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Effect of Inhibitors of Ribonucleic Acid and Protein Synthesis on the Cyclic Adenosine Monophosphate Stimulation of Plasmid ColE1 Replication

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Addition of cyclic adenosine 3'-5'-monophosphate (c-AMP) to growing *Escherichia coli* cells, colicinogenic for the plasmid ColE1, results in a fourfold stimulation in the rate of synthesis of the plasmid deoxyribonucleic acid (DNA). The stimulation is transient (30 min) and is succeeded by a brief period (30 min) of cessation of plasmid DNA replication. The stimulation of ColE1 DNA replication also occurs in chloramphenicol-treated cells. Rifampin inhibits ColE1 DNA replication in the presence or absence of c-AMP. Employing thymine starvation conditions to stop ColE1 DNA synthesis, it was found that c-AMP, added during the period of thymine starvation, effected a stimulation in the amount of subsequent replication which took place when replicating conditions were restored. The stimulatory effect of c-AMP under these conditions was not prevented by chloramphenicol but was completely eliminated when rifampin was present. Under these conditions, when rifampin was added after the effect of c-AMP was allowed to occur, subsequent replication of the plasmid could take place, but only one round of replication occurred. A model to account for the c-AMP effects is presented.

Colicinogenic factor E1 (ColE1) is a bacterial plasmid of molecular weight 4.2×10^6 which exists in multiple copies in strains of *Escherichia coli* (2). It can be isolated both as a covalently closed supercoiled deoxyribonucleic acid (DNA) molecule or in the form of relaxation complex consisting of supercoiled DNA and protein (6).

It has recently been demonstrated that there is a direct involvement of ribonucleic acid (RNA) in the replication of ColE1 DNA (3, 6, 7, 33) and certain other plasmid elements (17, 18, 21), bacteriophage DNA (4, 5, 10, 22, 27, 29, 30, 33), and the *E. coli* chromosome (19, 20). It has been proposed that in these various replication systems the RNA is serving a primer role for initiation of DNA replication. In the ColE1 system, covalently closed circular DNA possessing an average of 25 ribonucleotides per molecule at a single site in the majority of molecules has been purified from *E. coli* cells replicating ColE1 DNA in the presence of chloramphenicol (34). In addition to a primer role for RNA in the initiation of DNA synthesis, a segment of RNA has been demonstrated at the 5' terminus of newly synthesized fragments which appear to be generated during the discontinuous synthesis of the *E. coli* chromosome (31, 32).

Earlier studies (14, 23) with the ColE1 plasmid have shown that the nucleotide cyclic adenosine 3',5'-monophosphate (c-AMP) is required for the production of at least two ColE1 determined proteins, colicin E1 and the protein(s) of the relaxation complex. A stimulation of ColE1 DNA replication by the addition of c-AMP has also been reported (14, 24). A more detailed examination of the effect of c-AMP upon ColE1 DNA synthesis is the subject of this report.

MATERIALS AND METHODS

Materials. Reagents and enzymes and their sources are: Triton X-100, Bass Chemical Co.; sodium dodecyl sulfate, Fisher Scientific; CsCl (technical grade), American Potash and Chemical Co; c-AMP, Sigma Chemicals; egg white lysozyme (crystallized three times), ethidium bromide, thymine (Thy), thymidine (Tdr), 5-bromouracil (5-BU), 5-bromodeoxyuridine (5-BUdR), chloramphenicol (CM), and rifampin, Calbiochem Co; [*methyl*-^{3}H]Thy ([^{3}H]Thy, 18 to 50 Ci/mmol), [*methyl*-^{3}H]Tdr ([^{3}H]Tdr, 57 Ci/mmol), and [2-^{14}C]Thy([^{14}C]Thy, 55 to 58 mCi/mmol), New England Nuclear.

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Organism. The *E. coli* K-12 strain CR34 Thy$^-$, colicinogenic for the plasmid ColE1 (2), was used in all of the experiments.

Media. M9 medium, described by Roberts et al. (26), contained 0.2% glucose, 2 μg of Thy per ml, 1 to 10 μg of B_1 per ml, and 0.02% each of L-leucine and L-threonine. c-AMP, CM, and rifampin were employed in final concentration of 3×10^{-3} M, 150 μg/ml, and 50 μg/ml, respectively.

Growth, labeling conditions, and preparation of cleared lysates. All cultures were grown at 37 C. Measurements of cell mass were performed on a Klett-Summerson colorimeter (green filter). Measurements of cell number were performed with a celloscope particle counter employing a probe containing a 30-μm diameter opening.

In experiments in which the relative rate of ColE1 DNA synthesis in response to various treatments was measured, the cells were incubated while being shaken in broth culture containing [^{14}C]Thy (0.5 μCi/ml) and 2×10^8 to 4×10^8 cells per ml, harvested by centrifugation at 25 C, suspended in the same volume of fresh medium containing no label, and shaken for a period of 30 min, after which time [^{3}H]Thy (10 μCi/ml) was added. After 20 min of additional shaking (zero time), additions were made and samples were taken. Growth and incorporation were stopped by the addition of NaN_3 (5×10^{-2} M, final concentration), and the sample was frozen quickly in a dry ice-ethanol bath.

In experiments in which the amount of ColE1 synthesized after a period of thymine starvation was determined, the cells were pregrown in [^{14}C]Thy to a density of 5×10^8 cells per ml and harvested by centrifugation as described above. The cell pellet was suspended in medium lacking thymine, incubated with shaking, and after 10 min, additions, where noted, were made, and the cultures were allowed to incubate for 30 to 60 min. The cells were then pulsed with [^{3}H]Tdr (10 μCi/ml) and chased with a mixture containing either Thy (20 μg/ml) and Tdr (1 mg/ml) or 5-BU (20 μg/ml) and 5-BUdR (1 mg/ml) for the times noted. Samples taken were treated with NaN_3 and quickly frozen as described above.

After the samples were thawed and centrifuged, cleared lysates were made as described previously (14). In some instances a modification (27) of the lysing procedure was employed. The cells were resuspended at a density of 5×10^{10} to 8×10^{10} per ml in a solution containing 10% sucrose and 0.05 M tris(hydroxymethyl)aminomethane (pH 7.5). After the suspension was quickly frozen and thawed, NaCl (final concentration 0.1 M) and lysozyme (200 μg/ml) were added, and the cells were incubated at 0 C for 45 min. One volume of a mixture containing Triton X-100 (0.05%) and ethylendiaminetetraacetic acid (0.3 M, pH 7.5) was then added to 9 volumes of the treated cells, and the resulting crude lysate was centrifuged at 46,000 $\times$ *g* for 30 min. The supernatant fluid (cleared lysate) was saved.

Equilibrium centrifugation. Dye-bouyant density equilibrium centrifugation in cesium chloride-ethidium bromide has been described in detail (2). Isopycnic centrifugation was performed by the addition of 4.8 g of CsCl to 3.85 ml of a solution containing ColE1 DNA in TES (0.05 M tris(hydroxymethyl)aminomethane, 0.005 ethylendiaminetetraacetic acid, 0.05 M NaCl, pH 8.0) and centrifugation in a Beckman Ti50 rotor at 40,000 rpm for 44 h at 15 C.

Determination of the amount of DNA synthesis. Cleared lysates were treated with 0.2% sodium dodecyl sulfate at 23 C for 5 min and centrifuged in 5 ml of a 5 to 20% or 20 to 31% sucrose gradient containing TES and 0.5 M NaCl (8). Dropwise fractionation of the gradient onto Whatman no. 1 filter paper and counting of radioisotope have been described previously (8).

The relative amount of ColE1 DNA synthesis in the samples was determined from the ^{3}H/^{14}C counts per minute ratio of radioactivity which appeared in both the 23*S* and 17*S* positions in sucrose gradients. The relative amount of total cellular DNA synthesized was determined by the ^{3}H/^{14}C counts per minute ratio of radioactivity incorporated into crude lysates. To account for differences in the amount of [^{3}H]Tdr used in different pulse experiments, a normalized value of relative amount of ColE1 DNA synthesized was obtained by dividing the ^{3}H/^{14}C ratio for ColE1 DNA by the ^{3}H/^{14}C ratio for the total DNA of the same sample.

RESULTS

Effect of c-AMP on the rate of ColE1 DNA synthesis. Cells were grown for several generations in [^{14}C]Thy-containing medium, washed free of the label, and resuspended in [^{3}H]Thy-containing medium. Thirty minutes after the addition of [^{3}H]Thy the culture was split into two parts and c-AMP was added to one. Samples were taken from both portions and assayed for the amount of ColE1 and total cellular DNA synthesized.

It can be observed (Fig. 1A and 1B) that the addition of c-AMP resulted in a fourfold increase in the specific rate of ColE1 DNA synthesis. This large stimulation in rate of ColE1 DNA synthesis appeared to last approximately 35 min and was succeeded by the onset of complete cessation of plasmid replication. Sixty-five minutes after the addition of c-AMP, it was found that ColE1 DNA synthesis resumed at the rate observed where c-AMP was not added.

The effect of c-AMP upon the rate of increase of cell mass and cell numbers was also measured. It can be observed (Fig. 2A and 2B) that c-AMP did not affect the rate of cell division, nor did it affect a change in cell mass.

Effect of c-AMP upon ColE1 DNA synthesis in CM and rifampin. Since ColE1 DNA can replicate in the presence of CM (9), it was possible to determine whether cessation of protein synthesis would eliminate the stimulatory effect of c-AMP. A culture was grown as described in the previous experiment with

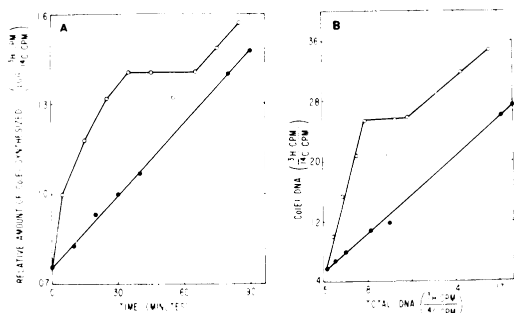

FIG. 1. Kinetics of stimulation of ColE1 DNA synthesis by c-AMP. Cells were uniformly labeled with [14C]Thy, washed free of the label, and suspended into a medium containing [3H]Thy. After 30 min of incubation (zero time), the culture was divided into two portions and c-AMP was added to one portion. Samples were taken at time points indicated and assayed for amount of both ColE1 DNA and total cellular DNA. Symbols: O, samples removed from cells growing in the presence of c-AMP; ●, samples removed from cells growing in the absence of c-AMP.

[14C]Thy, shifted to medium containing [3H]Thy and CM, and then split into two portions. c-AMP was added to one portion and the rate of ColE1 DNA synthesis was determined. A twofold increase in the rate of ColE1 DNA synthesis was observed in the c-AMP-containing culture (Fig. 3). In this case, as contrasted to the findings when CM was not employed, the rate of ColE1 DNA synthesis was constant in the presence of the nucleotide for the 1-h period examined.

The effect of rifampin upon the rate of ColE1 DNA synthesis in the presence and absence of c-AMP was also examined. It was observed (Fig. 4A) that the addition of rifampin to untreated cells resulted in an immediate and virtually complete cessation of ColE1 DNA synthesis. This result is in agreement with the findings of Clewell et al. (7). When the cells were treated with c-AMP, rifampin was again found to have caused an immediate and almost total shutdown of plasmid DNA synthesis (Fig. 4B).

The above findings indicate that the c-AMP-induced stimulation of ColE1 DNA synthesis is not dependent upon protein synthesis and that the requirement of RNA synthesis for plasmid replication is present under conditions of c-AMP-induced stimulation of plasmid replication.

Effect of c-AMP upon nonreplicating ColE1 DNA. Since, at any given time in a population of growing cells, greater than 90% of the plasmid molecules are not replicating, it was considered possible that the effects induced by c-AMP were taking place on nonreplicating molecules, resulting in an increased potential in the ability of a ColE1 molecule to undergo replication. To test the effect of c-AMP upon nonreplicating ColE1 DNA the cells were incubated in the absence of Thy, and during this time the nucleotide was added. The period of Thy starvation was followed by a short pulse with Tdr to allow subsequent replication. Since the period of the pulse was kept short enough to have allowed a maximum of one round of replication under normal conditions, the amount of replication subsequent to the Thy deprivation condition can be employed as an assay for the effect of c-AMP upon the mobilization of nonreplicating plasmid DNA for replication.

Cells were prelabeled with [14C]Thy, harvested, and suspended into medium without

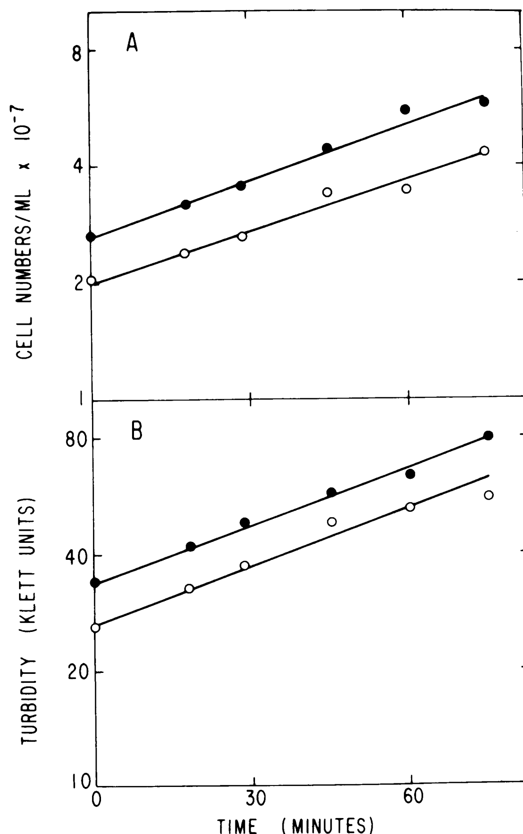

FIG. 2. Growth of CR34 (ColE1) cells in presence of c-AMP. Samples were taken from cells described in the legend to Fig. 1. (A) Cell numbers per milliliter were analyzed on a celloscope particle counter employing a probe with a 30 μm orifice. (B) Turbidity was measured on a Klett-Summerson colorimeter employing a green filter (540 nm). Symbols are described in the legend to Fig. 1.

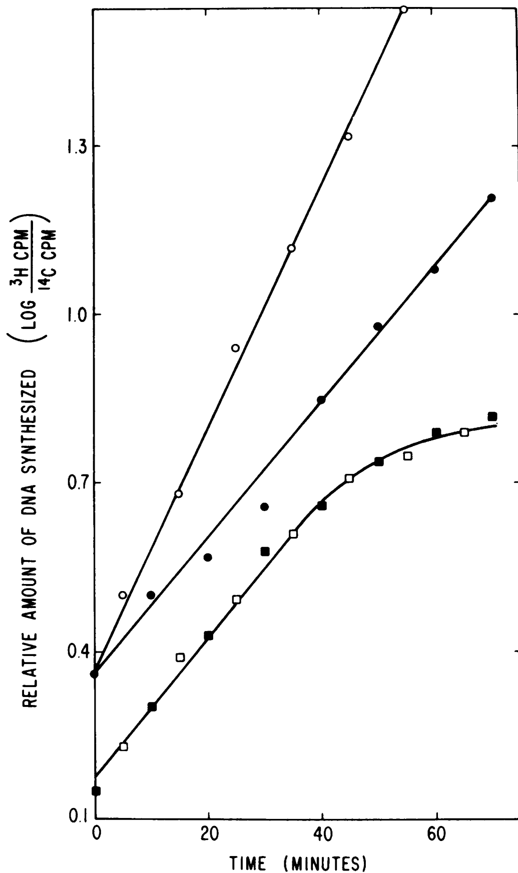

FIG. 3. *Kinetics of c-AMP-induced stimulation of ColE1 DNA synthesis in the presence of CM. The cells were grown and treated, and analysis of DNA synthesis was carried out as described in the legend to Fig. 1, except that CM was added to the culture 5 min prior to division of the culture and subsequent addition of c-AMP. ColE1 DNA* (○) *and total cellular DNA* (□) *in samples removed from culture growing in cyAMP; ColE1 DNA* (●) *and total cellular DNA* (■) *in samples removed from culture growing in the absence of c-AMP.*

label and with or without Thy. Each culture was split into two portions and c-AMP was added to one. After 60 min each culture that had not been exposed to c-AMP was given the nucleotide, and all the cultures were pulsed for 1 min with [^{3}H]Tdr and then subjected to a chase for periods of 2, 5, and 10 min. Samples taken after the pulse and throughout the chase period were analyzed for the amount of incorporation of label into ColE1 and total DNA (Table 1). By comparing the two cases in which the cells were not Thy-starved, it can be seen that c-AMP addition resulted in an increase (1.5-fold) in the amount of ColE1 DNA synthesized as determined by the values observed after a 1-min pulse and a 10-min chase. This relatively small stimulation effected by c-AMP is probably due to the fact that since replication was assayed 60 min after the addition of the nucleotide, the rate of ColE1 DNA synthesis would either have slowed down or ceased at the time of the pulse (Fig. 1).

In the case where the culture was subjected to Thy starvation without c-AMP, the subsequent amount of ColE1 DNA synthesized, as determined in either pulse or chase samples, was found to be slightly less than 1% of the total DNA made. The relative amount of ColE1 DNA produced under these conditions is the same as the amount made in the corresponding sample which had not been Thy-starved. For the culture Thy-starved in the presence of c-AMP, it can be seen that greater than 4% of the DNA subsequently synthesized in ColE1 DNA. In comparison to the corresponding values for cells Thy-starved without c-AMP, it is clear that the addition of c-AMP results in a 4- to 4.5-fold increase in the amount of plasmid DNA which can be replicated. These findings are in agreement with the notion that c-AMP effects an increase in the number of nonreplicating molecules that are capable of undergoing replication upon the readdition of Thy.

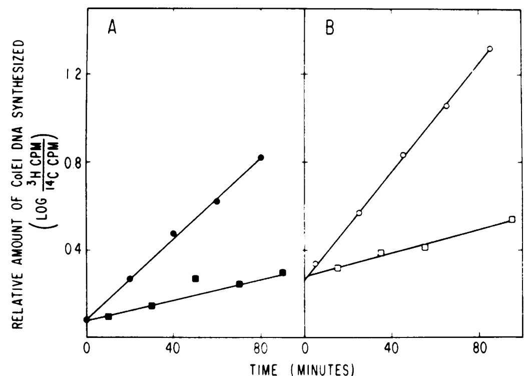

FIG. 4. *Effect of rifampin on ColE1 DNA synthesis in untreated and c-AMP-treated cells. A culture was uniformly prelabeled with [^{14}C]Thy, washed free of the label, resuspended in the [^{3}H]Thy-containing medium, and allowed to incubate for 30 min. CM was added and an additional 15-min incubation was allowed. The culture was then divided into two portions and c-AMP was added to one. Each portion was subsequently divided into two parts, and rifampin added to one part of each portion (zero time). Samples were taken at time points indicated and analyzed for amount of ColE1 DNA. Samples removed from cultures growing in CM and the following: (A) no additive* (●) *or rifampin* (■)*; (B) c-AMP* (○) *or c-AMP and rifampin* (□).

It can be observed (Table 1) that the values determined for ColE1 DNA synthesis after a 1-min pulse were up to fourfold lower than the corresponding values determined after the chase periods. The values determined for total DNA synthesis indicate that greater than 75% of the label was incorporated during the pulse period. The discrepancies between pulse and chase values for ColE1 DNA, especially in the cases of c-AMP-treated cells, is most likely due to the fact that radioactive label was measured in completed ColE1 DNA molecules (23*S* and 17*S* forms in sucrose gradients). Replicating intermediates of ColE1 DNA present at other positions in the sucrose gradients were not measured. Secondly, molecules undergoing replication in membrane fractions would not have been recovered by the methods employed. ColE1 DNA molecules partially replicated or present in membrane fractions at the end of the pulse are chased into membrane-free supercoils (28) and, thus, are determined as ColE1 DNA in the chase samples.

TABLE 1. *Simulation of ColE1 DNA synthesis by c-AMP after Thy starvation*

Prepulse conditions[a]	Sample taken after:	Relative amount of DNA synthesis ($^3H/^{14}C$)		
		Total[b]	ColE1[c]	ColE1 total
+ Thy	Pulse	1.4	1.5	1.0
	2-min chase	2.1	1.8	0.89
	5-min chase	2.4	2.2	0.96
	10-min chase	2.6	2.8	1.1
+ Thy + c-AMP	Pulse	1.4	1.2	0.80
	2-min chase	1.9	3.5	1.8
	5-min chase	2.2	3.4	1.6
	10-min chase	2.3	3.9	1.7
− Thy	Pulse	1.0	0.72	0.72
	2-min chase	1.1	1.0	0.91
	5-min chase	1.2	1.0	0.87
	10-min chase	1.5	1.2	0.80
− Thy + c-AMP	Pulse	0.81	2.1	2.6
	2-min chase	1.0	4.1	4.1
	5-min chase	1.0	4.4	4.4
	10-min chase	1.2	4.9	4.2

[a] Cells were grown to 5×10^8 cells per ml with [^{14}C]Thy, centrifuged at 23 C, suspended in medium with or without unlabeled Thy plus c-AMP (3×10^{-3} M final concentration), and incubated for 60 min. Cultures which did not receive c-AMP during the prepulse were given the nucleotide at the time of the pulse. The cultures were pulsed with [^{3}H]Tdr and chased.

[b] Determined on crude lysates of cells.

[c] Determined from sucrose gradient analysis.

Effect of c-AMP upon nonreplicating ColE1 DNA in the presence of CM. The experiment described above was repeated with either CM or c-AMP or both added during the period of Thy starvation. It was found that c-AMP produced a twofold increase in the amount of ColE1 DNA subsequently replicated whether or not CM was present during preincubation in the absence of Thy (Table 2). This result further demonstrates that the c-AMP-induced stimulation of either the replication itself or the potential for synthesis of ColE1 DNA is not mediated through the production of a protein.

Synthesis of ColE1 DNA in the presence of rifampin. Since ColE1 DNA replication is inhibited by rifampin, the effect of c-AMP upon replicating DNA treated with rifampin could not be tested. To test the effect of rifampin on nonreplicating DNA, either stimulated or not stimulated with c-AMP, cells were prelabeled with [^{14}C]Thy and then Thy-starved in the presence of CM with or without c-AMP for 40 min. Each culture was then divided into two portions; rifampin was added to one of the two portions in each case. After 5 min, the cells were

TABLE 2. *Stimulation of ColE1 DNA synthesis by c-AMP in presence of CM and after Thy starvation*

Prepulse conditions[a]	Sample taken after:	Relative amount of DNA synthesis[b] ($^3H/^{14}C$)		
		Total	ColE1	ColE1/total
A. − Thy	Pulse	4.5	4.1	0.93
	10-min chase	5.6	6.6	1.2
B. − Thy + c-AMP	Pulse	4.6	10.3	2.3
	10-min chase	5.1	12.8	2.5
C. − Thy + CM	Pulse	3.1	2.4	0.75
	10-min chase	4.8	6.6	1.4
D. − Thy + CM + c-AMP	Pulse	3.6	5.1	1.4
	10-min chase	4.7	10.7	2.3

[a] Cells were prelabeled with [^{14}C]Thy and Thy-starved as described in the footnotes to Table 1. c-AMP was used at 3×10^{-3} M final concentration; CM was at 150 μg/ml. At the time of the pulse, cells in case A received c-AMP and CM; cells in case B received CM; cells in case C received c-AMP.

[b] Determined as described in the footnotes to Table 1.

pulsed and chased, and ColE1 DNA synthesis was determined as described above. In addition, a culture was Thy-starved in the presence of CM and rifampin for 40 min, after which time c-AMP was added, followed 5 min later by a pulse and chase. In the case of the cultures not treated with rifampin, c-AMP was found to have stimulated a greater than twofold increase in plasmid replication (Table 3, lines A and C). Rifampin, given at the onset of Thy starvation, not only prevented the potential effect of c-AMP but also reduced the subsequent amount of ColE1 DNA synthesis to less than 30% of the control (Table 3, lines A and E). When the inhibitor was added to the culture 40 min after the onset of Thy starvation in the presence of c-AMP, it was observed that the subsequent amount of ColE1 DNA synthesis was less than that observed for the condition of c-AMP addition in the absence of rifampin but of the same order as that in which the cells were neither stimulated by c-AMP nor treated with rifampin (Table 3, lines A and D). The threefold difference in residual synthesis in prepulse conditions B and D with rifampin treatment is due most likely to an effect of c-AMP taking place prior to the addition of the inhibitor. This finding suggests that once c-AMP has acted upon nonreplicating ColE1 DNA, the plasmid molecule can replicate in the presence of rifampin. The lowered amount of replication observed in prepulse condition D, where the cells were Thy-starved in the presence of c-AMP and then treated with rifampin, as compared to prepulse condition C, where cells were treated only with c-AMP, may be due to the degradation of RNA in the presence of the inhibitor or to a partial reversal of the c-AMP effect under conditions of inhibition of RNA synthesis.

TABLE 3. *Effect of rifampin on c-AMP stimulation of ColE1 DNA synthesis after Thy starvation*

Prepulse conditions[a]	Relative amount of DNA synthesis[b] ($^3H/^{14}C$)		
	Total	ColE1	ColE1/ total
A. − Thy + CM	4.2	6.0	1.4
B. − Thy + CM + RIF	4.9	2.0	0.4
C. − Thy + CM + c-AMP	4.1	13.3	3.3
D. − Thy + CM + c-AMP + RIF	5.0	6.3	1.3
E. − Thy + CM + RIF + c-AMP	3.9	1.6	0.4

[a] Cells were prelabeled and Thy-starved as described in the footnotes to Table 1. Additions of c-AMP, CM, and rifampin (RIF) during Thy starvation were at 3×10^{-3} M, 150 μg/ml, and 50 μg/ml, respectively. In cases B and D, RIF was added after 40 min Thy starvation and 5 min prior to the pulse. In case E, RIF was added at the time of CM addition, and c-AMP was added 5 min prior to the pulse. Cells were pulsed with [^{3}H]Tdr for 2 min and chased for 10 min as described in the footnotes to Table 1.

[b] Determined as described in the footnotes to Table 1.

To test whether or not more than one round of replication of ColE1 DNA molecules occurs in the presence of rifampin after treatment with c-AMP (in the absence of Thy), cells were prelabeled with [^{14}C]Thy and then Thy-starved in the presence or absence of c-AMP for 40 min. The cultures were divided into two portions, and rifampin was added to one of the two portions in each case. After 2 min each culture was pulsed for 1 min with [^{3}H]Tdr and then chased with unlabeled 5-BUdR and 5-BU. Samples were taken at 5 and 65 min after the initial addition of [^{3}H]Tdr, harvested, and assayed for amount of DNA synthesis as determined by the relative amount of [^{3}H]Tdr incorporated. In both early and late samples, rifampin given to unstimulated cells resulted in a twofold reduction in the amount of ColE1 DNA synthesis (Table 4). On the other hand, rifampin given after exposure of the cells to c-AMP did not significantly affect the stimulation of plasmid DNA synthesis which subsequently did occur.

If the large amount of plasmid DNA produced in the presence of rifampin after prior treatment of the cells with c-AMP were the result of a single round of replication within the 1-min exposure to [^{3}H]Tdr, the ColE1 DNA synthesized should not contain BU and hence would appear in the light-banding region of a neutral cesium chloride density gradient. The supercoiled DNA in all of the samples was purified by equilibrium centrifugation in a cesium chloride-ethidium bromide density gradient and subjected to neutral cesium chloride equilibrium centrifugation (Fig. 5, where the letters A to H correspond to the samples designated in Table 4). In the 5-min samples of all the cultures, all of the replicated ColE1 DNA (^{3}H label) appear in the light region of the gradient, indicating that only a single round of replication had occurred within 5 min after resumption of DNA synthesis. In the 65-min samples of both the unstimulated and c-AMP-stimulated cultures not treated with rifampin, (Fig. 5B and F) ColE1 DNA appears in both the hybrid and heavy regions of the gradient, indicating more than one round of replication of some ColE1 molecules during the 65-min period in the presence of 5-BU and 5-BUdR. In the 65-min samples (both unstimulated and c-AMP-stimulated) treated with rifampin, the ^{3}H label appears only in the light-banding region. This indicates that all of the ColE1 DNA synthesized in the presence of rifampin after Thy starvation

TABLE 4. *Extent of ColE1 DNA synthesis in the presence of rifampin, CM, and c-AMP after Thy starvation*

Prepulse conditions[a]	Sample time of chase (min)	Relative amount of DNA synthesis[c] ($^3H/^{14}C$)		
		Total	ColE1	ColE1/total
− Thy + CM	(A)[b] 5	3.5	11.1	3.7
	(B) 65	5.7	22.7	4.0
− Thy + CM + RIF	(C) 5	4.2	8.3	2.0
	(D) 65	6.9	12.9	1.9
−Thy + CM + c-AMP	(E) 5	3.9	21.6	5.6
	(F) 65	6.3	42.8	6.8
− Thy + CM + c-AMP + RIF	(G) 5	5.1	25.2	5,0
	(H) 65	6.9	30.1	4.4

[a] Cells were prelabeled with [^{14}C]Thy, Thy-starved, and additions made as described in footnote to Table 3 except that rifampin (RIF), when employed, was added at 2 min prior to the pulse. Cells were pulsed with [^{3}H]Tdr for 1 min and chased with 1 mg of cold 5-BUdR and 20 μg of 5-BU per ml. Samples were taken at 5 and 65 min after the addition of [^{3}H]Tdr.

[b] Letters correspond to panels of Fig. 5.

[c] Determined as described in the footnotes to Table 1.

was made in the short interval of 1 min. Hence the effect of c-AMP is the two- to threefold stimulation of one round of ColE1 DNA replication in the presence of rifampin.

Extent of the c-AMP effect. In the examinations of the amount of stimulation of ColE1 DNA synthesis induced by c-AMP, it has consistently been found that the maximum stimulation in the presence of CM is not greater than twofold, whereas as much as a fourfold stimulation can take place in the absence of CM. It would appear, therefore, that fewer ColE1 DNA molecules can be stimulated to replicate by c-AMP in the presence of CM. This reduction could be due to the fact that factors other than the amount of RNA synthesis, such as the levels and activities of the replication machinery (enzymes, membrane replication sites, etc.) could limit the rate of ColE1 DNA synthesis after treatment with CM and hence mask the potential maximum effect of c-AMP. If such were the case, it is expected that exposure of the cells to CM for increasing periods of time would reduce the potential for the c-AMP stimulation. To test this, a culture was grown in the presence of [^{14}C]Thy to a density of 3.5×10^8 cells per ml, at which time CM was added. Samples, each containing 1.4×10^{10} cells, were taken at 0, 2.5, and 6.5 h after CM addition, harvested, and suspended into medium lacking Thy, with or without c-AMP (7×10^9 cells each), and containing CM. After 30 min each culture was pulsed with [^{3}H]Tdr and chased as described previously. In each sample the number of ColE1 DNA molecules per cell, the amount of ColE1 DNA synthesis during the pulse, and the number of plasmid molecules per cell which underwent replication in the pulse were determined.

From determinations of the amount of ^{14}C radioactivity incorporated, the amount of ColE1 DNA in each culture as a percentage of the total DNA was calculated. It can be observed that the level of ColE1 DNA rises from 1.5 to 7.85% of the total cellular DNA from 0 to 6.5 h after addition of CM to the cells (Table 5, column 3). The number of ColE1 DNA molecules per cell for M9-glucose-grown cells has been shown previously to be 24 (9). This number was used for the sample taken at time zero after the addition of CM to the culture (at this time 1.53% of the total DNA is ColE1 DNA). Correcting for the approximate twofold increase in the level of total cellular DNA in the samples taken after CM treatment and assuming that the population does not increase in cell number during CM treatment, the number of ColE1 DNA molecules per cell was calculated for the remaining samples. It can be seen (Table 5, column 4) that the number of plasmid molecules per cell increases from 24 to approximately 240 after 6.5 h of CM treatment.

The amount of ColE1 DNA synthesis was determined on the basis of the level of [^{3}H]Tdr radioactivity incorporated into plasmid DNA (Table 5, column 6). Employing the known specific radioactivity of the [^{3}H]Tdr (57.5 Ci/mmol) used in this experiment and 1,500 for the maximum number of Thy molecules per replicated strand, the minimum number of ColE1 DNA molecules per cell which underwent replication during the pulse could be determined from the measured incorporation of [^{3}H]Tdr into plasmid DNA (Table 5, columns 6 and 7). Since not all of the ColE1 molecules replicated would be uniformly labeled with [^{3}H]Tdr, the maximum value of 1,500 Thy molecules em-

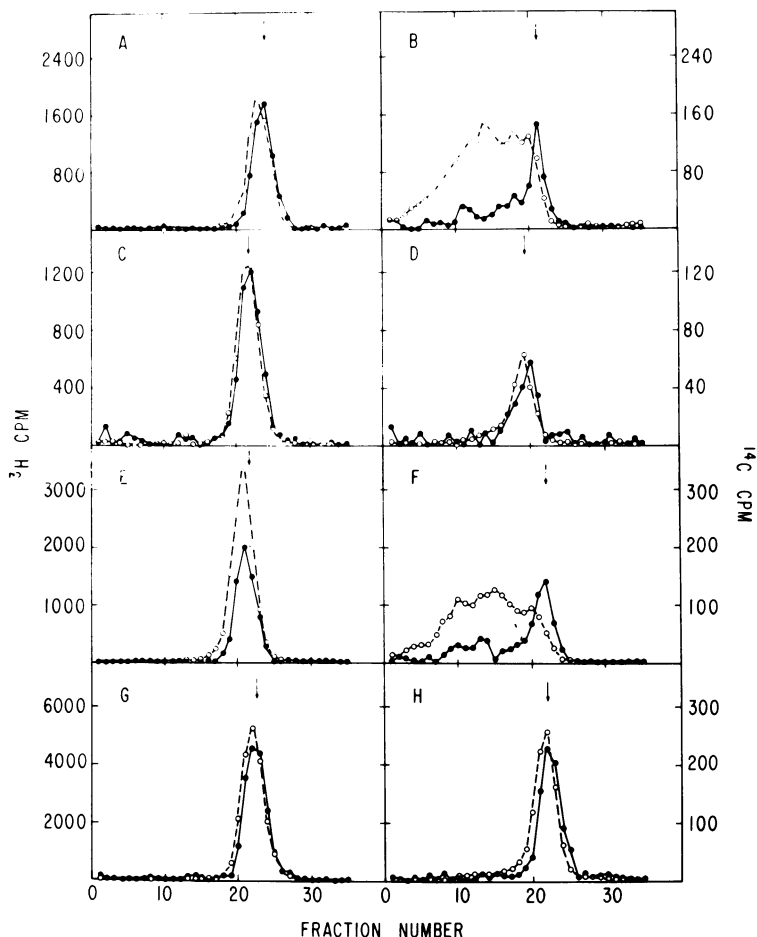

FIG. 5. *Density gradient profiles of ColE1 DNA synthesized in rifampin and BUdR. Growth, additions made during thymine starvation treatments, labeling and 5-BU and 5-BUdr chase conditions, and sample taking are described in the footnotes to Table 4. The DNA extracted from the samples was first subjected to cesium chloride-ethidium bromide equilibrium centrifugation. Fractions of each gradient containing ColE1 DNA were pooled, and the dye was removed by isopropyl alcohol extraction. The ColE1 DNA was then subjected to density gradient equilibrium centrifugation in cesium chloride. The profiles of this last centrifugation are shown. The segments A to H correspond to the samples extracted from cultures treated as described in the footnotes to Table 4. The arrows correspond to the position in each gradient of ^{32}P-labeled ColE1 DNA extracted from CR34 (colE1) grown in the absence of BU or BUdr. In each profile, denisty increases from right to left. ^{14}C label (●), ^{3}H label (○) in ColE1 DNA.*

ployed per replicated strand would yield an underestimate of the extent of ColE1 replication. It can be observed for each of the three samples taken (0. 2.5 and 6.5 h) that the addition of c-AMP results in a 1.7- to 2.0-fold increase in the amount of ColE1 DNA synthesis (Table 5, column 5). The increase in the ^{3}H/^{14}C level of 1.06 to 1.84 upon c-AMP treatment for the 0-h time sample is reflected in an increase in the average fraction of ColE1 DNA molecules per cell undergoing replication: 0.84/25.9 versus 0.45/24. The same degree of stimulation by c-AMP was also found in the 2.5-h sample in cells containing 115 to 136 ColE1 molecules. Furthermore, in this case (Table 5, column 6) the number of molecules that underwent replication without c-AMP treatment increased from the fraction in the corresponding zero time sample: 0.45/24.0 to 3.71/115.3, a 1.7-fold increase in the percentage of the plasmid molecules per cell (Table 5, column 7). In the presence of c-AMP in the 2.5-h sample, the average fraction of plasmid molecules per cell which underwent replication during the pulse was 9.36/136.2 or 6.9% of the ColE1 DNA per cell. This finding indicates

TABLE 5. *Extent of ColE1 DNA synthesis stimulated by c-AMP after replication in the presence of CM and after Thy starvation*

Time of addition of CM to culture[a] (h)	Preincubation conditions[b]	Total DNA as ColE1[c] (%)	Avg no. of ColE1 DNA molecules/cell[d]	ColE1 DNA synthesis after preincubation		
				$^3H/^{14}C$[e]	Avg no. of ColE1 DNA molecules/cell replicated[f]	ColE1 DNA molecules/cell replicated (%)
0	− Thy + CM	1.53	24.0	1.06	0.45	1.87
	− Thy + CM + c-AMP	1.60	25.9	1.84	0.84	3.25
2.5	− Thy + CM	3.66	115.3	1.83	3.71	3.21
	− Thy + CM + c-AMP	4.66	136.2	3.62	9.36	6.87
6.5	− Thy + CM	7.80	237.3	0.62	2.60	1.10
	− Thy + CM + c-AMP	7.85	247.3	1.12	5.25	2.12

[a] Cells were grown to 3.5×10^8 cells per ml, and CM (150 μg/ml) was added to the culture. Samples were taken at the times indicated.

[b] Samples were harvested and suspended in medium lacking Thy and containing CM (150 μg/ml) with or without c-AMP (3×10^{-3} M). Each batch contained 7×10^9 cells. Preincubation of all samples was performed for 30 min.

[c] Measurement of the percentage of ColE1 DNA/total cellular DNA was performed for the ^{14}C-labeled DNA.

[d] The number used for the cells grown under these conditions in the absence of CM was 24. For each of the remaining samples, the average number of ColE1 molecules per cell number was calculated from the following:

$$\frac{24 \times \%\ \text{total DNA as ColE1} \times {}^{14}\text{C-cpm total DNA in sample}}{1.53 \times {}^{14}\text{C-cpm total DNA in 0-h sample}}$$

where ^{14}C-cpm is the counts of ^{14}C per minute.

[e] Samples were pulsed for 1 min with [^3H]Tdr and chased with cold Tdr and cold Thy. $^3H/^{14}C$ for ColE1 was determined as described in the footnotes to Table 1.

[f] Average number of replicated ColE1 molecules per cell was

$$\frac{\text{total }{}^{3}\text{H-cpm incorporated into ColE1} \times 6.02 \times 10^{23}}{\text{sp act of }[{}^{3}\text{H}]\text{Tdr} \times \text{no. of Thy molecules per replicated strand} \times \text{no. of cells}}$$

where the sp act is measured in counts per minute per mole.

that, since the level of protein components of the replication apparatus could not have increased during the 2.5 h of CM treatment, there is sufficient activity and amount of these components to accomodate at least a 20-fold increase in the number of ColE1 DNA molecules which can undergo simultaneous replication. In the 6.5-h sample, the fraction of ColE1 DNA replication in cells not treated with c-AMP fell from the high level of 3.71/115.3 molecules per replicating cell seen at 2.5 h to 2.60/237.3, an actual 2.9-fold decrease in the average percentage of the plasmid molecules that replicate (Table 5, columns 4–7). Although the potential for ColE1 DNA synthesis is decreased after 6.5 h of CM treatment, it was observed that the amount of ColE1 DNA replication could be stimulated twofold by treatment with c-AMP (Table 5, columns 5–7).

DISCUSSION

The effect of c-AMP upon bacterial cells has been extensively investigated (13). c-AMP has been shown to act by binding to a protein termed catabolite gene activator which is subsequently involved in the initiation of messenger RNA synthesis in operons involved generally in the catabolism of carbohydrates and amino acids (1, 13, 25). In an earlier study, c-AMP was shown to cause a stimulation of ColE1 DNA synthesis (14). We have demonstrated that this stimulation is independent of protein synthesis (Table 2 and Fig. 3), but sensitive to the inhibition of RNA synthesis (Fig. 4B), suggesting that the increase in rate of ColE1 synthesis occurs through an effect of c-AMP on RNA synthesis.

The concept of a direct involvement of RNA

in ColE1 DNA replication has been advanced by Clewell et al. (7) because of the finding that the RNA polymerase inhibitor rifampin effects an immediate and complete shutdown of ColE1 DNA synthesis in cells replicating ColE1 DNA under conditions in which protein synthesis has been arrested. A short transcript of RNA can satisfy the requirements for a free 3'-OH which can serve to prime the DNA polymerase-directed incorporation of deoxyribonucleotides into DNA without the requirement for a preexisting nick in the supercoiled structure. DNA polymerase I has been shown to be essential for ColE1 DNA replication (15, 16). Furthermore, the findings reported by Fuke and Inselburg (11, 12) and observations made in this laboratory (L. Katz, P. H. Williams, R. W. Leavitt, and D. R. Helinski, manuscript in preparation) indicate that the covalently closed structure of the circular DNA is conserved in replicating intermediates of ColE1 DNA.

It has been shown that the replication of ColE1 DNA in the presence of CM in *E. coli* results in the formation of supercoiled molecules containing a short sequence of approximately 25 ribonucleotides convalently inserted into the newly synthesized complementary strands (34). A repair process for the subsequent removal of the RNA from the supercoiled DNA also has been demonstrated in intact cells (P. H. Williams, D. G. Blair and D. R. Helinski, manuscript in preparation). The nature of both the appearance of the inserted RNA and its removal are consistent with its role as a primer of ColE1 replication. These observations and the earlier findings that the synthesis of at least two ColE1 DNA products, colicin E1 and the relaxation complex protein(s), are sensitive to c-AMP-induced effects suggest that c-AMP affects ColE1 DNA replication by stimulating the synthesis of the ColE1-specific primer RNA, which is required for the initiation of plasmid replication.

The hypothesis that the stimulation of the rate of ColE1 DNA synthesis by c-AMP is mediated through a stimulation of ColE1 primer RNA synthesis would account for the increased ColE1 DNA replication rate upon addition of the nucleotide to either growing or CM-treated cells (Fig. 1 and 2) on the basis of synthesis of primer RNA for an increased number of nonreplicating molecules and the immediate replication of these newly primed plasmids. Priming of nonreplicating ColE1 DNA molecules takes place in the absence of Thy and in the presence of CM (conditions in which DNA and protein synthesis is arrested), but is prevented by rifampin, an inhibitor of RNA polymerase (Tables 1-3). However, it was found that when nonreplicating ColE1 DNA was primed (under conditions of Thy starvation in the presence of c-AMP), then rifampin added, most of these primed molecules were capable of undergoing one round of replication in the presence of the inhibitor after restoration of Thy to the cells (Tables 3 and 4, Fig. 5). The evidence suggests, therefore, that the primer RNA for ColE1 DNA replication can be synthesized under conditions where the DNA does not replicate and that it can serve for only one round of replication per molecule.

The nature of the factors limiting the replication of the ColE1 DNA molecules during the cell cycle is unknown. In addition to the priming of ColE1 DNA synthesis with RNA, such factors as restricted accessibility of membrane replication sites or the level of DNA polymerase I and other enzymes involved in plasmid replication may play a role in limiting the rate of ColE1 DNA replication. Under normal conditions of growth in M9-glucose-containing medium, the cells contain an average of 24 copies of the plasmid and duplicate this number every 60 min. Assuming replication of ColE1 DNA throughout the cell cycle, on the average an initiation of ColE1 DNA replication would occur every 2.5 min per cell. When the cells were exposed to CM for 2.5 h, a treatment resulting in the accumulation of greater than 100 ColE1 molecules per cell, presumably without an increase in the number of membrane replication sites or the level of replication enzymes, it was found that the number of ColE1 molecules which could undergo replication at one time was increased 10-fold per cell by the CM treatment alone and 20-fold by additional treatment with c-AMP (Table 5). In this case, the effect of c-AMP is not readily explained by a stimulation of synthesis of replication enzymes or membrane replication sites. Furthermore, if the rate of ColE1 DNA synthesis in c-AMP were limited by factors other than the amount of primed molecules, the addition of rifampin to c-AMP-stimulated cells would be expected to result in a delay in the shutdown of ColE1 DNA replication. When tested, however, a rapid cessation of ColE1 DNA synthesis was found (Fig. 4B). These observations suggest, therefore, that the cells contain sufficient membrane replication sites and replication enzymes to allow for substantial increases in the rate of ColE1 DNA synthesis for a limited time and that the maximum rate of ColE1 DNA replication is controlled to a significant extent by primer RNA

synthesis which is stimulated by, if not dependent upon, c-AMP.

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