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Effect of Growth Conditions on the Formation of the Relaxation Complex of Supercoiled ColE1 Deoxyribonucleic Acid and Protein in *Escherichia coli*

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Colicinogenic factor E1 (ColE1) is present in *Escherichia coli* strain JC411 (ColE1) cells to the extent of about 24 copies per cell. This number does not appear to vary in situations which give rise to twofold differences in the amount of chromosomal deoxyribonucleic acid (DNA) present per cell. If cells are grown in the absence of glucose, approximately 80% of the ColE1 molecules can be isolated as strand-specific DNA-protein relaxation complexes. When glucose is present in the medium, only about 30% of the plasmid molecules can be isolated as relaxation complexes. Medium shift experiments in which glucose was removed from the medium indicate that within 15 min after the shift the majority (>60%) of the plasmid can be isolated as relaxation complex. This rapid shift to the complexed state is accompanied by a two- to threefold increase in the rate of plasmid replication. The burst of replication and the shift to the complexed state are both inhibited by the presence of chloramphenicol. Inhibition of protein synthesis in log cultures by the addition of chloramphenicol or amino acid starvation allows ColE1 DNA to continue replicating long after chromosomal replication has ceased. Under these conditions, noncomplexed plasmid DNA accumulates while the amount of DNA that can be isolated in the complexed state remains constant at the level that existed prior to treatment. In the presence of chloramphenicol, there appears to be a random dissociation and association of ColE1 DNA and "relaxation protein" during or between rounds of replication.

The colicinogenic factor E1 (ColE1), a bacterial plasmid determining the production of the antibiotic protein colicin E1, has been isolated from gently lysed cells as a supercoiled circular deoxyribonucleic acid (DNA) molecule (molecular weight of 4.2×10^6) tightly complexed to a specific protein (5, 6). The isolated complex has the property of undergoing a unique phenomenon when treated with certain ionic detergents, proteases, heat, or alkali. In response to any one of these treatments, the twisted circular DNA in the complex undergoes a relaxation to the open circular state, and a strand-specific nick or gap is present in the open circular DNA product (2, 5, 6). Similar complexes have been found in the ColE2, ColE3, and ColIb plasmid systems (2, 7, 8), as well as the F_1 sex factor system (11) of *Escherichia coli*.

In this communication, data will be presented dealing with some physiological aspects of the ColE1 relaxation complex. It will be shown that the number of plasmid molecules in the complexed state differs depending on the medium employed for bacterial growth. Furthermore, it will be demonstrated that the number of plasmid molecules per host chromosome is not constant in different media but rather that there is a constant number per cell. Finally, data are presented on the synthesis of plasmid DNA and relaxation protein under conditions of medium shifts, and inhibition of protein synthesis.

MATERIALS AND METHODS

Materials. Reagents and sources were as follows:

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Brij 58, from Atlas Chemical; sodium deoxycholate (DOC) from Mann Research Laboratories; sodium dodecyl sulfate (SDS) from Fisher Scientific; Sarkosyl NL30 (sodium dodecyl sarcosinate) from the Geigy Chemical Company; egg-white lysozyme from Worthington Biochemical Corp.; CsCl (technical grade) from Penn Rare Metals, Division of Kawecki Chemical Company; chloramphenicol (CAP) from Parke, Davis and Company; ethidium bromide was a gift of the Boots Pure Drug Company Ltd., Nottingham, England; [*methyl*-^3H]thymine (18.2 Ci/mmole) and [2-^{14}C]thymine (55.2 mCi/mmole) from New England Nuclear Corp.

Bacteria and media. *E. coli* K-12 strains JC411 *thy*$^-$ (ColE1) (4) (a thymine-requiring strain isolated by D. Kingsbury) and CR34 (ColE1) (12) were utilized in the present study. Each strain harbors the ColE1 plasmid DNA which was transferred by conjugation from *E. coli* strain K-30. The tris(hydroxymethyl)aminomethane (Tris)-Casamino Acids medium used for these strains has been described in detail previously (5). Normally, 2 μg of thymine per ml was present in the medium. Unless otherwise specified, growth was performed at 37 C.

Preparation of cleared lysate. The lysing procedure has been described in detail previously (5, 6). It essentially involved the lysis of ethylenediaminetetraacetic acid (EDTA)-lysozyme spheroplasts with a detergent mixture of Brij 58 and DOC followed by a low-speed centrifugation of the lysate. The latter centrifugation removes about 95% of the total DNA leaving most of the plasmid DNA (both complexed and noncomplexed) in the supernatant fluid. This supernatant fluid is referred to as a cleared lysate.

Preparation and dye-buoyant centrifugation of Sarkosyl lysate. Two 15-ml log cultures of cells labeled separately with [^3H]thymine and [^{14}C]thymine were centrifuged, and the cell pellets were suspended together in 3.4 ml of 25% sucrose (containing 0.05 M Tris, *p*H 8.0). To this suspension was added 0.4 ml of lysozyme (5 mg/ml), followed by, after 5 min of incubation at 25 C, 0.8 ml of Na_2 EDTA (0.25 M, *p*H 8.0). After 5 min of further incubation, a 1-ml portion was removed for lysis with Brij 58 while the remainder was lysed by adding 2 ml of 2% Sarkosyl. The Sarkosyl lysate was centrifuged to equilibrium in CsCl-ethidium bromide, fractionated, and counted as described previously (6).

Sucrose density gradients. Sucrose density gradient centrifugation, dropwise fractionation of the gradients, and the counting of radioisotope were carried out as described in detail previously (6). Unless designated otherwise, the gradients contained TES (0.05 M NaCl, 0.005 M Na_2 EDTA, and 0.03 M Tris, *p*H 8.0). The high salt gradients contained 0.55 M NaCl, 0.005 M Na_2 EDTA, and 0.03 M Tris, *p*H 8.0.

Medium shifts. In experiments involving a medium shift, cells were harvested by centrifugation in a Sorvall centrifuge (SS39 rotor) at 25 C for 4 min at 10,000 rev/min; washed by resuspending in twice the original volume of the medium; recentrifuged and resuspended in the new medium. The entire procedure required 12 to 15 min. Cell growth during the shift was minimal as evidenced by less than a 10% increase in turbidity after the shift. Turbidity was measured with a Klett-Summerson colorimeter.

RESULTS

Effect of glucose on the level of complexed ColE1 DNA. It was observed that the percentage of ColE1 DNA in the form of a relaxation complex of supercoiled DNA and protein was not constant when purified from an *E. coli* strain growing in different media. To determine what effects glucose might have on the percentage of ColE1 DNA in the complexed state, the following experiment was performed. *E. coli* strain JC411 *thy*$^-$ (ColE1) cells were grown in two different 15-ml batches of Tris-Casamino Acids-glycerol medium. One batch contained in addition, 0.5% glucose and [^3H]-thymine (0.3 mCi); the other portion lacked glucose and contained [^{14}C]thymine (15 μCi). Each culture was harvested in log phase, after which the cells were suspended and mixed together in a solution of 25% sucrose (0.5 M Tris, *p*H 8.0). After preparation of lysozyme-EDTA spheroplasts, a 1-ml portion of the mixed suspension was lysed with a Brij 58-DOC detergent mix, whereas the remainder was lysed with Sarkosyl. A cleared lysate (see Materials and Methods) was prepared from the Brij 58-DOC lysate and subsequently analyzed by sucrose density gradient centrifugation. Prior to centrifugation, one portion was treated with SDS while another portion served as an untreated control. Since SDS converts only the supercoiled ColE1 DNA that is in the form of relaxation complex to the open circular state, the extent of conversion after the addition of SDS is a quantitative measure of the level of complexed ColE1 DNA. The result, which is shown in Fig. 1, indicates that, whereas the ColE1 DNA extracted from the cells grown in the absence of glucose (the ^{14}C-label) is in the form of a relaxation complex of supercoiled DNA and protein to the generally observed extent of about 82% (5), ColE1 DNA obtained from cells grown in the presence of glucose (the ^3H-label) was complexed to the extent of only about 33%.

This difference in the level of complexed ColE1 DNA was also observed upon examination of total cellular DNA in the Sarkosyl lysate. When the Sarkosyl lysate was centrifuged to equilibrium in an ethidium bromide-CsCl buoyant density gradient, the profile shown in Fig. 2 was obtained. It has been demonstrated previously (5) that either Sarkosyl or the equilibrium centrifugation proce-

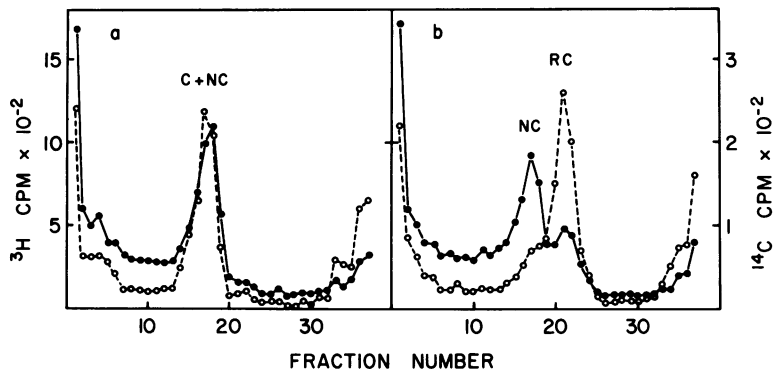

FIG. 1. *Sedimentation analysis of cleared lysates of cells grown in the presence and absence of glucose. Strain JC411 thy$^-$ (ColE1) cells grown as described in the text in the presence and absence of glucose were harvested and resuspended together. From a portion of the resuspended cells, a cleared lysate was prepared as described in Materials and Methods and diluted twofold with TES. One 0.3-ml portion was mixed with 0.1 ml of 1% SDS, while another 0.3-ml portion was mixed with 0.1 ml of TES to serve as a control. After incubation at 25 C for 10 min, the samples were sedimented (from right to left) through 5 to 20% sucrose density gradients containing 0.55 M NaCl in an SW50.1 rotor (15 C) at 50,000 rev/min for 135 min. (●) ^{3}H-labeled DNA prepared from cells grown in presence of glucose. (○) ^{14}C-labeled DNA prepared from cells grown in the absence of glucose. (a) Untreated cleared lysate; (b) cleared lysate treated with SDS. NC indicates noncomplexed ColE1 DNA; C indicates complexed ColE1 DNA; RC indicates the relaxed state of complexed ColE1 DNA.*

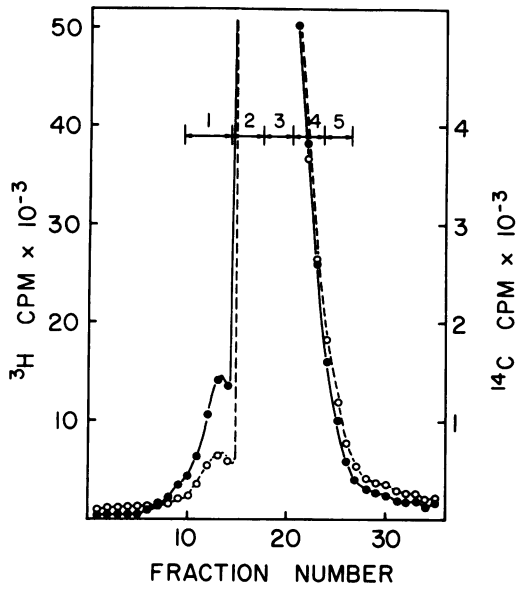

FIG. 2. *Dye-buoyant density gradient centrifugation of a Sarkosyl lysate of cells grown in the presence and absence of glucose. A portion of the mixed cell suspension described in Fig. 1 was lysed with Sarkosyl and centrifuged to equilibrium in a CsCl-ethidium bromide gradient in a Ti 60 rotor (15 C) at 44,000 rev/min for 60 hr. (●) ^{3}H-labeled DNA prepared from cells grown in the presence of glucose; (○) ^{14}C-labeled DNA prepared from cells grown in the absence of glucose. The numbers 1 to 5 designate regions where fractions were pooled for subsequent analysis on sucrose density gradients.*

dure itself promotes the relaxation of complexed ColE1 DNA, with the result that noncomplexed DNA appears in the dense band characteristic of covalently closed circular DNA (13) while the relaxed DNA which remains associated with protein appears in the "light" portion of the large chromosomal DNA band (6). When DNA from the various pools indicated in Fig. 2 were dialyzed and analyzed separately on sucrose gradients (Fig. 3), the majority of the ^{14}C-labeled ColE1 DNA was found in the open circular 17*S* form and in the "light" portion of the noncovalently closed band (Table 1). However, the ^{3}H-labeled ColE1 DNA appeared predominantly as 23*S* supercoiled DNA in the satellite band of the equilibrium gradient. Table 2A shows that the proportions of relaxed and supercoiled molecules correspond closely to that observed in the SDS-treated cleared lysate (Fig. 1). Table 2B summarizes the results of a similarly performed experiment, except that the isotopes were reversed. Essentially identical results were obtained. The reduction in the amount of plasmid DNA in the complexed state thus appears to be related to the presence of glucose. Although in these experiments glycerol was present in addition to glucose, in other experiments where glycerol was not present similar results were obtained. It was further shown that glycerol itself is not involved in determining the amount of plasmid DNA in the complexed state, since cells grown in a

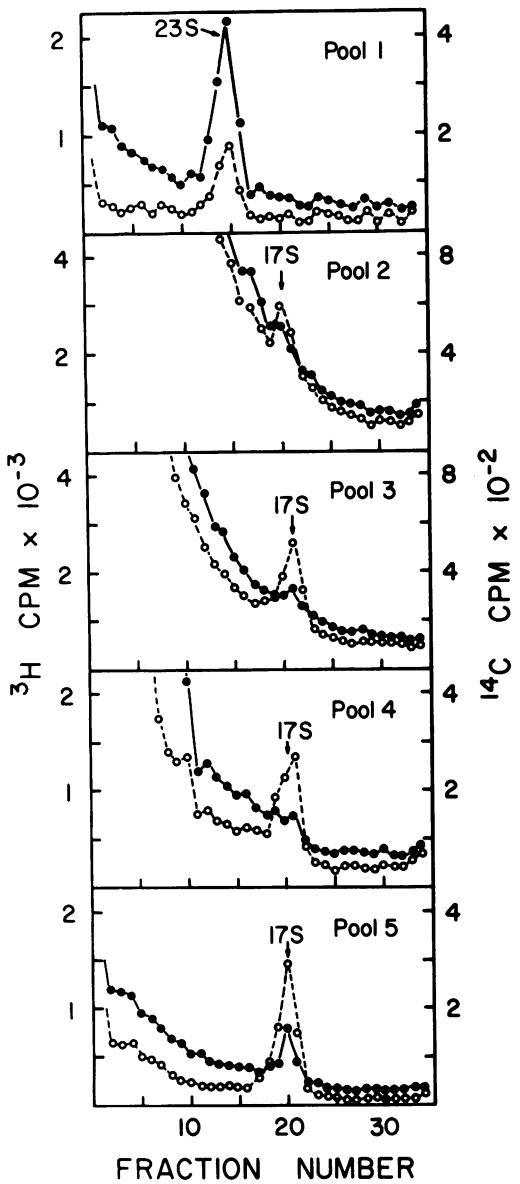

FIG. 3. *Sedimentation analysis of the fractions pooled after dye-buoyant density centrifugation of the Sarkosyl lysate of cells grown in the presence and absence of glucose. Sedimentation was through 5 to 20% sucrose density gradients as described in Fig. 1.* (●) *^{3}H-labeled DNA prepared from cells grown in the presence of glucose;* (○) *^{14}C-labeled DNA prepared from cells grown in the absence of glucose.*

Casamino Acids medium (lacking glucose) yield identical results regardless of whether or not glycerol is present.

The percentage of ColE1 DNA molecules in the complexed state is not a function of the growth rate of the cells. Experiments in which a rich medium lacking glucose (Casamino Acids-yeast extract) was used to allow a relatively rapid growth rate (45-min doubling time) yielded data indicating a typically high percentage of ColE1 DNA in the form of a relaxation complex. In contrast, cells growing relatively slowly (90-min doubling time) in a

TABLE 1. *Relative amounts of supercoiled and relaxed plasmid DNA recovered after dye-buoyant density centrifugation of a Sarkosyl lysate*[a]

Pool	Counts/min in pool	Percent plasmid DNA[b]	Amt of plasmid DNA (counts/min)	
			Supercoiled	Relaxed
A. ^{3}H counts				
1	49,027	13.76	6,746	
2	683,099	0.08		546
3	269,262	0.27		727
4	110,641	0.27		298
5	30,462	2.10		639
Total	1,142,491	0.78	6,746	2,210
B. ^{14}C counts				
1	2,468	17.08	421	
2	87,710	0.42		368
3	32,468	2.31		750
4	11,924	4.59		547
5	3,856	19.70		759
Total	138,426	2.06	421	2,424

[a] DNA prepared and analyzed as described in Fig. 2.

[b] Based on the amount of plasmid DNA in each pool expressed as a percentage of the total counts recovered in each sucrose density gradient.

TABLE 2. *Percentage of complexed and noncomplexed ColE1 DNA obtained from cleared and Sarkosyl lysates*[a]

Determination	Plasmid DNA in cleared lysate (%)		Plasmid DNA in crude Sarkosyl lysate (%)	
	Complexed	Noncomplexed	Complexed	Noncomplexed
A. Glucose present (^{3}H)	33[b]	67[b]	29[c]	71[c]
Glucose absent (^{14}C)	82[b]	18[b]	88[c]	12[c]
B. Glucose present (^{14}C)	21	79	20	80
Glucose absent (^{3}H)	88	12	87	13

[a] Same preparation of cells was used for both analyses as described in Fig. 1 and 2 and the text.

[b] Data taken from Fig. 1.

[c] Data taken from Table 1.

minimal medium but in the presence of glucose always yielded a low percentage of plasmid DNA in the complexed state.

In addition to the striking effect of the presence of glucose on the percentage of plasmid DNA in the complexed state, it became apparent from the above experiments that the percentage of total DNA that is plasmid DNA in the case of glucose-grown cells was about one-half that found in the case when glucose was absent (Table 1). This observation is also reflected in data of Fig. 1a in which the ratio of ^{3}H to ^{14}C in the ColE1 DNA peak (ratio of 3.9) is only about one-half that of the chromosomal DNA (ratio of 7.4) indicated by the ratio of counts in the crude lysate. This observation has been reproduced a number of times with similar results. From four experiments, average values of 0.93 and 1.72 for the percentage of the total DNA representing plasmid have been obtained when glucose was present and absent, respectively. It was determined, on the basis of cell counts of cultures of equal turbidity (using both the Petroff-Hauser counting chamber and viable cell counting), that cells grown in a glucose-glycerol-Casamino Acids medium (doubling time of 45–60 min) have approximately twice the mass of cells grown in a glycerol-Casamino Acids medium (doubling time of 80–100 min). On the basis of the specific activity of isotope in the DNA [as determined by the method of Burton (3)], the total amount of DNA present per cell was estimated to be 16.9×10^{-15} g/cell for cells growing in the presence of glucose and 8.0×10^{-15} g/cell for cells growing in the absence of glucose. The amount of DNA present can be expressed as 4.0 and 1.9 genome equivalents per cell, respectively, assuming that the size of the *E. coli* genome is 2.5×10^{9} daltons (or 4.2×10^{-15} g). These values are higher than those reported by Cooper and Helmstetter (9) for *E. coli* strain B/r cells growing with doubling times of 50 and 90 min.

Nevertheless, cells growing in Casamino Acids-glucose medium, although approximately twice the size of cells growing in Casamino Acids-glycerol medium, also have approximately twice as much chromosomal DNA per cell. The fact that the ratio of plasmid DNA to chromosomal DNA in the former situation is about one-half that in the latter case indicates that, independent of the amount of chromosomal DNA per cell, cells grown in the presence of glycerol or glucose have the same amount of plasmid DNA per cell. We have previously estimated that, under conditions where glucose was absent, the number of plasmid molecules per chromosomal genome equivalent is approximately 12 (6). Since there are about two genome equivalents per cell under these conditions, the average number of plasmid molecules per growing cell should therefore be 24 in Tris-Casamino Acids medium irrespective of the presence of glucose.

Immediate effects of the removal of glucose. A 30-ml culture of strain JC411 *thy*$^{-}$ (ColE1) cells was grown for several generations in a medium containing 0.5% glucose and [^{14}C]thymine (15 μCi). While in log phase the culture was divided into three equal portions, pelleted, and resuspended into three different media (20 ml each). All three media contained equal amounts of [^{3}H]thymine (0.8 mCi) but no ^{14}C-isotope. In addition, one of the media, the control, contained 0.5% glucose and thus resembled the prelabeling conditions. In the other two, glucose was absent, and in one case CAP (30 μg/ml) was present. All three cell suspensions were incubated for 15 min, at which time they were rapidly chilled on ice. Cleared lysates were prepared, treated or not treated with SDS, and analyzed on sucrose gradients. The results are shown in Fig. 4. On the basis of the extent of SDS-induced relaxation of the ColE1 DNA, it is clear that in the case where glucose was removed the majority (>60%) of the plasmid DNA was found in the complexed state. This case is in contrast to those in which CAP was present or glucose was maintained in the medium. The ratio of ^{3}H to ^{14}C provides a measure of the relative extents of DNA replication after the medium shift. The ratios in each case for plasmid and chromosomal DNA are shown in Table 3. It is evident that the removal of glucose from the medium resulted in greater than a onefold increase in the rate of synthesis of plasmid DNA compared to that of the parallel culture where glucose was present. When CAP was present, an increase in the rate of DNA synthesis was not observed. In all cases, the rate of replication of chromosomal DNA did not vary.

When cells labeled with [^{3}H]thymine were grown in the presence of glucose for several generations and then starved for thymine for as long as 1 hr, no difference was observed in the amount of complexed DNA compared to that of the prestarved state. However, if glucose was removed during the thymine starvation, the plasmid DNA was then found predominantly (~90%) in the complexed state. The presence of CAP inhibited this shift.

To determine what happens in the reverse situation, a 30-ml culture of strain JC411 *thy*$^{-}$ (ColE1) cells was grown for several generations

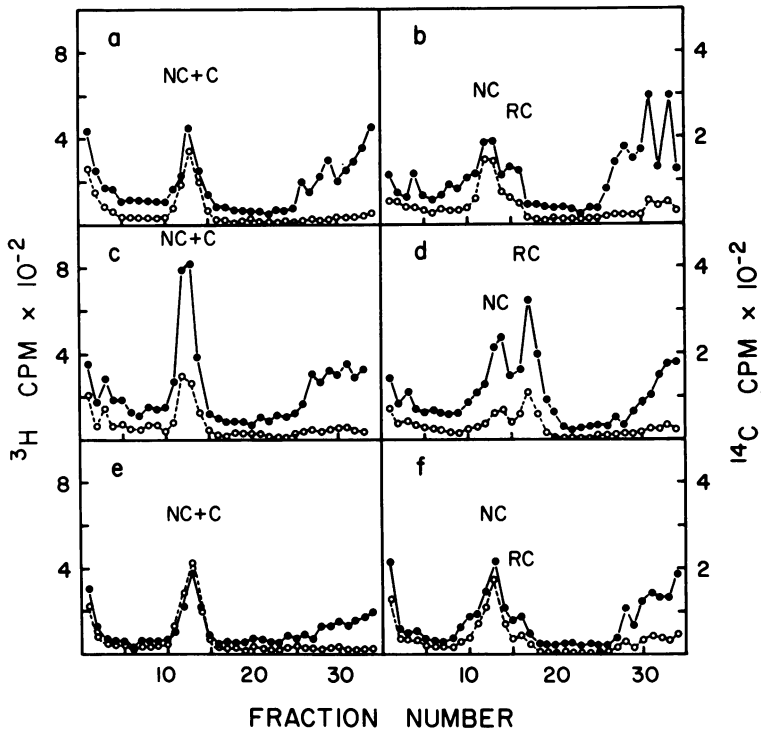

FIG. 4. *Sedimentation analysis of cleared lysates to determine the effect of the removal of glucose from the medium. Cleared lysates were prepared from cells grown in the presence of glucose and then shifted to a medium lacking glucose in the presence and absence of CAP as described in the text. The cleared lysates were incubated in the absence and presence of SDS and centrifuged (from right to left) as described in Fig. 1.* (●) *^{3}H-labeled DNA synthesized after the medium shift;* (○) *^{14}C-labeled DNA present prior to the medium shift. Cleared lysates were prepared from the following cells. (a) Cells shifted into a medium identical (with the exception of the isotope) to the preshift medium; (b) cells shifted to a medium lacking glucose; (c) cells shifted to a medium lacking glucose but where CAP was present; d, e, and f correspond to the samples a, b, and c, respectively, except that these samples were treated with SDS prior to centrifugation. The designations NC, C, and RC are described in Fig. 1.*

TABLE 3. *Effect of the immediate removal of glucose from the medium on the rate of replication of ColE1 DNA*[a]

Growth condition	^{3}H counts per min/^{14}C counts per min	
	Chromosomal DNA	Plasmid DNA
Glucose maintained	2.31	2.38
Glucose removed	2.68	6.42
Glucose removed, chloramphenicol present	2.00	1.64

[a] The data were taken from the experiment described in Fig. 4. Chromosomal DNA counts were obtained from the crude lysates used for the preparation of cleared lysates in this experiment.

in the absence of glucose (Casamino Acids and glycerol present) and in the presence of [^{3}H]-thymine (0.6 mCi). In log phase, the culture was divided into two 15-ml portions and glucose (final concentration of 0.5 M) was added to one portion. After 15 min, the cells were chilled on ice and cleared lysates were prepared and analyzed on sucrose density gradients. The results are shown in Fig. 5. Treatment with SDS produced a similar amount of relaxation in each case; thus, the addition of glucose did not appear to affect significantly the amount of plasmid DNA in the complexed state during the 15-min time period of the shift. Similar experiments have shown that even after 30 min there is no noticeable effect of the addition of glucose on the level of plasmid DNA in the complexed state. These data suggest that the protein of the relaxation complex is stable and probably is diluted to a lower level only as a result of cell division. Similarly performed double-labeling experiments indicate that replication during the first 15 min after glucose addition proceeds at a

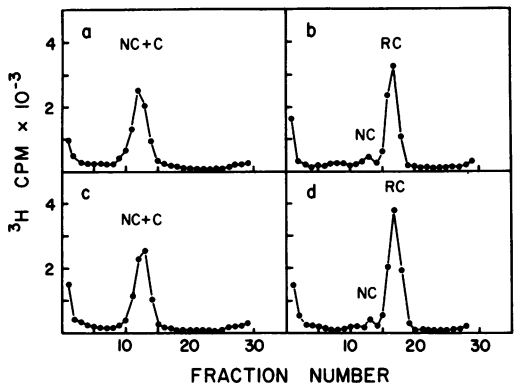

FIG. 5. *Sedimentation analysis of cleared lysates to determine the effect of the addition of glucose to the medium. Cleared lysates were prepared from cells grown in the absence of glucose and then shifted to a medium containing glucose as described in the text. The cleared lysates were incubated in the absence and presence of SDS and centrifuged (from right to left) through a 5 to 20% sucrose density gradient as described in Fig. 1. Cleared lysates were prepared from the following cells. (a) Cells grown in the absence of glucose; (b) cells grown in the absence of glucose and then in the presence of glucose for 15 min.; c and d correspond to the samples a and b, respectively, except SDS was added to these samples prior to centrifugation as described in Fig. 1.*

rate not detectably different from that of a control culture with glucose remaining absent.

Effect of inhibition of protein synthesis on plasmid DNA synthesis. A 40-ml culture of strain CR34 (ColE1) cells was grown for several generations in glycerol-Casamino Acids medium containing [^{14}C]thymine (30 μCi). In log phase, the cells were pelleted and resuspended in 80 ml of medium containing [^{3}H]-thymine (1.6 mCi) in place of the [^{14}C]thymine. A 20-ml sample of the freshly resuspended cells was removed to serve as a control while 30 μg of CAP/ml was added to the remainder. The control cells were allowed to grow for one generation (90 min) and then quickly chilled on ice. From the CAP-treated cells, 20-ml samples were removed and placed on ice after 90, 180, and 360 min. A cleared lysate was prepared from each sample, treated with SDS, and analyzed by sucrose density gradient centrifugation.

As shown in Fig. 6a, the majority of the ColE1 DNA in the one-generation control cells were in the complexed state as expected. In addition the ^{3}H to ^{14}C ratio at this point represents an extent of DNA replication approximately equivalent to a doubling of the amount of DNA that was present prior to the medium shift. Fig. 6b through d indicate that, while replication of plasmid DNA was occurring, the relative proportion of noncomplexed ColE1 DNA increased dramatically. Fig. 7 illustrates that, while the synthesis of chromosomal DNA was essentially at a halt at a level equivalent to about 60% that of the one-generation control, the plasmid DNA continued to replicate at an approximately linear rate. By 360 min, the extent of plasmid DNA replication represented slightly more than a complete doubling of ColE1 DNA. The ratio of ^{3}H to ^{14}C within the complexed and noncomplexed DNA was not significantly different for the time points taken, suggesting that the association and dissociation of plasmid DNA into and from the complexed state is a reversible and random event with respect to unreplicated and newly replicated plasmid DNA. Using the total amount of ^{14}C-labeled plasmid DNA (noncomplexed plus complexed) in each gradient (Fig. 6) as a normalizing factor, it can be seen (Fig. 8) that the amount of ^{3}H-labeled DNA appearing in the complexed state reaches a nearly constant value, while the amount in the noncomplexed state continues to increase. This observation is consistent with the notion that the amount of protein with which plasmid DNA can become associated to form complex remains at a constant level in the presence of CAP and is relatively stable. In a separate but similarly performed experiment, a sample of cells was analyzed after 18 hr of incubation in the presence of CAP. At this time, the amount of ColE1 DNA synthesized was almost equivalent to three times the amount found in the one-generation control cells. A concentration of 12 μg of CAP/ml was equally as effective in the inhibition of the synthesis of the supercoiled DNA-protein complex.

In consideration of the differences in the percentage of plasmid DNA molecules in the complexed state depending on whether glucose is present in the medium, it was of interest to determine the effect of inhibition of protein synthesis on ColE1 DNA synthesis in cells grown in the presence of glucose. The experiment was carried out essentially as described for the analagous experiment in the absence of glucose described above except that since the doubling time is shorter in the presence of glucose the one-generation control represented a 60-min time period; and samples of the CAP-treated culture were removed after 60, 120, and 240 min. As in the previous case, the relative amount of plasmid DNA found in the complexed state decreased dramatically during CAP-treatment (Fig. 9). It is apparent that

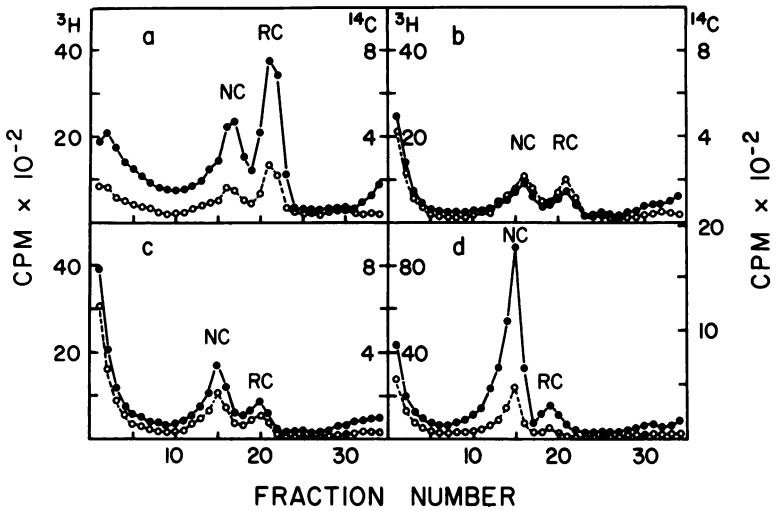

FIG. 6. *Sedimentation analysis of cleared lysates prepared from cells grown either in the absence or presence of CAP. Cells were grown in the presence of [^{14}C]thymine in the absence of glucose and then shifted to a medium containing CAP and [^{3}H]thymine and lacking [^{14}C]thymine as described in the text. Cleared lysates were prepared, treated with SDS as described in Fig. 1, and centrifuged through 5 to 20% sucrose density gradients in an SW65 rotor (15 C) at 50,000 rev/min for 135 min. (●) ^{3}H-labeled DNA synthesized after the medium shift; (○) ^{14}C-labeled DNA synthesized prior to the medium shift. Cleared lysates were prepared from the following cells. (a) Cells grown in the presence of [^{14}C]thymine and shifted to a medium identical (with the exception of the isotope) to the preshift medium for one generation (90 min); (b), (c), and (d) cells shifted to a medium containing CPA for 90, 180, and 360 min, respectively. The designations NC and RC are described in Fig. 1.*

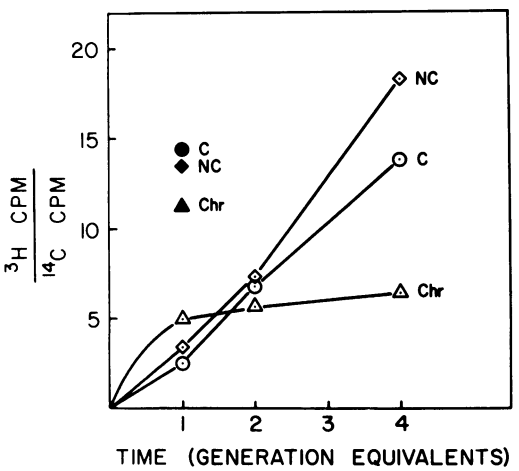

FIG. 7. *Extent of replication of DNA found in the complexed and noncomplexed state in cells grown in either the absence or presence of CAP. The ratios of ^{3}H to ^{14}C were calculated from the data presented in Fig. 6. The values obtained for chromosomal DNA were based on the amount of ^{3}H and ^{14}C in the crude lysates. NC indicates noncomplexed ColE1 DNA; C indicates complexed ColE1 DNA; and Chr indicates chromosomal DNA. The solid points represent DNA from the one-generation control cells; the open points correspond to the CAP-treated cells.*

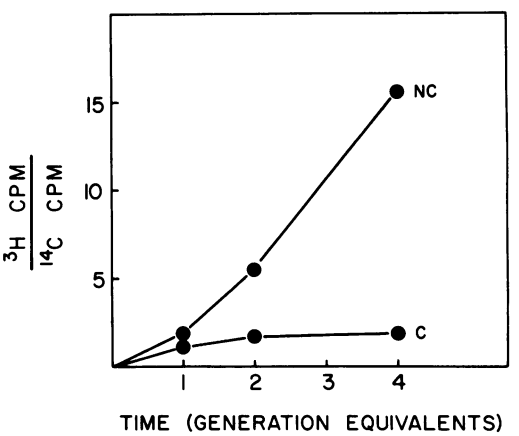

FIG. 8. *Relative amounts of incorporation of [^{3}H]-thymine into complexed and noncomplexed ColE1 DNA in cells grown in the presence of CAP. The data were obtained from the experiment described in Fig. 6. The NC and C designations are described in Fig. 1.*

this change was due to an accumulation of noncomplexed plasmid DNA, while the amount of DNA in the complexed state remained at a constant level (Fig. 10). Once again the ratio of ^{3}H to ^{14}C of the plasmid DNA

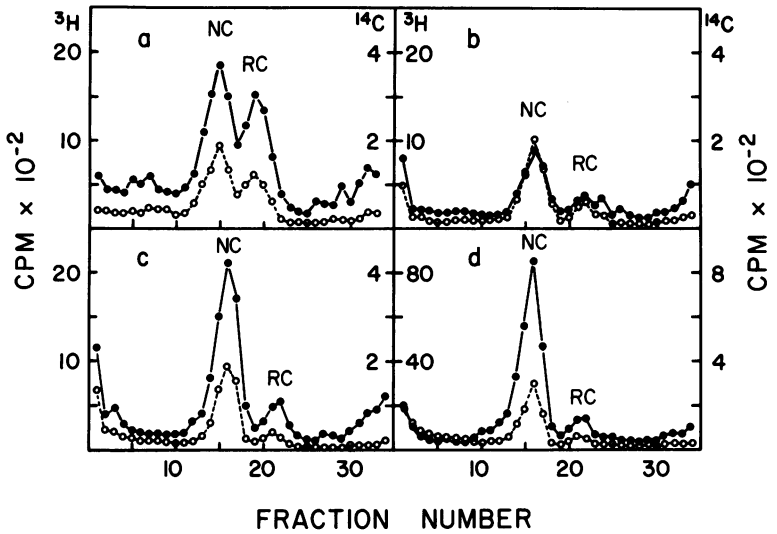

FIG. 9. *Sedimentation analysis of cleared lysates prepared from cells grown in the presence of glucose and either in the absence or presence of CAP. Cells were grown as described in Fig. 6 except that glucose was present in the growth medium. Cleared lysates were prepared, treated with SDS, and centrifuged (from right to left) through 5 to 20% sucrose density gradients in an SW50.1 rotor (15 C) for 100 min. (●) [^{3}H]thymine-labeled DNA synthesized after the medium shift; (○) [^{14}C]thymine-labeled DNA synthesized prior to the medium shift. Cleared lysates were prepared from the following cells. (a) Cells grown in the presence of [^{14}C]-thymine and shifted to a medium identical (with the exception of the isotope) to the preshift medium; (b), (c), and (d) cells shifted to a medium containing CAP for 60, 120, and 240 min, respectively. The NC and RC designations are described in Fig. 1.*

in the complexed and noncomplexed states is similar (Fig. 11). In contrast to the previous experiment where glucose was not present, it is observed from the ^{3}H to ^{14}C ratios (Fig. 10) that at the end of 240 min the extent of plasmid DNA replication represented a 2.5-fold increase over that found in the one-generation control. In this and other similarly performed experiments, the rate of plasmid DNA synthesis appears to increase during the first few hours of treatment. As indicated in Fig. 10, the replication rate after the first hour was two- to threefold greater than that observed during the first hour.

Additional experiments have indicated that plasmid replication continues under these conditions for approximately 10 hr to a point representing a five- to sixfold increase in the number of plasmid molecules per cell. Experiments with CAP concentrations as high as 280 μg/ml yielded similar results.

Experiments involving amino acid starvation as a means of inhibiting protein synthesis provided results very similar to those from the CAP experiments. Upon removal of Casamino Acids, plasmid DNA replication continued and noncomplexed DNA accumulated. When glucose was absent in such an experiment, the

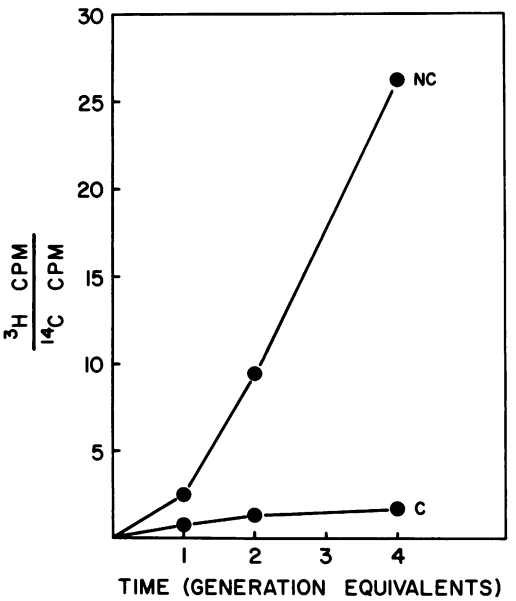

FIG. 10. *Relative amounts of incorporation of [^{3}H]-thymine into complexed and noncomplexed ColE1 DNA in cells grown in the presence of CAP. The data were obtained from the experiment described in Fig. 9.*

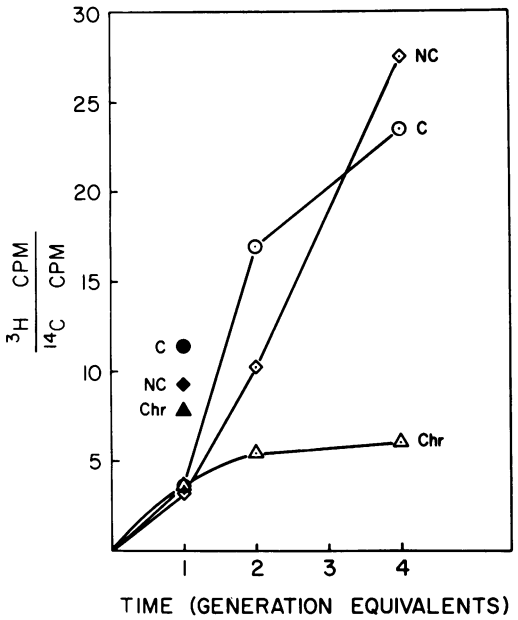

FIG. 11. *Extent of replication of DNA found in the complexed and noncomplexed states in cells grown in the presence of glucose and either in the absence or presence of CAP. The ratios of ^3H to ^{14}C were calculated from the data presented in Fig. 9. The values obtained for chromosomal DNA were based on the amount of ^3H and ^{14}C counts in the crude lysates. NC indicates noncomplexed ColE1 DNA; C indicates complexed ColE1 DNA; Chr indicates chromosomal DNA. The solid points represent the one-generation control cells; the open points correspond to the CAP-treated cells.*

extent of replication was limited to approximately one doubling of plasmid DNA. However, when glucose was present, replication continued to the extent of more than a threefold increase in plasmid DNA. In the latter case, the rate of plasmid replication was about twofold faster than that observed in a one-generation control culture, thus confirming an observation previously reported by Bazaral and Helinski (1).

DISCUSSION

ColE1 DNA is present in host *E. coli* cells to the extent of approximately 24 copies per growing cell. This value does not change under conditions where the amount of chromosomal DNA per cell varies. This finding indicates a fundamental difference between ColE1 DNA and chromosomal DNA with regard to the control and maintenance of the number of these replicons.

The percentage of ColE1 DNA that can be isolated as a relaxation complex of supercoiled DNA and protein is much lower when the cells are grown in a medium containing glucose than in a medium lacking glucose. When cells are grown in the presence of glucose for several generations and then shifted to a medium lacking glucose, there is a rapid increase in the percentage of ColE1 DNA that is found in the complexed state. This increase is accompanied by a two- to threefold increase in the rate of synthesis of ColE1 DNA. Although both phenomena are inhibited if CAP is present, these data do not necessarily indicate a direct relationship between relaxation complex and the replication of ColE1 DNA. In fact, a burst of plasmid DNA synthesis might be expected if a rapid decrease in cell mass were to occur in order to maintain a constant number of plasmid molecules per cell. DNA synthesis is not required for the synthesis of the plasmid DNA-protein complex since thymine starvation did not prevent the shift to the complexed state when glucose was removed. Experiments utilizing nalidixic acid to inhibit DNA synthesis yield similar results (Clewell, *unpublished data*).

In experiments in which cells were grown in the absence of glucose and then shifted to a medium containing glucose, no noticeable difference could be detected in the amount of complexed plasmid DNA present after 15 min. This supports the notion that the protein in the complex is stable physiologically and any decrease in its level is the result of dilution due to cell division.

The effect of glucose suggests the involvement of catabolite repression in the regulation of synthesis of complexed ColE1 DNA. This is supported by recent data demonstrating that the effect of glucose can be reversed by exogenous cyclic AMP (Katz, Kingsbury, and Helinski, *manuscript in preparation;* Clewell, *unpublished data*).

Inhibition of protein synthesis by CAP treatment or starvation for amino acids resulted in a gradual halt in chromosomal DNA synthesis consistent with completion of a round of replication. In contrast, however, plasmid DNA synthesis continued to various extents depending upon the particular conditions employed. In all cases, the synthesis of the protein component(s) of the relaxation complex was inhibited, whereas the protein present before inhibition appeared to be stable. Plasmid DNA that was synthesized accumulated as noncomplexed, covalently closed molecules.

On the basis of data obtained from density-

shift and amino acid starvation experiments, it was previously proposed (1) that a random selection of ColE1 DNA molecules to be replicated in a cell takes place by a mechanism involving a limited number, or activity, of a stable replicator(s) and that this replicator would be subject to regulatory control by the metabolic state of the cell. It has been demonstrated that ColE1 DNA interacts specifically with a protein substance (relaxation protein), the level of which depends on the metabolic state of the cell. It has been suggested (5, 6) that this protein may be involved in the initiation of plasmid DNA replication on the basis of the assumption that a covalently closed circular DNA molecule must at some time acquire a nick to allow separation of the template strands during semiconservative replication and the potential role of the relaxation complex in the nicking event. It was also proposed that plasmid molecules found in the complexed state represent a "repressed" (or resting) condition with regard to replication. This was based on the assumption that if the travel time for replication of a ColE1 DNA molecule is similar to that of the host chromosome, then it would take approximately 4 to 7 sec to complete a round of ColE1 DNA replication. Thus, in a randomly growing culture at any particular instant, the vast majority of plasmid molecules are not in the process of replication. It is conceivable that the ColE1 DNA complex is part of an inactive replicator that is activated in vivo by a specific signal. During replication of ColE1 DNA in the presence of CAP or under conditions of starvation for amino acids, it has been shown that plasmid replication continues and the ColE1 DNA freely associates with or dissociates from the protein component(s) of the relaxation complex as predicted (1) for the proposed replicator.

The timing of the ColE1 DNA initiation event does not appear to be tightly coupled to the stage of replication of the host chromosome since the initiation of plasmid DNA synthesis continues long after chromosomal DNA synthesis has been stopped by CAP treatment. The increase in replication rate during amino acid starvation (in the presence of glucose) (1) would also appear to reflect a replication control process that at least under certain conditions can be uncoupled from the control process for chromosomal DNA replication. However, it seems reasonable that under normal growth conditions there is coordination between host chromosome and plasmid DNA synthesis since a constant number of plasmid copies per cell is maintained during growth. It is possible that the inhibition of protein synthesis may in fact produce a condition that more closely resembles the process of plasmid replication during conjugal transfer of ColE1 DNA.

Although the ColE1 DNA relaxation complex appears to have certain properties that suggest a role for this complex in the initiation of plasmid DNA replication, direct evidence for this role has not been obtained. Using the extended CAP treatment which allows continued synthesis of plasmid DNA in the absence of chromosome synthesis, it has been possible to mutagenize ColE1 DNA selectively and obtain conditional plasmid DNA replication mutants (Kingsbury and Helinski, *manuscript in preparation*). An investigation is currently underway to determine if any of these plasmid DNA replication mutants are altered with respect to the properties of the relaxation complex of supercoiled ColE1 DNA and protein.

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