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Influence of Protein and Ribonucleic Acid Synthesis on the Replication of the Bacteriocinogenic Factor Clo DF13 in *Escherichia coli* Cells and Minicells

E. VELTKAMP, W. BARENDSEN, AND H. J. NIJKAMP

Department of Molecular Genetics, Biological Laboratory, Free University, Amsterdam-Buitenveldert, The Netherlands

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The influence of ribonucleic acid (RNA) and protein synthesis on the replication of the cloacinogenic factor Clo DF13 was studied in *Escherichia coli* cells and minicells. In chromosomeless minicells harboring the Clo DF13 factor, Clo DF13 deoxyribonucleic acid (DNA) synthesis is slightly stimulated after inhibition of protein synthesis by chloramphenicol or puromycin and continues for more than 8 h. When minicells were treated with rifampin, a specific inhibitor of DNA-dependent RNA polymerase, Clo DF13 RNA and DNA synthesis appeared to stop abruptly. In cells, the Clo DF13 factor continues to replicate during treatment with chloramphenicol long after chromosomal DNA synthesis ceases. When rifampin was included during chloramphenicol treatment of cells, synthesis of Clo DF13 plasmid DNA was blocked completely. Isolated, supercoiled Clo DF13 DNA, synthesized in cells or minicells in the presence of chloramphenicol, appeared to be sensitive to ribonuclease and alkali treatment. These treatments convert a relatively large portion of the covalently closed Clo DF13 DNA to the open circular form, whereas supercoiled Clo DF13 DNA, isolated from non-chloramphenicol-treated cells or minicells, is not significantly affected by these treatments. These results indicate that RNA synthesis and specifically Clo DF13 RNA synthesis are involved in Clo DF13 DNA replication and that the covalently closed Clo DF13 DNA, synthesized in the presence of chloramphenicol, contains one or more RNA sequences. De novo synthesis of chromosomal and Clo DF13-specific proteins is not required for the replication of the Clo DF13 factor. Supercoiled Clo DF13 DNA, isolated from a polA107 (Clo DF13) strain which lacks the 5′ → 3′ exonucleolytic activity of DNA polymerase I, is insensitive to ribonuclease or alkali treatment, indicating that in this mutant the RNA sequences are still removed from the RNA-DNA hybrid.

The cloacinogenic factor DF13 (Clo DF13) is a bacterial plasmid determining the production of the extracellular antibiotic protein cloacin DF13. The Clo DF13 factor originates from *Enterobacter cloacae* (34); however, *Escherichia coli* can serve as the host as well. The non-transmissible Clo DF13 factor, a relatively small circular deoxyribonucleic acid (DNA) molecule (molecular weight 6×10^6) with a contour length of 3.0 μm (36), is present in *E. coli* cells to the extent of 15 copies per cell (25a). The Clo DF13 DNA directs the synthesis of at least four messenger ribonucleic acid (mRNA) species and eight proteins (25, 25a). The function of only one protein, cloacin DF13, is known. Cloacin DF13 inhibits protein synthesis of sensitive cells by a specific cleavage of 16*S* ribosomal RNA (rRNA) near its 3′ terminus (17, 18). This action of cloacin DF13 resembles closely that of colicin E3; in some other aspects its action corresponds to that of Col E1 (4, 17, 18, 32).

With respect to the replication, there is a remarkable difference between transmissible and non-transmissible plasmids. Whereas transmissible plasmids seem to require DNA polymerase III for their replication (15), Clo DF13 shows, like other non-transmissible plasmids such as Col E1 and minicircular DNA of *E. coli* 15, a unique dependence on DNA polymerase I (13, 16, 22, 23, 36a). DNA polymerase II (36a) and DNA polymerase III (15, 16, 36a) seem not to be required for the replication of these latter plasmids. Whereas inhibition of protein synthesis by chloramphenicol (CAP) blocks the replication of chromosomal DNA and DNA of transmissible plasmids (2), de novo protein

synthesis appeared not to be required for the replication of the non-transmissible plasmid Col E1 (6), minicircular DNA of *E. coli* 15 (27), or Clo DF13 (this report). RNA synthesis is a prerequisite for the replication of chromosomal DNA and for DNA of transmissible as well as non-transmissible plasmids (3, 5, 7, 8, 12, 20, 24, 26, 27, 33, 35). It has been shown that rifampin-sensitive RNA synthesis is required for the initiation of chromosomal DNA replication at the fixed origin (26). The synthesis of "Okazaki-pieces" seems to require rifampin-insensitive RNA primers (35). It has been shown that Col E1 DNA, synthesized in the presence of CAP, contains one or more ribonucleotides (3). RNA primer sequences must be rapidly removed from the DNA-RNA hybrid. It has been suggested that the 5′ → 3′ exonucleolytic activity of DNA polymerase I is involved in the degradation of RNA primers (31, 38). The 5′ → 3′ exonucleolytic activity, however, seems not to be essential for the maintenance of the DNA polymerase I-dependent Clo DF13 factor in *E. coli* (36a).

The influence of RNA and protein synthesis on the replication of the Clo DF13 factor in *E. coli* cells and minicells was studied. Minicells are small, spherical bodies produced by an aberrant cell division of an *E. coli* mutant strain (1). They contain little if any chromosomal DNA, but may contain plasmid DNA. Minicells were reviewed extensively by Curtiss and Frazer (R. Curtiss III and A. C. Frazer, Curr. Top. Microbiol. Immunol., 1973, in press). It has been demonstrated that Clo DF13 DNA segregates into minicells of a Clo DF13-harboring, minicell-producing *E. coli* mutant (25). Clo DF13-harboring minicells are able to synthesize Clo DF13 DNA, RNA, and protein (this report; 25, 25a). The minicell system enables us to study specifically the role of Clo DF13 RNA and protein synthesis in the replication of Clo DF13 DNA. Synthesis of Clo DF13 DNA in cells continues when protein synthesis is inhibited by CAP, but is completely blocked when RNA synthesis is inhibited by rifampin. Similar results were obtained with minicells, indicating that Clo DF13 RNA synthesis, but not de novo Clo DF13 protein synthesis, is involved in the replication of the Clo DF13 factor. Supercoiled Clo DF13 DNA, isolated from cells and minicells after CAP treatment, is sensitive to ribonuclease and alkali treatment, indicating the presence of RNA sequences. Supercoiled Clo DF13 DNA, synthesized in cells or minicells cultured without CAP, appeared to be insensitive to ribonuclease and alkali treatment. Similar results were obtained with purified Clo DF13 DNA synthesized in a *polA107* (Clo DF13) strain which lacks the 5′ → 3′ exonucleolytic activity of DNA polymerase I.

MATERIALS AND METHODS

Bacterial strains. The minicell-producing strain *E. coli* K-12 P678-54 (1), obtained from K. J. Roozen, has the following markers: *thr leu lacY minA gal minB str*^{r} *thi* (for *min* genotype see reference 30). A P678-54 strain, harboring the cloacinogenic factor DF13 (Clo DF13), was isolated in our laboratory (25). *E. coli* K-12 KMBL 1789 (*arg thy pheA bio polA107*), obtained from B. W. Glickman, was made cloacinogenic as described previously (36a).

Media. The media used were as follows: (i) brain heart infusion (BHI; Oxoid, 3.7%) and (ii) minimal medium consisting of: $KH_2PO_4 \cdot 2H_2O$, 0.3%; $Na_2HPO_4 \cdot 2H_2O$, 0.7%; NH_4Cl, 0.1%; NaCl; 0.05%, $CaCl_2$, 0.001%; $MgSO_4$, 0.025%; and glucose, 0.5%. This medium was supplemented with 0.5% Casamino Acids (Difco) unless otherwise indicated. Demineralized water was used as solvent. When required, other supplements were used at the following final concentrations (micrograms per milliliter): thiamine-hydrochloride, 1; threonine, 40; and leucine, 20.

Growth and labeling of bacteria. *E. coli* P678-54 (Clo DF13) and KMBL 1789 (Clo DF13), grown overnight at 37 C in BHI medium, were inoculated 1/100 into fresh BHI medium and allowed to grow to an optical density of 0.35 at 660 nm. In the absence of inhibitors, cells were labeled with 10 μCi of [*methyl*-^{3}H]thymidine per ml in the presence of 300 μg of deoxyadenosine per ml. When inhibitors were used, 3 h after the addition of 150 μg and/or 100 μg of rifampin per ml, cultures were labeled with 10 μCi of [*methyl*-^{3}H]thymidine per ml in the presence of 300 μg of deoxyadenosine per ml.

Purification of minicells. The minicell purification was performed by the method of Roozen et al. (30) with minor modifications. A culture of strain P678-54 (Clo DF13) or P678-54 was grown in 1.6 liters of BHI medium at 37 C. The culture was centrifuged in the cold for 30 min at 4,000 rpm in an MSE18 rotor (6 × 250 ml). The supernatant was then centrifuged for 15 min at 12,000 rpm in a rotor (6 × 250 ml). The pellets were suspended in 6 ml of cold, sterile, buffered saline with gelatine (BSG; see reference 11). Minicells were isolated from these suspended pellets by three successive sedimentations through 40-ml, 5 to 20% (wt/vol) sucrose gradients in BSG. These gradients were centrifuged at 3,700 rpm for 30 min in a Christ minifuge. After the third sucrose gradient, the minicells were suspended in minimal medium at an absorbancy of 0.2 at 620 nm. The purified minicell fraction usually contained less than 100 contaminating bacterial cells per 10^8 minicells. All seperations were done at approximately 4 C.

Radioactive labeling of minicells. DNA synthesis in minicells was assayed by the incorporation of [*methyl*-^{3}H]thymidine into cold trichloroacetic acid-

insoluble material. Purified minicells were suspended in minimal medium, supplemented with thiamine, threonine, and leucine, to an absorbancy of 0.2 at 620 nm. After a 15-min preincubation at 37 C, 30 μCi of [^{3}H]thymidine per ml and 300 μg of deoxyadenosine per ml were added to the cultures. RNA synthesis in minicells was determined by the incorporation of [^{3}H]uridine into cold trichloroacetic acid-insoluble material. Purified minicells, suspended and preincubated as described above, were labeled with 65 μCi of [^{3}H]uridine per ml. Protein synthesis in minicells was assayed by the incorporation of [^{14}C]leucine into hot acid-insoluble material. Purified minicells were suspended as described above, except that leucine was omitted from the medium. After 15 min of incubation at 37 C, 20 μCi of [^{14}C]leucine per ml was added to the cultures. For following DNA, RNA, and protein synthesis, samples (100 μliters) were collected at various times after labeling onto Whatman 3 MM filter discs. Where specified, inhibitors were added at the beginning of the preincubation to the following final concentrations (micrograms per milliliter): rifampin, 100; CAP, 150; and puromycin, 30.

Isolation of Clo DF13 DNA. Covalently closed Clo DF13 DNA was purified from labeled cells before and after incubation with CAP (150 μg/ml) for 18 h. Cells were lysed by the method of Clewell and Helinski (9), with Brij-58 (final concentration, 1%, wt/vol) as detergent. The crude lysate was centrifuged at 48,000 × *g* for 30 min in an MSE 75 ultracentrifuge. This centrifugation step pellets the bulk of the chromosomal DNA, leaving plasmid DNA in the supernatant. DNA in the supernatant fraction (cleared lysate) was fractionated by the dye-buoyant density centrifugation procedure of Radloff et al. (29) as described previously (36a). Fractions containing supercoiled Clo DF13 DNA were pooled from the dye-buoyant density gradient. The ethidium bromide was removed from the pooled fractions by a single passage through a column (0.5 by 3.5 cm) of analytical-grade Dowex-50 resin. CsCl was then removed by dialysis at 4 C for 12 h against TESP buffer (0.05 M tris(hydroxymethyl)-aminomethane [Tris], 5 mM ethylenediaminetetraacetic acid [EDTA], 0.05 M NaCl, and 0.05 M K_2HPO_4, pH 8.0). For isolation of Clo DF13 DNA synthesized in minicells, ^{3}H-labeled minicells were lysed, before and after incubation in the presence of CAP (150 μg/ml), as described above. After centrifugation of the crude lysate for 20 min at 40,000 × *g*, the supernatant was dialyzed at 4 C for 4 h against TESP buffer.

RNase treatment. A 200-μliter sample of supercoiled Clo DF13 DNA, isolated from cells or minicells, was incubated by the method of Blair et al. (3) with 100 μliters of a solution containing 3 mg of pancreatic ribonuclease A (RNase A) per ml and 10,000 U of RNase T_1 per ml in 10 mM Tris (pH 7.5) for 30 min at 37 C. The RNase solution was heated at 100 C for 10 min before use to inactivate deoxyribonuclease. After RNase incubation, 100 μliters of a solution containing 5 mg of Pronase per ml in TES (0.05 M Tris, 5 mM EDTA, 0.05 M NaCl, pH 8.0) was added to the reaction mixture. Incubation was continued for 10 min at 37 C. The Pronase had been digested for 60 min at 37 C. After Pronase treatment, the reaction mixture was layered over a 4.0-ml, 5 to 20% (wt/vol) sucrose gradient prepared in 0.05 M Tris-5 mM EDTA-0.55 M NaCl (pH 7.5). After centrifugation for 130 min at 30,000 rpm in an SW6 x 5 Ti-rotor of an MSE 75 ultracentrifuge at 4 C, 100-μliter fractions were collected from the top of the tube into scintillation vials.

Alkali treatment. Alkali treatment of isolated Clo DF13 DNA involved the addition of 150 μliters of phosphate buffer (0.2 M K_2HPO_4, pH 13.0) to 50 μliters of purified DNA. After incubation for 90 min at 37 C, the reaction mixture was neutralized by the addition of 200 μliters of 1.0 M Tris (pH 7.5). The neutralized mixture was then analyzed by centrifugation in a 4.0-ml, 5 to 20% (wt/vol) sucrose gradient containing 0.05 M Tris, 1.05 M NaCl, and 5 mM EDTA (pH 8.0). After centrifugation for 110 min at 50,000 rpm and 15 C in an SW6 x 5 Ti-rotor of an MSE 75 ultracentrifuge, 100-μliter fractions were collected from the top of the tube for determination of radioactivity.

Determination of radioactivity. All radioactive samples except those from the sucrose gradients were collected onto Whatman 3 MM filter discs and precipitated with cold 10% trichloroacetic acid. After being washed successively with 5% trichloroacetic acid, ethanol-ether and ether, the filters were dried and placed in scintillation vials containing 10 ml of toluene-2,5-diphenyloxazole (PPO)-1,4-bis-(5-phenyloxazolyl)-benzene (POPOP). Filters with protein samples were treated with 10% trichloroacetic acid at 80 C instead of cold 10% trichloroacetic acid. Samples of sucrose gradients were counted with 10 ml of Dioxan containing PPO, POPOP, naphthalene, and 10% water. Scintillation counting was performed in a Nuclear-Chicago Mark I or Mark II liquid scintillation counter.

Chemicals. Reagents and sources were as follows. Puromycin and Dowex H^+ (type 50WX 8, 50 to 100 mesh) were obtained from Serva. Beef pancrease RNase A (code RASE) and RNase T_1 (code RT_1) were purchased from Worthington Biochemical Corp. Rifampin was obtained from Lepetit. CAP was from Sigma Chemical Co. [*methyl*-^{3}H]thymidine (specific activity 15,000 mCi/mmol), [^{3}H]uridine (specific activity 2,000 to 5,000 mCi/mmol) and [^{14}C]leucine (specific activity 50 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, England. Pronase (B grade, free from nucleases) was from Calbiochem. RNase-free sucrose was purchased from Serva and autoclaved before use. The sources of all other chemicals used were as described previously (36a).

RESULTS

Influence of RNA and protein synthesis on Clo DF13 DNA replication in cells. *E. coli* P678-54 (Clo DF13) was grown at 37 C in BHI medium to an absorbancy at 660 nm (A_{660}) of 0.30. The culture was then divided into three

equal portions (A, B, and C). Culture A was labeled immediately with 10 μCi of [^{3}H]thymidine per ml for 1 h. Before being labeled with 10 μCi of [^{3}H]thymidine per ml, culture B was incubated for 3 h at 37 C in the presence of 150 μg of CAP per ml and culture C was incubated for 3 h at 37 C in the presence of 150 μg of CAP per ml and 100 μg rifampin per ml. ^{3}H-labeled DNA was isolated from the cultures by the lysozyme-Brij 58 technique. The resultant "cleared lysates" were analyzed in cesium chloride-ethidium bromide gradients. By this procedure, supercoiled Clo DF13 DNA (peak II in Fig. 1) is separated from the chromosomal DNA and from the open circular Clo DF13 DNA (peak I in Fig. 1). In contrast to chromosomal DNA, the Clo DF13 plasmid continues to replicate in the presence of CAP (Fig. 1).

To investigate the possibility that during CAP treatment Clo DF13 DNA with aberrant *S* values is synthesized, fractions of peak II (Fig. 1A and 1B) were pooled and analyzed by sucrose gradient centrifugation as described before (36a). No differences in *S* values between non-CAP-treated Clo DF13 DNA and CAP-treated Clo DF13 DNA were observed (gradients not shown). When rifampin was included during CAP treatment, no incorporation of label into Clo DF13 DNA was observed (Fig. 1C). Because the synthesis of Clo DF13 DNA does not depend on de novo protein synthesis, as demonstrated by its insensitivity towards CAP (Fig. 1B), the rifampin effect cannot result from a block of the transcription of mRNAs which code for proteins involved in Clo DF13 DNA replication. Therefore, we conclude that RNA synthesis itself is required for Clo DF13 DNA synthesis. That the synthesis of Clo DF13 RNA is involved specifically in Clo DF13 DNA replication was shown by experiments with *E. coli* minicells.

Influence of RNA and protein synthesis on Clo DF13 DNA replication in minicells. It has been shown previously that the cloacinogenic factor Clo DF13 segregates into minicells of the Clo DF13-harboring *E. coli* strain, P678-54, and that this Clo DF13 factor is the only DNA present in these otherwise chromosomeless minicells (25). These properties make the minicell system very valuable for studying replication, transcription, and translation of the Clo DF13 plasmid. To investigate the effects of transcriptional and translational inhibitors on the replication of the Clo DF13 DNA, the ability of Clo DF13-containing minicells to incorporate [^{3}H]thymidine in the presence and absence of CAP, puromycin, or rifampin was determined.

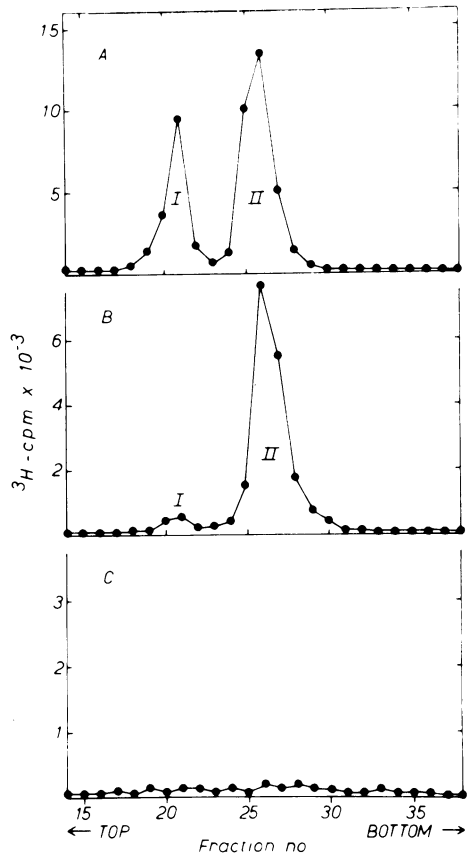

FIG. 1. *Effects of CAP and rifampin on the synthesis of Clo DF13 DNA in E. coli cells. ^{3}H-labeled DNA, synthesized in E. coli P678-54 (Clo DF13), was isolated and analyzed by dye buoyant density centrifugation as described in Materials and Methods. (A) Profile obtained from a culture labeled for 1 h with [^{3}H]thymidine. (B) Profile obtained from a culture incubated for 3 h in the presence of 150 μg of CAP per ml before being labeled for 1 h with [^{3}H]thymidine. (C) Profile obtained from a culture incubated for 3 h in the presence of 150 μg of CAP per ml and 100 μg of rifampin per ml before being labeled for 1 h with [^{3}H]thymidine.*

The incorporation of [^{3}H]thymidine by minicells harboring the Clo DF13 factor is not reduced by protein synthesis inhibitors such as CAP and puromycin, but actually is slightly stimulated (Fig. 2). The presence of rifampin in the incubation medium inhibits the synthesis of DNA drastically. Chromosomal DNA synthesis due to contaminating cells present in the minicell preparation is unlikely (less than 100 cells per 10^8 minicells). To exclude this possibility, however, identical experiments were performed

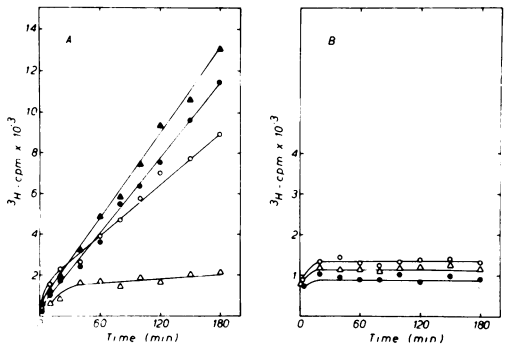

FIG. 2. *Effects of CAP, puromycin, and rifampin on the synthesis of Clo DF13 DNA in E. coli minicells. Minicells of P678-54 (Clo DF13) and P678-54 were purified and suspended in minimal medium. After 15 min of preincubation at 37 C, 30 μCi of [^3H]thymidine per ml was added, and samples were taken at intervals for determination of radioactivity. (A) Incorporation of [^3H]thymidine by CloDF13 plasmid-containing minicells in the absence (○) of inhibitors and in the presence of CAP (●), puromycin (▲), or rifampin (△). (B) Incorporation of [^3H]thymidine by plasmid-less minicells in the absence (○) of inhibitors and in the presence of CAP (●) or rifampin (△). Inhibitors, when present, were added at the start of the preincubation at the following final concentrations (micrograms per milliliter): CAP, 150; puromycin, 30; and rifampin, 100.*

with plasmidless minicells, isolated from strain P678-54, with a similar grade of purity. Only a low background level of incorporation is observed in noncloacinogenic minicells (Fig. 2B). The presence of CAP or rifampin does not affect this background level. From this result it can be concluded that the incorporation of DNA precursors by Clo DF13-containing minicells is due to the presence of the Clo DF13 plasmid.

It has been shown by Kool et al. (25) that Clo DF13-containing minicells are capable of protein synthesis. To determine whether CAP and puromycin do affect protein synthesis in minicells as in cells, the ability of Clo DF13-containing minicells to incorporate [^{14}C]leucine in the presence or absence of CAP and puromycin was measured. The results (Fig. 3) show that the presence of CAP or puromycin in the incubation media reduced the incorporation to its initial background level. From these results we conclude that Clo DF13 DNA synthesis does not depend on de novo synthesis of Clo DF13-specific proteins. Similar experiments were conducted to assess the effects of rifampin, puromycin, and CAP treatment on RNA synthesis in minicell preparations. Treatment of Clo DF13-containing minicells with rifampin caused an almost complete reduction in the incorporation of [^3H]uridine into Clo DF13 RNA. The rate of incorporation was not reduced, but slightly stimulated, by treatment of minicells with CAP or puromycin (Fig. 4). It has been demonstrated by RNA-DNA hybridization experiments that RNA synthesized in minicells is Clo DF13 specific (25a). From the results obtained from the minicells experiments, we conclude the following. (i) Minicells harboring the Clo DF13 plasmid are able to synthesize DNA, RNA, and protein. (ii) De novo synthesis of Clo DF13-specific proteins is not a requirement for Clo DF13 DNA synthesis. (iii) Clo DF13 RNA synthesis is a prerequisite for the replication of the Clo DF13 factor.

Conversion of Clo DF13 DNA, isolated from cells and minicells, by ribonuclease and alkali treatment. The experiments described

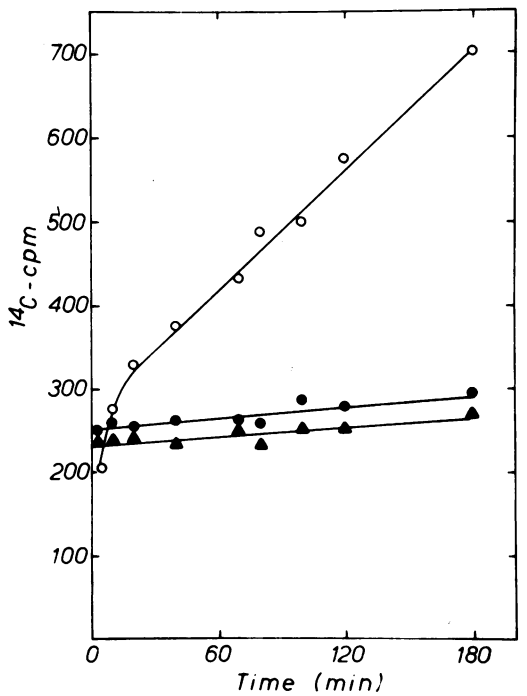

FIG. 3. *Effects of CAP and puromycin on the incorporation of [^{14}C]leucine by plasmid-containing minicells. Minicells of p678-54 (Clo DF13) were purified and suspended in minimal medium to an A_{620} of 0.20. After 15 min of preincubation at 37 C, 20 μCi of [^{14}C]leucine per ml was added, and samples were taken at intervals for determination of radioactivity. Inhibitors were added at the start of the preincubation. Symbols: ○, no inhibitors present; ●, 150 μg of CAP per ml present; ▲, 30 μg of puromycin per ml present.*

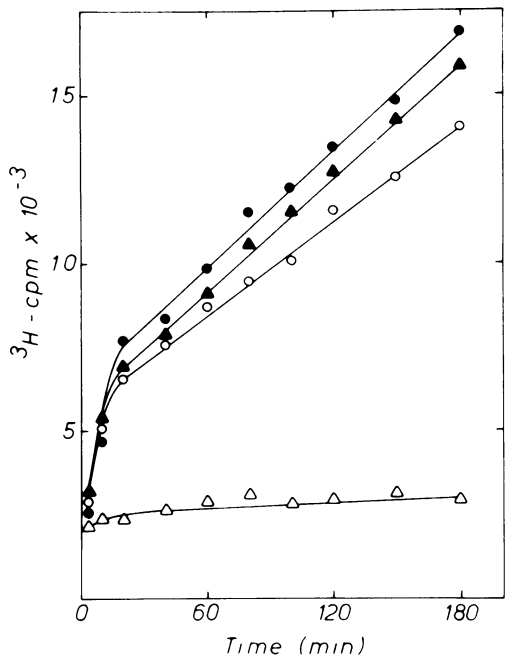

FIG. 4. *Effects of CAP, puromycin, and rifampin on the ability of plasmid-containing minicells to incorporate [^3H]uridine into RNA. Minicells of P678-54 (Clo DF13) were purified and suspended as described in Materials and Methods. After preincubation for 15 min at 37 C, 65 μCi of [^3H]uridine per ml was added, and samples were taken at intervals for determination of radioactivity. Inhibitors, when present, were added at the start of the preincubation. Symbols: ○, no inhibitor present; ●, 150 μg of CAP per ml present; ▲, 30 μg of puromycin per ml present; and △, 100 μg of rifampin per ml present.*

in the previous sections suggest than RNA synthesis is involved in the replication of the Clo DF13 plasmid. When RNA serves as a primer in the Clo DF13 replication, then there must exist, at least for a short time, RNA-DNA duplexes in the Clo DF13 DNA molecule. It has been shown that Col E1 DNA synthesized in the presence of CAP contains one or more ribonucleotides (3). To investigate whether RNA sequences are also present in Clo DF13 DNA, it was decided to test the sensitivity of supercoiled Clo DF13 DNA, synthesized in the presence or absence of CAP, to alkaline and RNase hydrolysis. DNA duplexes containing a RNA-DNA hybrid fragment are sensitive to alkaline and ribonuclease hydrolysis. Incubation of supercoiled Clo DF13 DNA isolated from CAP-treated cells, with ribonucleases A and T_1 converts more than 25% of the Clo DF13 DNA from a covalently closed form (sedimenting as 23.5*S*) to an open circular form (sedimenting as 18.5*S*) (Fig. 5B, Table 1), whereas supercoiled Clo DF13 DNA isolated from untreated cells is not affected by RNase treatment (Fig. 5A and Table 1).

The sensitivity of supercoiled Clo DF13 DNA to alkali treatment was examined by incubating CAP-treated Clo DF13 DNA and non-CAP-treated Clo DF13 DNA at pH 13.0. After 90 min of incubation at 37 C, the pH was reduced to 8.0 and the reaction mixtures were analyzed by sucrose gradient centrifugation. After centrifugation, the percent supercoiled Clo DF13 DNA of total Clo DF13 DNA recovered from the gradients was determined (Table 1). Incubation of CAP-treated Clo DF13 DNA at pH 13 induces a loss of the covalently closed form, whereas non-CAP-treated Clo DF13 DNA is scarcely (1.9% conversion) affected by the high pH conditions.

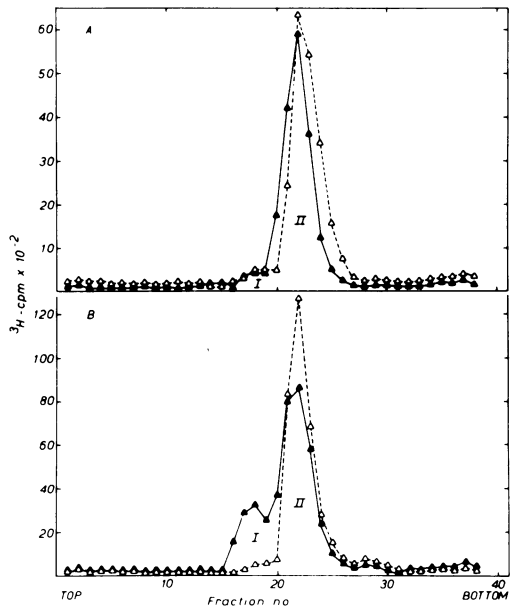

FIG. 5. *Sensitivity of covalently closed Clo DF13 DNA, isolated from cells, to RNase. Supercoiled Clo DF13 DNA, purified from CAP-treated (B) and untreated (A) P678-54 (Clo DF13) cells, was incubated for 30 min at 37 C in the presence (▲) and absence (△) of pancreatic RNase A (1 mg/ml) and RNase T_1 (3,000 U/ml). After Pronase treatment, the reaction mixtures were layered over 5 to 20% sucrose gradients. Centrifugation conditions were as described in Materials and Methods. Peak I ($s_{20,w}$ value of 18.5 S) represents open circular Clo DF13 DNA; peak II ($s_{20,w}$ value of 23.5S) represents supercoiled Clo DF13 DNA. S values are related to the following markers: ØX174 DNA (18.4S) and 17S and 26S rRNA.*

TABLE 1. *Conversion of supercoiled Clo DF13 DNA by ribonuclease or alkali treatment*

Preparation	Conversion of supercoiled Clo DF13 DNA[a]	
	RNase treatment[d]	Alkali treatment[d]
A. Non-CAP Clo DF13 DNA[b]	0	1.9
CAP Clo DF13 DNA[c]	25.3	42.1
B. Non-CAP Clo DF13 DNA[b]	0	2.6
CAP Clo DF13 DNA[c]	20.7	49.2

[a] Supercoiled Clo DF13 DNA is expressed as a percentage of total Clo DF13 DNA recovered from the gradients.

[b] Non-CAP Clo DF13 DNA indicates purified Clo DF13 DNA from untreated cells (A) or minicells (B).

[c] CAP Clo DF13 DNA indicates purified Clo DF13 DNA from chloramphenicol-treated cells (A) or minicells (B).

[d] Procedures are described in the text.

Identical experiments were performed with purified Clo DF13 DNA synthesized in the presence and absence of CAP in minicells. Minicells contain little if any chromosomal DNA; therefore, isolated DNA from minicells was not purified by CsCl-EB gradients after the clearing centrifugation step, but directly used for incubation experiments with RNases and alkali treatment. Treatment of Clo DF13 DNA, isolated from CAP-treated minicells, with ribonuclease A and T_1 induces a conversion of the plasmid DNA from the covalently closed form to an open circular form of about 20% (Fig. 6B and Table 1). The covalently closed form of non-CAP-treated Clo DF13 DNA is not significantly affected by this treatment (Fig. 6A and Table 1). The different response of non-CAP- and CAP-treated Clo DF13 DNA, synthesized in minicells, on alkaline treatment is shown in Table 1B. About 50% of the CAP-treated Clo DF13 DNA is nicked during incubation at high pH, in contrast to non-CAP-treated Clo DF13 DNA which is only affected slightly by this treatment. These results suggest that, in the presence of CAP, supercoiled RNA containing Clo DF13 DNA is synthesized in cells and minicells.

Role of the 5′ → 3′ exonucleolytic activity of DNA polymerase I in the removal of RNA primer sequences. It has been shown that the Clo DF13 plasmid could be maintained in an *E. coli polA107* strain (36a). The *polA107* strain (KMBL1789), isolated by Glickman et al. (14), possesses the polymerase and 3′ → 5′ exonucleolytic activities but lacks the 5′ → 3′ exonucleolytic activity of DNA polymerase I (19). To investigate the possibility that the 5′ → 3′ exonucleolytic activity of DNA polymerase I is involved in the removal of the RNA fragment from the Clo DF13 DNA, we examined the sensitivity of purified, covalently closed Clo DF13 DNA, isolated from a *polA107* (Clo DF13) strain, to RNase and alkali. Assuming that the 5′ → 3′ exonuclease is responsible for degradation of RNA primer sequences, one would expect that, in this *polA107* strain, Clo DF13 DNA structures containing a short RNA-DNA fragment accumulate. However, supercoiled Clo DF13 DNA isolated from a *polA107* (Clo DF13) strain, cultured without CAP appeared to be insensitive to RNase or alkali treatment (Fig. 7A). This result indicates that in a *polA107* strain RNA sequences are still removed from the Clo DF13 DNA molecule. Clo DF13 DNA molecules, synthesized in the presence of CAP in *polA107* (Clo DF13) bacteria, are sensitive to RNase treatment (Fig. 7B).

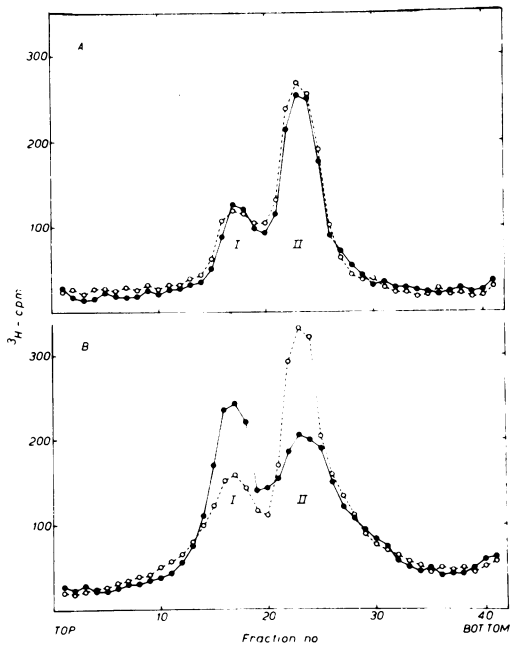

FIG. 6. *Sensitivity of Clo DF13 DNA, isolated from minicells, to RNase. Clo DF13 DNA, isolated from chloramphenicol-treated (B) and untreated (A) P678-54 (Clo DF13) minicells, was tested for its sensitivity to RNase A and T_1 as described in the legend of Fig. 5. A 300-μliter portion of each reaction mixture was analyzed on 5 to 20% sucrose gradients. Symbols: ●, RNase-treated Clo DF13 DNA; ○, untreated Clo DF13 DNA.*

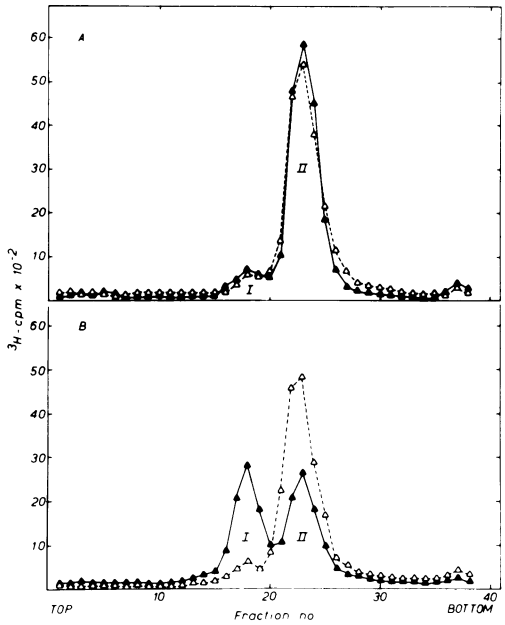

FIG. 7. *Sensitivity of supercoiled Clo DF13 DNA, isolated from polA107 (Clo DF13) cells, to RNase. Covalently closed Clo DF13 DNA isolated from CAP-treated (B) and untreated (A) polA107 (Clo DF13) cells, was tested for its sensitivity to RNase A and T_1 as described in the legend of Fig. 5. A 300-μliter portion of each reaction mixture was analyzed on 5 to 20% sucrose gradients. Symbols: ▲, RNase-treated Clo DF13 DNA; △, untreated Clo DF13 DNA.*

DISCUSSION

We studied the influence of RNA and protein synthesis on the replication of the cloacinogenic factor DF13 in *E. coli* cells and minicells. The minicell system has proved to be a valuable system for specifically studying plasmid replication, transcription, translation, and the interactions between these processes. We took advantage of this system to study the role of Clo DF13 RNA and protein synthesis in the replication of the Clo DF13 factor. Synthesis of Clo DF13 DNA in cells continues when protein synthesis is inhibited by CAP long after chromosomal DNA synthesis had ceased. Inhibition of RNA synthesis by rifampin, however, blocks Clo DF13 DNA synthesis completely. When minicells are treated with CAP or puromycin, Clo DF13 protein synthesis is inhibited, but the incorporation of DNA precursors into Clo DF13 DNA is slightly stimulated. Rifampin, an inhibitor of DNA-dependent RNA polymerase, inhibits Clo DF13 RNA synthesis as well as Clo DF13 DNA synthesis in minicells. From these results we conclude that Clo DF13 RNA synthesis, but not de novo Clo DF13 protein synthesis, is involved in the replication of the Clo DF13 factor. This involvement can be interpreted in two ways. Synthesis of RNA may be required for synthesizing a short RNA segment which serves as a primer in DNA synthesis (20, 35), or transcription may facilitate DNA replication by making the DNA initiation site more accessible to the replication machinery (11).

Recently, it has been shown that Col E1 DNA, synthesized in the presence of CAP, contains one or more ribonucleotides (3). The presence of RNA sequences in Clo DF13 DNA, synthesized in the presence and absence of CAP, was examined. During extensive treatment of cells or minicells with CAP, Clo DF13 DNA molecules which are sensitive to RNase or alkali accumulate. Treatment of supercoiled Clo DF13 DNA, synthesized in the presence of CAP with ribonuclease or alkali induces a loss of the covalently closed structure of a relative large portion of the Clo DF13 DNA molecules. These results indicate the presence of RNA sequences in CAP-treated Clo DF13 DNA. As suggested by Blair et al. (3), the long incubation period in the presence of CAP may block the degradation of the RNA segment which is normally removed after having served as primer in DNA synthesis.

Assuming that a RNA primer is involved in Clo DF13 replication, a question was raised of how the RNA primer sequences are removed. From the observation that homogeneous DNA polymerase I from *E. coli* is capable of degrading the RNA strand of a RNA-DNA hybrid during the course of in vitro DNA synthesis, Roychoudhury and Kössel (31) have suggested that DNA polymerase I itself is involved in the removal of RNA primers. That DNA polymerase I might function in this proces is also postulated by Wovcha and Warner (38), since this enzyme exhibits a 5′ → 3′ exonucleolytic activity which selectively attacks a DNA strand containing uracil instead of thymine. In addition, DNA polymerase I has been shown to be a prerequisite for the replication of Clo DF13 factor (36a). We therefore investigated a possible role of the 5′ → 3′ exonucleolytic activity of DNA polymerase I in the removal of RNA primer sequences. The sensitivity of supercoiled Clo DF13 DNA, isolated from a *polA107* (Clo DF13) strain, to ribonuclease and to alkali treatment was determined. Covalently closed Clo DF13 DNA, synthesized in this *polA107* strain which lacks the 5′ → 3′ exonucleolytic activity of DNA polymerase I, appeared to be insensitive to these treatments. Apparently no RNA sequences are present in supercoiled Clo DF13 DNA isolated from

the *polA107* (Clo DF13) strain. This observation indicates that either the 5′ → 3′ exonucleolytic activity of DNA polymerase I is not involved in degradation of primer RNA or, besides this nuclease, another enzyme(s) exists which is responsible for the removal of RNA primer sequences. It seems most likely that the RNA is on the 5′ end of the chain, and therefore the 3′ → 5′ exonucleolytic activity of DNA polymerase I is probably not relevant. We cannot exclude, however, alternative explanations, e.g., that the remaining activity of the mutant enzyme present in the *polA107* strain (19) could account for degradation of ribonucleotides, or that the mutant retains in vivo its 5′ → 3′ exonucleolytic activity.

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