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Replication of Colicin E1 Plasmid DNA Added to Cell Extracts*

(intermediates/electron microscopy/RNA synthesis/protein synthesis)

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ABSTRACT **Closed-circular DNA of colicin E1 plasmid can undergo a round of semiconservative replication when added to an extract of *Escherichia coli*. Extracts of cells that do not carry the plasmid are able to perform complete replication of the plasmid. Replication requires *de novo* RNA synthesis but not protein synthesis.**

In a previous paper (1) it has been reported that extracts prepared from *Escherichia coli* carrying colicin E1 plasmid (Col E1) are able to support a round of semiconservative replication of endogenous Col E1 DNA. In this paper evidence is presented that these extracts, as well as extracts made from bacteria that do not carry the plasmid, are capable of carrying out a complete round of replication of exogenous, closed-circular Col E1 DNA.

MATERIALS AND METHODS

Materials. Most of the materials used have been described in the previous papers (1–4). *E. coli* YS1 *thr leu thi str minA end* is an *end* derivative of P678-54, and YS10 is a derivative of YS1 carrying Col E1 (1). Restriction endonuclease *Eco*R1 and Pronase were obtained from Miles Laboratories and Sigma Chemical Co., respectively. Actinomycin D, streptolydigin and puromycin, and Pronase were the products of Merck Co., Upjohn Co., and Nutritional Biochemical Corp., respectively. L-[*4,5*-^{3}H]Leucine was obtained from New England Nuclear.

Preparation of Cell Extracts. Extracts were prepared as described from cells incubated with chloramphenicol for 2 hr before harvesting (1).

Preparation of Col E1 DNA. For preparation of nonlabeled, closed-circular Col E1 DNA, A745 (Col E1) *thy* bacteria were grown exponentially in a Casamino acids medium containing 5 μg/ml of thymine (1). The culture was treated with chloramphenicol for 2 hr, and a cleared lysate was prepared (5). DNA was purified by two cycles of CsCl/ethidium bromide density gradient centrifugation (4). Less than 5% of the total closed-circular molecules contained a ribonucleotide sequence (6), as judged by the stability against alkali treatment (1). The DNA preparation was dialyzed against buffer A [50 mM K_2HPO_4, 50 mM NaCl, 1 mM EDTA, and 50 mM Tris·HCl (pH 8.0)].

For preparation of ^{32}P-labeled, closed-circular Col E1 DNA, A745 (Col E1) *thy* bacteria were grown in a medium containing 1% Casamino acids (phosphate concentration was reduced by precipitation of ammonium magnesium phosphate), 0.5% (w/v) NaCl, 1 mM $MgCl_2$, 20 μg/ml each of L-tryptophan and thymine, 1% (w/v) glucose, 0.16 mM K_2HPO_4, and [^{32}P]H_3PO_4 (100 mCi/mmol). The bacteria were treated with chloramphenicol for 2 hr, and a cleared lysate was prepared. Closed-circular DNA was isolated by CsCl/ethidium bromide density gradient centrifugation followed by neutral sucrose gradient centrifugation (1). When preferable, the ^{32}P-labeled DNA was diluted with nonlabeled DNA. ^{3}H-Labeled, closed-circular DNA was similarly prepared from a cleared lysate of A745 (Col E1) *thy* bacteria grown in a Casamino acids medium supplemented with 5 μg/ml of thymidine and 20 mCi/mmol of [*methyl*-^{3}H]thymidine and treated with chloramphenicol for 2 hr.

One microgram of Col E1 DNA corresponds to approximately 1.4×10^{11} molecules or 750 pmol of incorporated dTMP.

Treatment of Closed-Circular DNA with Enzymes. ^{3}H-Labeled, closed-circular DNA (100 μg/ml) in buffer A was treated with a mixture of 100 μg/ml of RNase A (ribonucleate 3-pyrimidino-oligonucleotidohydrolase; EC 3.1.4.22) and 50 μg/ml of RNase T1 (ribonucleate 3′-guanylo-oligonucleotidohydrolase; EC 3.1.4.8) for 1 hr at 37° and then with 200 μg/ml of Pronase for 5 hr at 37°. After the addition of sodium lauroyl sarkosinate (0.2%), the mixture was shaken with an equal volume of water-saturated phenol. DNA in the aqueous phase was centrifuged in a CsCl/ethidium bromide density gradient. More than 90% of DNA was recovered in the heavy band.

Assay of Incorporation of L-[^{3}*H*]*Leucine into Hot Trichloroacetic Acid-Insoluble Material.* Incorporation of L-[^{3}H]leucine was measured with the standard reaction mixture (1) (300 μl) containing a YS1 extract that was passed through a Sephadex G-25 column (2), 8 μg/ml of closed-circular DNA, 10 μM L-[^{3}H]leucine (1100 cpm/pmol), 0.1 mM puromycin, and with or without 10% glycerol and 2 mM spermidine. The reaction mixtures with the same compositions except that L-[^{3}H]leucine was replaced by nonlabeled L-leucine and [α-^{32}P]dTTP (160 cpm/pmol) were simultaneously incubated. After incubation for 60 min, hot trichloroacetic acid-insoluble ^{3}H radioactivity and cold trichloroacetic acid-insoluble ^{32}P radioactivity were measured.

Other Methods. Assay of DNA synthesis, density gradient centrifugation analysis, electron microscopic examination, and preparation of the reference [^{3}H]DNA were performed as described (1, 3).

Abbreviation: Col E1, colicin E1 plasmid.

* This paper is the fifth of a series. The fourth is ref. 4.

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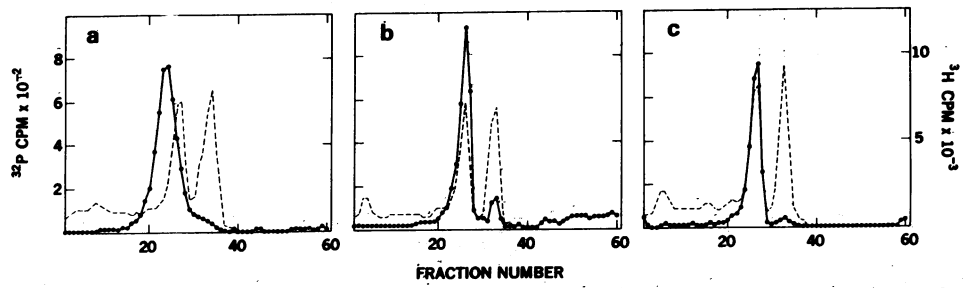

FIG. 1. Sedimentation analysis of ^{32}P-labeled, closed-circular DNA incubated with a YS10 extract. ^{32}P-Labeled, closed-circular DNA (0.01 μg; 5 $\times$ 10^4 cpm) was incubated at 30° with a YS10 extract in the standard reaction mixture (600 μl) with 10% glycerol and 2 mM spermiidine and with (c) or without (a and b) rifampicin (10 μg/ml, 13 μM). DNA was extracted after incubation for 75 min and centrifuged in neutral sucrose gradients at 45,000 rpm for 150 min at 10° in a Beckman SW50.1 rotor. In (b), DNA was heated at 90° for 90 sec before centrifugation. (●——●) ^{32}P-labeled DNA; (– – –) ^{3}H-labeled reference closed-circular and open-circular Col E1 DNA.

RESULTS

Replication of Col E1 DNA Added to Extracts of Cells That Carry Col E1. Col E1 DNA in YS10(Col E1)$^+$ extracts is converted to early replicative intermediates that sediment at approximately 26 S by incubation in the presence of 10% glycerol and 2 mM spermidine (2). The intermediates contain a newly synthesized DNA fragment(s) that is dissociated from parental DNA by heating at 90° for 90 sec (2). Closed-circular Col E1 DNA, added to a reaction mixture containing a YS10 extract, glycerol, and spermidine, was also converted to molecules sedimenting at 26 S (Fig. 1). More than 80% of the labeled exogenous DNA was converted to 26S molecules by incubation for 75 min (Fig. 1a). After heating at 90° for 90 sec, the labeled product sedimented with the closed-circular reference DNA (Fig. 1b). The formation of 26S molecules was inhibited by rifampicin (Fig. 1c) as similar conversion of endogenous DNA in YS10 extracts (2). Rifampicin is known to block initiation of Col E1 DNA replication (2). When a similar experiment was carried out in the presence of BrdUTP instead of dTTP, the density of the labeled exogenous DNA measured by CsCl density gradient centrifugation was increased by roughly one tenth of the difference of the light density and the hybrid density (data not shown). The density of these molecules corresponds to that of the molecules containing BrdUMP labeled 6S DNA (1, 2). These results show that exogenous DNA was efficiently converted to the early replicative intermediates in the extracts in the presence of 10% glycerol and 2 mM spermidine.

Evidence of the completion of a round of semiconservative replication of exogenous Col E1 DNA in a YS10 extract is presented in the following experiments. ^{3}H-Labeled, closed-circular molecules were incubated for 90 min and 180 min in a reaction mixture that contained a YS10 extract, BrdUTP, and [α-^{32}P]dATP in the absence of glycerol and spermidine. The ^{32}P-labeled, newly synthesized DNA formed a band at the half-heavy position in CsCl density gradients, and the ^{3}H-labeled, exogenous DNA formed a band at the light position with a considerable skew toward the heavy side (Fig. 2a and b). Recentrifugation of the DNA in the half-heavy fractions

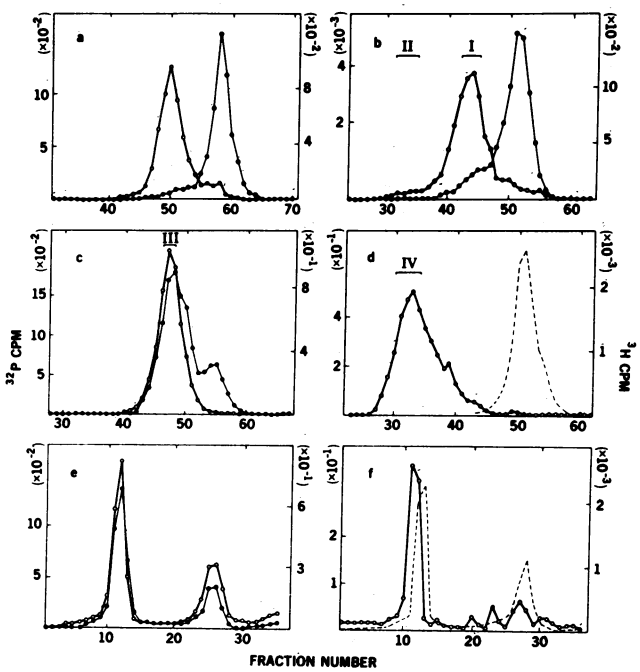

FIG. 2. Density labeling experiment of replication of exogenous and endogenous Col E1 DNA in a YS10 extract. The reaction mixture (1.6 ml) contained 50 μM each of dATP, dGTP, dCTP, and BrdUTP, 200 μM each of the four ribonucleoside triphosphates, 2 mM NAD, 7.5 mM $MgCl_2$, 25 mM potassium phosphate buffer (pH 7.4), 50 mM KCl, 20 Ci/mmol of [α-^{32}P]dATP, ^{3}H-labeled, closed-circular DNA (0.2 μg; 1.4 $\times$ 10^5 cpm), and 0.8 ml of an extract. DNA was extracted from half of the reaction mixture after incubation for 90 min. To the remaining half, an equal volume of the same reaction mixture without ^{3}H-labeled DNA was added, and incubation was continued for an additional 90 min before extraction of DNA. The DNA samples were centrifuged in CsCl density gradients at 36,000 rpm for 60 hr at 15° in a SW50.1 rotor. After centrifugation, approximately 4.5 ml of each sample was fractionated into 80 portions. Panels a and b show the patterns obtained with the DNA from the reaction mixtures incubated for 90 and 180 min, respectively. The fractions indicated by the brackets in panel b were combined (samples I and II), recentrifuged in CsCl density gradients for 60 hr, and similarly fractionated (panels c and d, respectively). ^{3}H-Labeled reference DNA was added to sample II before centrifugation. The fractions indicated in panels c and d were pooled (sample III and IV), dialyzed, and centrifuged in alkaline sucrose gradients at 45,000 rpm for 105 min at 5° (panels e and f, respectively). ^{3}H-Labeled reference DNA was added to sample IV before centrifugation. O, ^{32}P-labeled, newly formed DNA; ●, ^{3}H-labeled parental DNA; – – –, ^{3}H-labeled reference DNA. In panel f, ^{32}P-labeled molecules sedimented slightly faster than the corresponding molecular species of the reference DNA due to incorporation of BrdUMP (7).

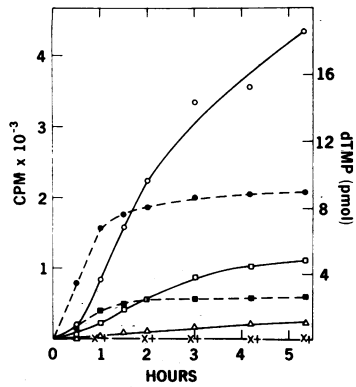

FIG. 3. Kinetics of incorporation of dTMP into acid-insoluble material. Standard reaction mixtures (450 μl each) containing a YS1 extract (150 μl), various concentrations of closed-circular DNA, and [α-^{32}P]dTTP were incubated in the presence (filled symbols) or absence (open symbols) of 10% glycerol and 2 mM spermidine. At the times indicated, acid-insoluble radioactivity in a 50 μl aliquot of each sample was determined. The concentrations of closed-circular DNA added were 8 μg/ml (●, ○), 1.6 μg/ml (■, □), and 0.32 μg/ml (△). To one of the mixtures without glycerol and spermidine no DNA was added (×), and to another 8 μg/ml of DNA and 10 μg/ml of rifampicin were added (+).

tion (Fig. 2c) showed replication of the exogenous DNA to half-heavy DNA that consisted almost exclusively of circular monomeric molecules (Fig. 2e). From these results it can be concluded that closed-circular Col E1 DNA added to an extract of cells that are carrying Col E1 can complete a round of semiconservative replication.

^{32}P-Labeled full-heavy DNA was synthesized during incubation (Fig. 2b and d). The full-heavy DNA was composed almost exclusively of circular monomeric molecules (Fig. 2f). This result indicates that Col E1 DNA molecules can complete more than one round of replication in the reaction mixture.

Incorporation of dTMP Dependent on the Addition of Col E1 DNA to Extracts of Cells That Do Not Carry Col E1. When a freshly prepared YS1(Col E1)$^-$ extract was incubated with added closed-circular DNA in the standard reaction mixture, labeled dTMP was incorporated into acid-insoluble material (Fig. 3). No incorporation was observed without the addition

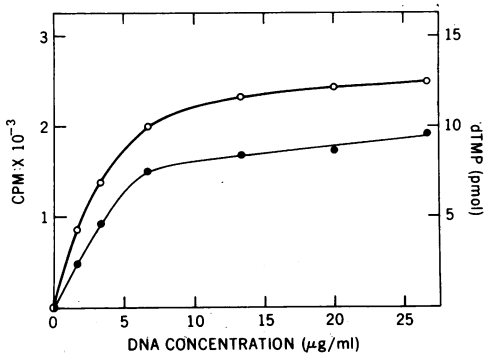

FIG. 4. Relation between the amount of dTMP incorporated and the amount of closed-circular DNA added to the standard reaction mixture containing a YS1 extract. Experimental conditions were the same as those described in the legend to Fig. 3 except that various amounts of closed-circular DNA, as indicated on the abscissa, were added to the reaction mixtures which were incubated for 45 min in the presence of 10% glycerol and 2 mM spermidine (●) or for 90 min in their absence (○).

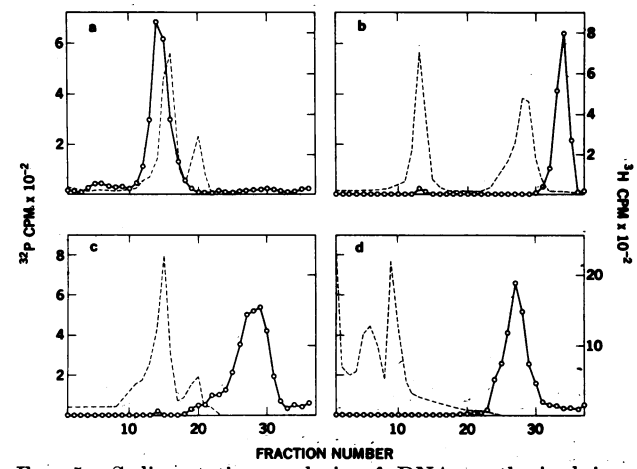

FIG. 5. Sedimentation analysis of DNA synthesized in a reaction mixture containing closed-circular DNA, a YS1 extract, glycerol, and spermidine. The standard reaction mixture (600 μl) containing 8 μg/ml of closed-circular DNA, a YS1 extract (200 μl), and [α-^{32}P]dTTP was incubated in the presence of 10% glycerol and 2 mM spermidine for 45 min. Without further treatment (a) or after heating at 90° for 90 sec (c), the DNA preparations were centrifuged in neutral sucrose gradients at 45,000 rpm for 150 min at 10°. The DNA preparation was also centrifuged in alkaline sucrose gradients at 45,000 rpm for 105 min (b) or 33,000 rpm for 15 hours (d) at 5°. ○, ^{32}P-labeled DNA; – – –, ^{3}H-labeled reference DNA.

of Col E1 DNA. An initial slow rate of incorporation was followed by almost linear incorporation for more than 3 hr. The linear rate of incorporation was roughly proportional to the concentration of the added DNA up to 5 μg/ml (Figs. 3 and 4). The incorporation was completely inhibited by rifampicin (Fig. 3). The initial rate of incorporation was increased by the addition of glycerol and/or spermidine. The optimum concentration of glycerol was approximately 10% and that of spermidine was 2–4 mM. Ten percent glycerol or 2 mM spermidine separately showed approximately 40 and 80%, respectively, of the maximum stimulation that was attained by the addition of both 10% glycerol and 2 mM spermidine. Incorporation in the presence of 10% glycerol and 2 mM spermidine (Fig. 3) or 10% glycerol alone stopped after about 60 min, while that in the presence of 2 mM spermidine continued more than 3 hr (data not shown). Activity of extracts assayed in the presence of glycerol and spermidine or spermidine alone was fairly stable against storage at −20°,

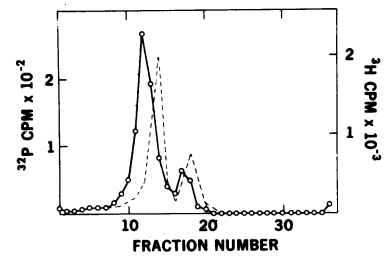

FIG. 6. Sedimentation analysis of ^{32}P-labeled, closed-circular DNA added to a reaction mixture containing a YS1 extract. The reaction mixture (300 μl) containing ^{32}P-labeled, closed-circular DNA (5 μg; 5 × 10^3 cpm), a YS1 extract (100 μl), 10% glycerol, and 2 mM spermidine was incubated for 45 min. DNA was extracted and centrifuged in a neutral sucrose gradient at 45,000 rpm for 150 min at 10°. ○, ^{32}P-labeled DNA; – – –, ^{3}H-labeled reference DNA.

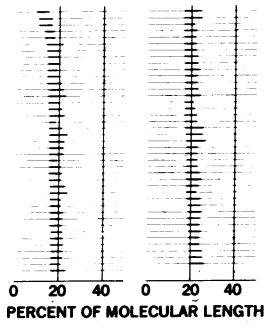

FIG. 7. Line diagram showing the location of loops (thicker lines) in 81 randomly selected replicative intermediates formed from exogenous closed-circular DNA. 26S DNA molecules were prepared under the condition described in the legend to Fig. 5a, except that 5 μg/ml of closed-circular DNA heated at 93° for 5 min in buffer A was added to the reaction mixture instead of the unheated DNA. They were converted to linear forms by treatment with endonuclease *Eco*R1 (3).

but that assayed in the absence of these chemicals was unstable. The kinetics of dTMP incorporation by a YS1 extract assayed with the addition of 6 μg/ml of closed-circular DNA was similar to the kinetics obtained with a YS10 extract containing endogenous DNA made in an identical manner.

Treatments of closed-circular DNA with RNases, Pronase, sodium lauroyl sarkosinate, and phenol as described in *Materials and Methods* did not affect the template activity of the molecules in the presence or absence of glycerol and spermidine.

Synthesis of Replicative Intermediates in Extracts of Cells That Do Not Carry Col E1. The DNA was labeled for 45 min with [α-^{32}P]dTMP in a reaction mixture containing a YS1 extract, closed-circular DNA, glycerol, and spermidine. In a neutral sucrose gradient, the labeled DNA sedimented in a single peak of approximately 26 S (Fig. 5a). Heating at 90° for 90 sec changed the sedimentation rate of the labeled DNA to approximately 6 S (Fig. 5c). In an alkaline gradient, the labeled DNA formed a peak at approximately 6 S (Fig. 5b and d). Most of the ^{32}P-labeled, closed-circular DNA (5 μg/ml) was converted to 26S molecules after 45 min of incubation (Fig. 6).

The similarity of the molecules synthesized from the endogenous and exogenous DNAs was further revealed by electronmicrographic examination of the products. A majority of molecules in the 26S band formed from the exogenous DNA

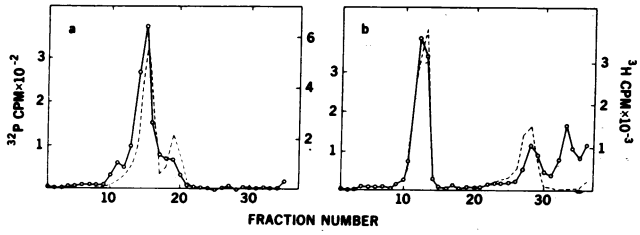

FIG. 8. Sedimentation analysis of DNA synthesized in a reaction mixture containing closed-circular DNA and a YS1 extract in the absence of both glycerol and spermidine. The standard reaction mixture (300 μl) containing 10 μg/ml of closed-circular DNA, a YS1 extract (100 μl), and [α-^{32}P]dTTP was incubated for 90 min. DNA was extracted and centrifuged in a neutral sucrose gradient at 45,000 rpm for 150 min at 10° (a) or an alkaline sucrose gradient at 45,000 rpm for 105 min at 5° (b). O, ^{32}P-labeled DNA; – – –, ^{3}H-labeled reference DNA.

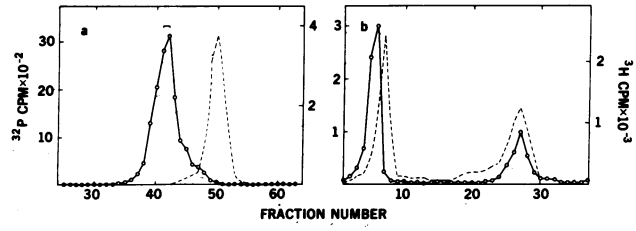

FIG. 9. Density labeling experiment of replication of exogenous closed-circular DNA in a YS1 extract. The BrdUTP-containing reaction mixture (0.8 ml) was as described in the legend to Fig. 2, except that the YS10 extract and ^{3}H-labeled DNA were replaced by 0.4 ml of a YS1 extract and 10 μg of nonlabeled, closed-circular DNA. Incubation was for 90 min. DNA was centrifuged in a CsCl density gradient and fractionated (a) as described in the legend to Fig. 2. DNA at the half-heavy peak was dialyzed and centrifuged in an alkaline sucrose gradient at 45,000 rpm for 120 min at 5° (b). O, ^{32}P-labeled DNA; – – –, ^{3}H-labeled reference DNA.

contained a small replication loop. The position of the loop in these molecules was determined by treating them with restriction endonuclease *Eco*R1 that cuts the molecules at a single unique site (3). As shown in Fig. 7, one of the branch points of the loop was located at 16.9 ± 1.9% (0.37 ± 0.04 μm) of the molecular length (2.22 ± 0.18 μm) from an end of the molecule. The location of the branch point is exactly the same as that observed with 26S molecules formed from the endogenous DNA (3). The length of the replicated regions was 5.4 ± 1.1% (0.12 ± 0.02 μm) of the molecular length and similar to that of the endogenous intermediates (3). Among 81 molecules, 28 molecules had a loop with two double-stranded branches and 38 molecules had one each of single- and double-stranded branches. For the rest of the molecules, identification of the strandedness of the branches was ambiguous.

Formation of Completely Replicated Molecules in Extracts of Cells That Do Not Carry Col E1. DNA synthesized in a reaction mixture containing a YS1 extract and exogenous Col E1 DNA in the absence of added glycerol and spermidine was next examined. In a neutral gradient, approximately 90% of DNA labeled during 90 min of incubation sedimented at the same rate as closed-circular reference DNA or 26S intermediates (Fig. 8a), and in an alkaline gradient approximately 55, 15, and 25% of the labeled DNA were in collapsed closed-circular molecules, single-stranded molecules of unit length, and 6S molecules, respectively (Fig. 8b). The distribution of labeled DNA among these species is similar to that observed with the labeled DNA formed from the endogenous DNA in a YS10 extract (1).¶ Completely replicated molecules isolated by neutral sucrose density gradient centrifugation after heating at 90° for 90 sec of the labeled DNA had a similar superhelix density as the parental DNA (data not shown). The DNA labeled for 90 min with [α-^{32}P]dAMP in the presence of BrdUTP formed a peak at the half-heavy position in a CsCl density gradient (Fig. 9a). The half-heavy molecules were exclusively circular monomers (Fig. 9b). These results prove

¶ Completely replicated molecules were formed in the presence of spermidine. However, the ratio of the amount of dTMP incorporated into completely replicated molecules to that into early replicative intermediates was lower in the presence than in the absence of spermidine. This ratio varied with the preparations of extracts and concentrations of spermidine.

TABLE 1. *Effects of addition of inhibitors of RNA or protein synthesis on incorporation of dTMP*

Addition	Concentration (M)	Incorporation of dTMP (pmol) With glycerol and spermidine, for 45 min	Incorporation of dTMP (pmol) Without glycerol and spermidine, for 90 min
None		6.25 (100)	8.05 (100)
Rifampicin	2×10^{-6}	0.16 (3)	0.12 (2)
Actinomycin D	2×10^{-6}	0.07 (1)	0.01 (0.1)
Streptolydigin	2×10^{-4}	2.06 (33)	1.05 (13)
Chloramphenicol	2×10^{-4}	6.37 (102)	6.04 (75)
Puromycin	1×10^{-4}	6.06 (97)	7.16 (89)

The standard reaction mixture (150 μl) containing a YS1 extract, closed-circular DNA (8 μg/ml), [α-^{32}P]dTTP (360 cpm/pmol), and an inhibitor was incubated in the presence or absence of 10% glycerol and 2 mM spermidine. The amount of incorporation of dTMP in a 50 μl aliquot was presented. The values in parentheses represent the ratio of incorporation with and without an inhibitor, multiplied by 100. All the reaction mixtures contained approximately 1.5×10^{-4} M chloramphenicol derived from the bacterial culture medium.

that exogenous Col E1 DNA can complete a round of semiconservative replication in a reaction mixture containing an extract of cells that do not carry Col E1.

Effects of Inhibition of RNA and Protein Synthesis on Replication of Col E1 DNA. The fact that Col E1 DNA completes a round of replication in extracts of cells that do not carry the plasmid means either that a product(s) specified by the plasmid is not essential for replication or that such a product(s) is synthesized in the reaction mixture and utilized. We first tested the effect of inhibitors of RNA and protein synthesis on Col E1 DNA replication. As shown in Table 1, rifampicin and actinomycin D blocked Col E1 DNA synthesis at very low concentrations, and streptolydigin also inhibited the DNA synthesis. These results show that RNA synthesis *de novo* is necessary for replication *in vitro* of exogenous as well as endogenous Col E1 DNA (1, 2). On the other hand, the addition of chloramphenicol and puromycin, inhibitors of protein synthesis, showed little effect on Col E1 DNA synthesis. Furthermore, the sedimentation pattern in an alkaline sucrose gradient of DNA labeled during 90 min of incubation without glycerol and spermidine in the presence of 0.5 mM puromycin was similar to the pattern (Fig. 8b) of DNA labeled in the absence of the inhibitor. We next examined protein synthesis under the condition of active Col E1 DNA synthesis. In 300 μl of the reaction mixture containing a Sephadex-treated YS1 extract and puromycin as described in *Materials and Methods*, 58 or 36 pmol of dTMP was incorporated into DNA during 60 min of incubation in the presence or absence of glycerol and spermidine, respectively. Under the comparable conditions, no detectable amount of L-leucine (less than 0.3 pmol) was incorporated into protein. We conclude that protein synthesis *de novo* is not necessary for Col E1 DNA replication in cell extracts.

DISCUSSION

Closed-circular Col E1 DNA added to cell extracts can initiate and complete a round of semiconservative replication. Extracts of cells that do not carry the plasmid are able to perform complete replication of the plasmid. *De novo* RNA synthesis but not protein synthesis is necessary for the replication.

Col E1 DNA replication in the *in vitro* system depends entirely on bacterial functions. Several bacterial gene products were implicated in the plasmid replication *in vivo* (10–14). The present system should be useful for determination of the functions of these gene products.

Utilization of RNA without translation suggests functioning of the RNA as a primer for Col E1 DNA synthesis. Association of RNA to 6S DNA fragments of early replicative intermediates has been reported (2).

Mutants of Col E1 that cannot be maintained in bacteria growing at an elevated temperature have been isolated (8, 9). Since no plasmid protein is essential for the replication *in vitro*, the isolation of these mutants suggests a mechanism of regulation of plasmid replication *in vivo* for which these mutants are defective. Alternatively, the plasmid might express a function(s) that is essential for its replication *in vivo* but dispensable or replaceable by a bacterial function(s) in the *in vitro* system. It may be worth mentioning that the structure and the origin and direction of replication of the molecules replicating in the *in vitro* system and those of the molecules replicating in bacteria showed no significant difference (ref. 3; Fig. 7).

We acknowledge helpful comments on the manuscript by our colleagues.

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