.77
JC411 (ColE1)	2.08	2.41	1.41	2.55
HfrH (ColE1)			0.85	0.83
CA7900 (ColE1)			0.46	0.52
5336 (ColE1)			0.76	0.82
CR34 (ColE2)	1.82	2.16	1.66	2.94
JC411 (ColE2)	1.30	1.44	0.79	0.89
HfrH (ColE2)			1.14	1.39
CA7900 (ColE2)			0.79	0.87
5336 (ColE2)			0.85	0.81

[a] Cultures employed in this experiment are identical to the cultures described in Tables 1, 2, and 3. The percentage of Col factor DNA was determined as the percentage of counts of Col factor in cleared lysates of the total counts of DNA in the lysed spheroplasts.

To determine the kinetics of stimulation of ColE1 DNA synthesis by c-AMP, a culture of CR34 (ColE1) was uniformly labeled with [^{14}C]thymine. After 30 min of incubation, the culture was split into two portions, and c-AMP was added to one portion. Samples were taken at 0, 15, and 30 min after the addition of c-AMP and analyzed for the amount of total cellular DNA and ColE1 DNA appearing in cleared lysates. It was seen (Fig. 6) that c-AMP does not affect the rate of synthesis of total cellular DNA (95–98% chromosomal DNA). In the case of ColE1 DNA, better than a twofold increase in the rate of synthesis had occurred for the period of 30 min after the addition of the nucleotide. It appeared that the stimulation in rate took place immediately upon the addition of c-AMP. After 30 min approximately 3% of the newly synthesized DNA was ColE1 DNA in the case in which the nucleotide was added, as compared to 1% in the case of its absence (insert to Fig. 6).

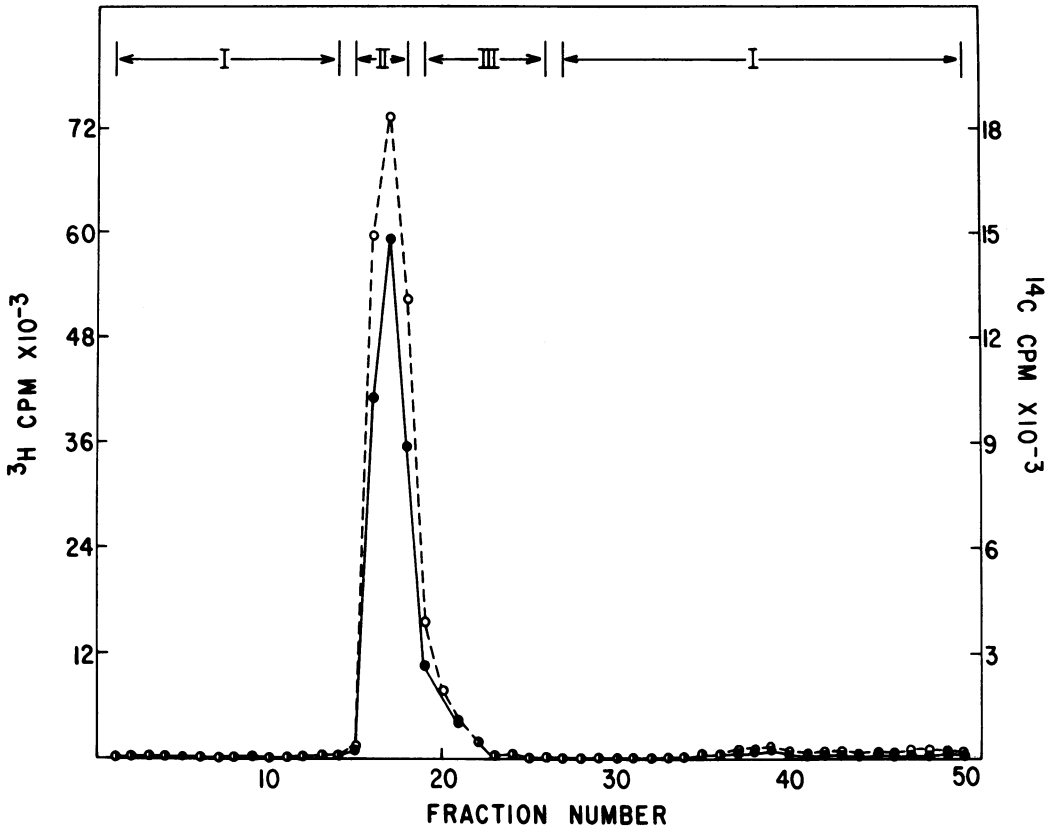

FIG. 5. *Dye-buoyant density gradient analysis of ColE1 and chromosomal DNA. The pellet after lysis of a suspension of cells consisting of a mixture of CR34 (ColE1) grown in the presence and absence of c-AMP (2×10^{-3} M) was resuspended, treated with SDS and Pronase, and subjected to cesium chloride-ethidium bromide equilibrium centrifugation as described in Materials and Methods. After fractionation of the gradient, the fractions were pooled as shown. Symbols:* (○) *^{3}H-thymine labeled DNA from cells grown in the presence of c-AMP;* (●) *[^{14}C]thymine-labeled DNA from cells grown in the absence of c-AMP.*

TABLE 10. *Effect of c-AMP on total cellular levels of ColE1 DNA*[a]

Preparation[b]	^{3}H Counts per minute		^{14}C Counts per minute	
	Col factor	Chromosome	Col factor	Chromosome
Lysed spheroplasts	4,282,394		2,646,673	
Cleared lysate	69,692	194,239	18,228	93,149
Resuspended pellet[c]				
Pool I	833	35,608	989	20,160
Pool II	27,134	3,848,243	1,716	1,748,061
Pool III	2,508	162,125	395	142,441
Total	100,167	4,240,215	21,3282	2,003,811
Col/chromosome	2.39%		1.06%	

[a] CR34 (ColE1) growing in M9-glucose was split into two portions. [^{3}H]Thymine plus c-AMP was added to one portion, and [^{14}C]thymine was added to the other. After 20 min, growth was stopped, the fractions were mixed together, and the cells were harvested.

[b] The lysed spheroplasts, cleared lysate, and resuspended pellet were prepared as described in Materials and Methods.

[c] The various pools are described in Fig. 5.

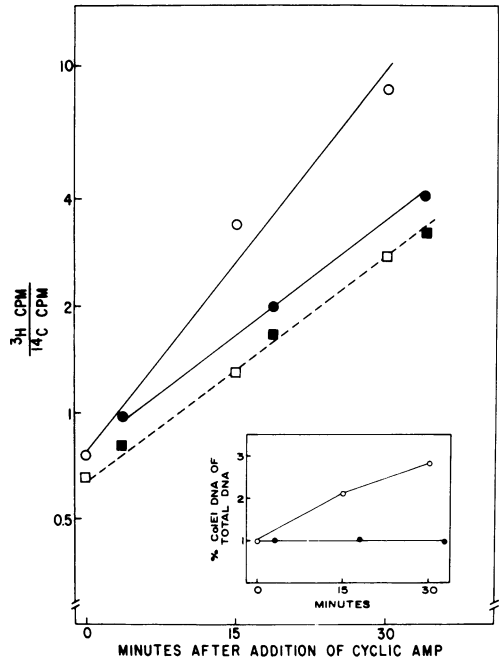

FIG. 6. *Kinetics of stimulation of ColE1 DNA synthesis by c-AMP. CR34 (ColE1) was uniformly labeled with [^{14}C]thymine, washed free of the isotope, and suspended into homologous medium containing [^3H]thymine. After 30 min the culture was divided into two portions, and c-AMP (2×10^{-3} M) was added to one portion. Samples taken at the times indicated were assayed for the amount of both ColE1 DNA and total cellular DNA as described in Materials and Methods. $^3H/^{14}C$ ratio of (○) ColE1 DNA and (□) total cellular DNA from cells grown with c-AMP; of (●) ColE1 DNA and (■) total cellular DNA from cells grown without c-AMP. The insert shows the percentage of total [^3H]thymine-labeled DNA as ColE1 DNA at times indicated. Symbols: (○) culture grown in presence of c-AMP; (●) culture grown in absence of c-AMP.*

DISCUSSION

Dependency on c-AMP and the CGA protein have been employed as criteria to establish the process termed "catabolite repression" as the mechanism which controls the synthesis of the bacterial enzymes involved in the metabolism of sugars such as lactose, galactose, L-arabinose, and mannitol (11). Furthermore, in most of these systems, exogenous c-AMP has been observed to reverse the partial catabolite repression which is caused by glucose (13, 17). Both dependency upon c-AMP and CGA protein for synthesis and a stimulation by exogenous c-AMP of the synthesis of the ColE1 relaxation complex were observed. These findings indicate that the "glucose effect" upon production of the ColE1 relaxation complex, first described by Clewell and Helinski (7) and also reported in this study, is exerted through catabolite repression.

Since the ColE1 relaxation complex is not synthesized as a unit but is formed upon assembly of supercoiled ColEl DNA and relaxation proteins (Fig. 4), it was possible to ask which of the following processes is dependent upon the c-AMP-CGA system: synthesis of the ColE1 DNA, synthesis of the relaxation proteins, or assembly of the DNA and proteins. If a c-AMP-CGA system were required for ColE1 DNA replication, maintenance of the ColE1 factor in both the 5336 (in the absence of exogenous cyclic AMP) and in CA7900 strains should not have occurred. It was found that these strains could, in fact, maintain ColE1 and that the replication of the plasmid in the 5336 host was not dependent upon exogenous c-AMP. It should be noted, however, that the CA7900 and 5336 mutant strains may be sufficiently leaky to allow synthesis of enough of a c-AMP-CGA-dependent protein required for ColE1 DNA replication. On the other hand, the observation of the loss of production of relaxation complex upon removal of c-AMP from 5336 ColE1 indicates that synthesis of the relaxation protein is dependent upon c-AMP. The low levels of relaxation complex in the CGA-deficient strain, CA7900 ColE1, further indicates that the effect of c-AMP is mediated through the CGA protein.

Without a direct method of examining the cells for relaxation proteins it is not possible to distinguish whether it is the synthesis of the relaxation proteins themselves or the synthesis of a factor required for the association of the relaxation proteins and the DNA which is dependent upon the c-AMP-CGA system. Though the latter case cannot be ruled out, it is considered less likely since it has been demonstrated that the relaxation proteins can dissociate from and reassociate to ColE1 DNA in cells in which protein synthesis has been arrested by CAP treatment (7). Furthermore, Lovett and Helinski (unpublished results) have demonstrated an enrichment of radioactive labeling of relaxation proteins in the presence of exogenous c-AMP.

With the criteria described above, uninduced synthesis of colicin E1 was also shown to be dependent upon the c-AMP-CGA system. Thus, a similar control process is involved in the synthesis of at least two plasmid-related proteins, relaxation proteins and colicin E1. Whether their structural genes comprise an operon remains to be determined.

Nakazawa and Tamada (15) have recently demonstrated that c-AMP stimulates the mi-

tomycin C-induced synthesis of colicin E1. These findings are corroborated in this study. Since induction of colicin E1 synthesis requires the stimulation of ColE1 DNA synthesis (8), they have proposed that the augmented level of induced colicin E1 production in the presence of c-AMP is the consequence of gene amplification resulting from the effect of the nucleotide.

Although stimulation of ColE1 DNA synthesis by c-AMP was found (Table 9, Fig. 6), the discovery that colicin E1 production is mediated through the c-AMP-CGA system (Table 5) suggests that the increased production of colicin E1 in the presence of c-AMP is due to increased transcription of the colicin genes rather than simply gene amplification.

In contrast to ColE1, no dependency upon the c-AMP-CGA system for synthesis of either the ColE2 relaxation complex itself, the relaxation proteins, or colicin E2 was found. A stimulation by c-AMP of the production of ColE2 complex was found in only one (CR34 (ColE2)) of the five strains tested. In this case the stimulation was observed to be as great as that observed in the case of CR34 (ColE1). Without demonstrating a dependency of synthesis upon the c-AMP-CGA system, the mechanism through which the stimulation takes place cannot be determined.

Production of a heat-relaxable CR34 (ColE2) complex also was found to be stimulated by c-AMP. It does not appear that this change in the properties of the complex is due to an increase in the numbers of ColE2-complexed molecules each with low sensitivity to heat. As in the case of the ColE1 complex, the ColE2 complex is formed upon the association of relaxation proteins and supercoiled ColE2 DNA (Table 8). In the absence of added c-AMP, very few of the relaxation proteins in association with the DNA are heat activatable; rather, heating tends to destroy the capability of inducing a single-stranded lesion in the DNA upon treatment with relaxation-inducing agents such as SDS or Pronase (4). In the presence of c-AMP, it was found that replicated ColE2 DNA molecules became complexed with protein capable of inducing relaxation by either SDS and heat treatment at a rate greater than plasmid molecules in the culture which had not undergone the replication event. It is postulated, therefore, that the component which renders heat relaxability is synthesized in the presence of c-AMP and is associated with the other protein components of the complex prior to association with the ColE2 DNA.

The observed stimulations of both ColE1 and ColE2 DNA synthesis in the presence of c-AMP was an unexpected finding. A stimulation in the rate of replication of an extrachromosomal DNA element in bacteria by c-AMP has not been reported previously. Approximately a two-fold increase in the rate of replication of ColE1 DNA was observed (Fig. 6). Further experiments have indicated that the stimulation is transient in that, 30 min after addition of nucleotide, the rate of synthesis declines sharply (Katz and Helinski, in preparation). Experiments are in progress to determine the nature of the burst of ColE1 replication induced by c-AMP.

Whatever the nature of the effect, however, it is clear that the stimulation is on the replication of the ColE1 factor itself (Fig. 6). Therefore, the apparent stimulation of the proportion of Col factor DNA by c-AMP, shown in Table 9, is not due to a reduction in the amount of chromosomal DNA per cell. Such an effect has been described by Clewell and Helinski (7) as the basis for the apparent increase in the percentage of ColE1 DNA in the total DNA when cells are shifted from media containing glucose to media containing glycerol. Upon such medium shifts, 1.5 and 1.9-fold increases in the percentage of Col factor DNA in the total DNA in JC411 (ColE1) and JC411 (ColE2) respectively, were found (Table 9). It has been shown previously (7) that after the medium shift, the cells contain less chromosomal DNA with no change in content of ColE1 DNA. In the present study the addition of c-AMP did not change the rate of cellular DNA synthesis (Fig. 6), the cell mass, or cell numbers (unpublished results). Thus, the increase in the rate of Col factor DNA synthesis is reflected by an increased number of Col factor DNA molecules per cell during the stimulation period.

The amount of ColE1 relaxation complex present in the cell, therefore, is dependent upon the relative rates of synthesis of its two major components which appear to be under different types of control. The complex is not synthesized de novo as a unit but is formed by association between a supercoiled DNA and the relaxation proteins. Formation of the complex appears to depend solely upon the availability of the relaxation proteins and not upon ColE1 DNA replication. Under normal conditions of cell growth in glucose medium, the rate of synthesis of relaxation proteins, relative to the rate of synthesis of ColE1 DNA, is sufficient to produce enough molecules to saturate approximately 50% of the DNA. If synthesis of the protein is blocked, by either addition of CAP or by growth at low temperature, the ColE1 DNA synthesized will be found as supercoiled DNA unassociated with relaxation proteins. If, after the

growth at low temperature, synthesis of the protein components is allowed to resume without the further synthesis of ColE1 DNA, eventually all the preexisting supercoiled DNA will become complexed.

Although the amount of ColE1 relaxation complex is dependent upon the relative rate of production of relaxation proteins, the timing of formation of the complex after the ColE1 DNA synthesis must be due to other factors. When grown under the conditions in which the cells contained an excess of relaxation proteins, very few of the supercoiled ColE1 DNA synthesized within 1 min were found to be complexed, but became complexed upon further incubation of the cells (Fig. 5). The complexing event has been found to be independent of protein synthesis (Fig. 3). Recent findings have indicated that the supercoiled ColE1 DNA formed upon replication differ in some properties from supercoiled DNA extracted from the cell after long incubation. It is possible that the newly replicated ColE1 supercoiled DNA is modified prior to association with the relaxation proteins and ultimate formation of the relaxation complex. This hypothesis helps to explain the findings that no more than 85% of the ColE1 DNA molecules can be isolated in the complexed state when extracted from cells growing under conditions in which an excess of relaxation protein is present. If one assumes a 3- to 5-min period for possible modification of a newly synthesized Col factor and subsequent association of the DNA molecules with the relaxation proteins, and a continuous replication period of the plasmid DNA throughout the cell cycle, it can be calculated that 15% of the total Col factor per cell will be unavailable for complex formation at any given time even under growth conditions which permit a high rate of synthesis of the relaxation proteins.

An in vivo function of the relaxation complexes has not yet been established. It has been proposed that, in response to a signal, the relaxation complex is activated and a nick or gap appears in the DNA as a required step in the replication of the plasmid (5, 7). The findings presented here do not either support or entirely rule out this model. If this model is correct, since the rate of replication of ColE1 DNA was not found to be reduced in the 5336 (ColE1) strain when grown under conditions in which little relaxation complex was present, it appears that very few copies of the relaxation proteins are required to initiate ColE1 DNA replication.

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