Proc. Nat. Acad. Sci. USA
Vol. 72, No. 3, pp. 829–833, March 1975

Replicative Intermediates of Colicin E1 Plasmid DNA in Minicells

(pulse labeling/single-stranded DNA/RNA/centrifugation analysis/electron microscopy)

ATSUHIRO OKA AND JOSEPH INSELBURG

Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire 03755

Communicated by Walter H. Stockmayer, December 6, 1974

ABSTRACT **Pulse-labeled colicin E1 plasmid (Col E1) DNA in minicells was examined to characterize replicating molecules. Replication of Col E1 DNA principally occurred in covalently closed circular molecules and involved the synthesis of variable length single-stranded DNA fragments that were dissociable from the template DNA and ultimately incorporated into the completely replicated molecules. RNA was found to be associated with some of these newly synthesized fragments. Replicating molecules containing different size displacement loops were observed by electron microscopy.**

Colicin E1 plasmid (Col E1) DNA has been shown to undergo semiconservative replication in the chromosomeless minicells of *Escherichia coli* strain P678-54 (Col E1) (1). The replicative intermediates of Col E1 DNA isolated from minicells exist as open circular (oc) and covalently closed circular (ccc) molecules containing two branch points, three branches, and no free ends (2–4). The band position of pulse-labeled Col E1 DNA in a CsCl/ethidium bromide (EthBr) density gradient suggested that the replicating ccc form is the principal replicative intermediate (3, 5). The examination of pulse-labeled Col E1 DNA in minicells described here indicates that Col E1 DNA replication proceeds in ccc molecules and involves the synthesis of single-stranded DNA fragments of variable lengths that are dissociable from the template DNA and are ultimately incorporated into the completely replicated molecules. Some of these newly synthesized single-stranded DNA fragments were found to contain an RNA component that is not heat-dissociable at 90°. The semiconservative replication of Col E1 DNA *in vitro*, involving the synthesis of RNA containing single-stranded DNA fragments on ccc templates, has recently been reported (6, 7).

An electron micrographic examination of the replicating Col E1 DNA indicated that a small percentage of the molecules contain displacement-loops of various sizes similar to those observed in replicating mitochondrial DNA (8).

MATERIALS AND METHODS

Bacterial Strains. The *Escherichia coli* K12 strains used were W3350*thy*$^-$, the minicells producing strain P678-54 (9), and their Col E1 colicinogenic derivatives, W3350*thy*$^-$ (Col E1) and P678-54 (Col E1) (1).

Reagents. [*Methyl*-^{3}H]Thymidine (52.7 Ci/mmol) and $H_3{}^{32}PO_4$ (5 mCi/ml in 0.02 M HCl) were obtained from New England Nuclear Corp. Restriction endonuclease *Eco*R1 was a gift of Dr. T. Maniatis.

Pulse Labeling of Col E1 DNA. Minicells were purified as described from P678-54 (Col E1) grown in Tris/casamino acid/glucose medium (1), suspended in 1/1000 volume of the medium containing 250 μg/ml of deoxyadenosine, and incubated for 20 min at 37° prior to the addition of [^{3}H]dT at 100 μCi/ml. After 60 sec, the labeling was stopped by the addition of four times the volume of cold buffer [50 mM Tris·HCl (pH 8.0)/5 mM EDTA/50 mM NaCl] containing 50 mM NaN_3, and cells were collected by centrifugation. DNA was extracted as described (3).

Preparation of Reference Col E1 DNA. ^{3}H- or ^{32}P-labeled Col E1 DNA was prepared from W3350*thy*$^-$ (Col E1) that was labeled in the presence of chloramphenicol (180 μg/ml) (10) in Tris/casamino acid/glucose or low-phosphate Tris/casamino acid/glucose medium (1). ccc Col E1 DNA was isolated by CsCl/EthBr bouyant density gradient centrifugation from "cleared lysates" (10). By heating ccc Col E1 DNA in 0.3 M NaOH/0.2 M K_2HPO_4 for 10 min in a boiling-water bath followed by neutralization, single-stranded DNA fragments with an average size of 15 S, corresponding to approximately 44% of a Col E1 DNA, were prepared as a single-stranded DNA density marker.

Sucrose Density Gradient Analyses. Sucrose solutions were made in Tris/EDTA/NaCl buffer or in 0.3 M NaOH/1.0 M NaCl/5 mM EDTA. The samples were centrifuged at 45,000 rpm for 165 min at 10° or for 105 min at 5° for neutral or alkaline sucrose gradient analysis, respectively, in a Beckman SW50.1 rotor. Unit-length single-stranded linear Col E1 DNA, prepared by heat denaturation of *Eco*R1-treated ccc monomers (11, 12), sedimented along with 23S ccc Col E1 DNA (13) in the neutral sucrose density gradient. The relation between the sedimentation coefficient of single-stranded DNA and its molecular weight was determined by using Col E1 and bacteriophage lambda DNA as the reference (14).

Bouyant Density Gradient Analyses. Cs_2SO_4 density gradients were prepared by adding the DNA samples to 2.7 g of Cs_2SO_4, 0.1 M Tris·HCl (pH 8.0), 0.01 M EDTA, and 0.02% Sarkosyl in a total volume of 4.5 ml in polyallomer tubes, and centrifuging them in a Beckman SW50.1 rotor at 36,000 rpm at 15° for 72 hr. Neutral CsCl and CsCl/EthBr density gradient centrifugation were performed as described (1, 2). All gradient fractions were collected from the tube bottom.

DNA·DNA Membrane Hybridization Methods and Electron Microscope Techniques have been described (15, 16).

RESULTS

A portion of pulse-labeled Col E1 DNA in minicells that is enriched with replicating ccc DNA molecules sediments faster than the completely replicated ccc Col E1 DNA in a neutral sucrose density gradient and bands between the positions of

Abbreviations: Col E1, colicin E1 plasmid; EthBr, ethidium bromide; ccc, covalently closed circular; oc, open circular.

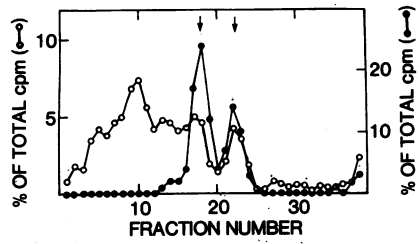

FIG. 1. Conversion of rapidly sedimenting molecules to completely replicated Col E1 DNA molecules. Minicells, pulse-labeled for 60 sec, were divided into two equal portions. One was immediately lysed and the other was washed, incubated for an additional hour with 200 μg/ml of nonradioactive dT, and then lysed. Both DNA samples were sedimented through a neutral sucrose gradient. The two separate gradients were plotted on one figure to facilitate comparison of sedimentation characteristics. Total ^3H cpm before (○) and after chase (●) were 1012 and 1216, respectively. The left and right arrows show the peak position of ccc and oc Col E1 [^{32}P]DNA, respectively.

ccc and oc Col E1 DNA in a CsCl/EthBr density gradient (3, 4). This pulse-labeled material from sucrose gradients will be referred to as rapidly sedimenting pulse-labeled material.

That the rapidly sedimenting pulse-labeled DNA is a precursor of completely replicated Col E1 DNA was examined by pulse-labeling Col E1 DNA in minicells with [^3H]dT for 60 sec and then either immediately extracting the DNA or allowing it to replicate for 1 hr in the absence of [^3H]dT. Both samples were analyzed by neutral sucrose density gradient centrifugation. The rapidly sedimenting pulse-labeled molecules were converted to completely replicated ccc or oc Col E1 DNA after the chase (Fig. 1). The total radioactivity in DNA before and after the chase was not significantly changed. The result indicates that most rapidly sedimenting pulse-labeled molecules were precursors of the completely replicated molecules.

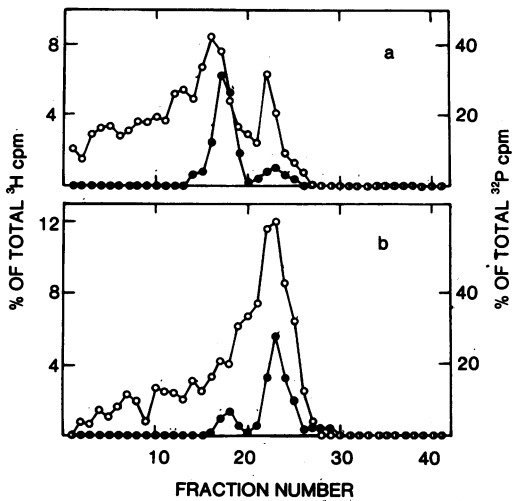

FIG. 2. DNase I treatment of replicating Col E1 DNA molecules. Pulse-labeled Col E1 [^3H]DNA was banded in a CsCl density gradient, and the fractions containing DNA were pooled, dialyzed, mixed with the reference Col E1 [^{32}P]DNA, and analyzed by neutral sucrose gradient centrifugation before (a) or after (b) treatment with 10^{-3} μg/ml of DNase I at 37° for 20 min in 10 mM Tris·HCl (pH 7.5) containing 5 mM $MgCl_2$. The nuclease treatment was terminated by the addition of EDTA. Total ^3H (○) cpm were 990 for (a) and 889 for (b), and total of ^{32}P (●) cpm were 702 for (a) and 625 for (b).

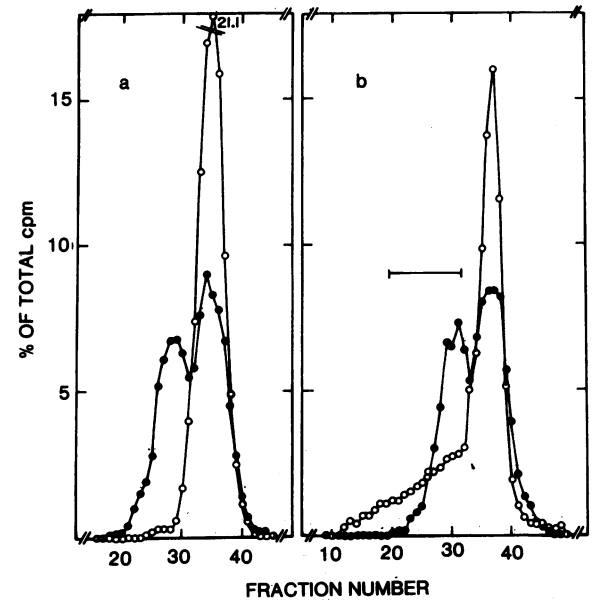

FIG. 3. CsCl equilibrium centrifugation of pulse-labeled Col E1 DNA before or after heat treatment at 90°. Pulse-labeled minicells were lysed before or after a (1 hr) chase, as described in the legend of Fig. 1. DNA samples were heat-treated in Tris/EDTA/NaCl buffer for 3 min at 90° or left untreated and examined by CsCl density gradient centrifugation. Single- and double-stranded Col E1 [^{32}P]DNA were added as density markers. (a) Unheated DNA before the chase; (b) heated DNA before the chase. The number of total fractions of (a) and (b) were 57 and 60, respectively. Total cpm of ^3H (○) and ^{32}P (●), respectively, were 7935 and 2458 in (a) and 7715 and 2330 in (b).

DNase I treatment of rapidly sedimenting pulse-labeled molecules under conditions that caused nicking of about 80% of ccc Col E1 DNA without its fragmentation, induced approximately 60% of those molecules similar to those in Fig. 1 to sediment in a neutral sucrose density gradient in the region between the ccc and oc monomer Col E1 DNA (Fig. 2). This alteration in sedimentation behavior by single-strand nicking suggests that most of the replicating DNA exists as ccc molecules.

In order to test whether the pulse-labeled DNA in replicating molecules is covalently associated with or only hydrogen-bonded to their template molecules, pulse-labeled DNA was banded in a CsCl density gradient before or after heating at 90° for 3 min. This treatment can release short single-stranded DNA fragments but not unit-length single-stranded DNA from the parental DNA template (unpublished data). A dense shoulder of ^3H-label appeared in the gradient after the heat treatment (Fig. 3b). When the pulse-labeled DNA was chased for 1 hr, after removal of [^3H]dT, in an excess of nonradioactive dT and then heated, almost no material denser than double-stranded DNA was seen in the CsCl density gradients. (The results are identical to that in Fig. 3a.) That the heat-released materials in the dense shoulder was newly synthesized single-stranded Col E1 DNA was determined by showing that the material that was sensitive to DNase I digestion specifically annealed to Col E1 DNA. The heat-released material annealed to Col E1 DNA on membrane filters at an efficiency of 59%, which was

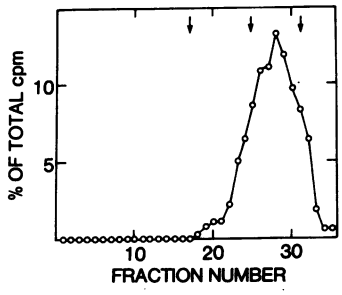

FIG. 4. Sedimentation analysis of newly synthesized single-stranded DNA released from replicating Col E1 DNA molecules by heat treatment. Fractions containing the heat-released materials denser than double-stranded DNA from a preparation similar to that shown in Fig. 3b were pooled and dialyzed against Tris/EDTA/NaCl buffer followed by neutral sucrose gradient centrifugation. Total ^{3}H cpm was 722. The left arrow corresponds to the position of unit-length single-stranded linear Col E1 DNA and ccc Col E1 [^{32}P]DNA. The middle and right arrows, respectively, are 12 S and 5 S, where single-stranded molecules of 27% and 4% of a unit-length Col E1 DNA molecule would sediment.

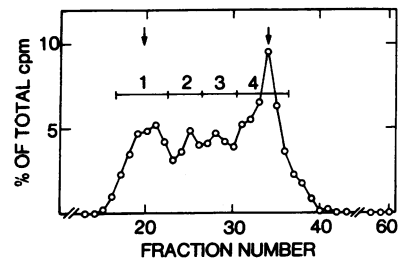

FIG. 5. CsCl/EthBr equilibrium centrifugation of pulse-labeled Col E1 DNA. Pulse-labeled minicells were lysed and banded in a CsCl/EthBr density gradient. The total of ^{3}H cpm in 2 μl aliquots of each fraction was 1754. The left and right arrows show the peak positions of ccc and oc Col E1 [^{32}P]DNA, respectively.

reduced to 5% by the addition of 20 μg of nonradioactive Col E1 DNA and was unaffected (53% annealing) by the addition of 20 μg of *E. coli* DNA in the same annealing mixture, while 51% of reference Col E1 and 0.3% of *E. coli* [^{3}H]DNA annealed to Col E1 DNA on filters. The density of the reisolated material in the dense shoulder was unchanged after it was heated at 100° and remained exactly the same as reference single-stranded [^{32}P]DNA placed in the same CsCl density gradients, which were centrifuged for 72 hr in an SW50.1 rotor. It was, therefore, considered that the material in the dense shoulder of the CsCl density gradient represents newly replicated single-stranded Col E1 DNA that is associated with the Col E1 DNA replicative intermediates. The heat-releasable single-stranded DNA fragments obtained from the dense shoulder of the CsCl density gradient have sedimentation coefficients of 5 to 13 S in a neutral sucrose density gradient (Fig. 4), which corresponds to approximately 4–32% of a unit Col E1 DNA length.

The greater the degree of replication of ccc replicating molecules, the closer they band to the density position of oc DNA in a CsCl/EthBr density gradient (5). In order to determine from which replicating molecules single-stranded Col E1 DNAs were released by heating, pulse-labeled DNA was banded in a CsCl/EthBr density gradient and the gradient

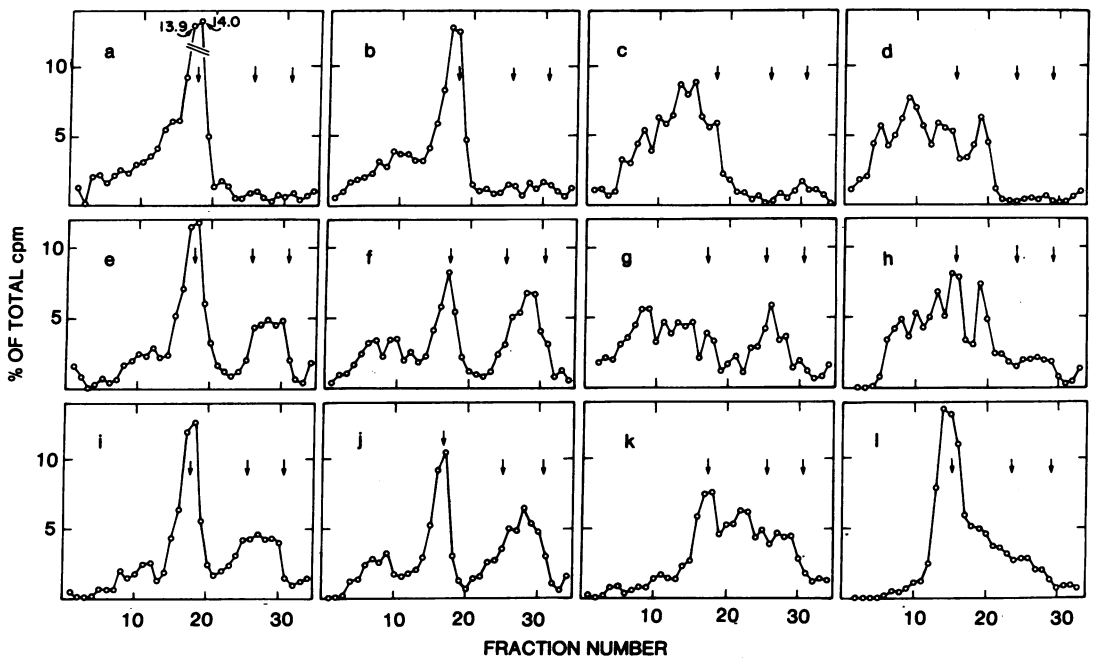

FIG. 6. Neutral sucrose gradient centrifugation analyses of replicating Col E1 DNA before or after heat treatment. Pulse-labeled Col E1 DNA, banded in the CsCl/EthBr density gradient shown in Fig. 5, was pooled into fractions 1, 2, 3, and 4 and each pool was dialyzed into Tris/EDTA/NaCl buffer and examined by neutral sucrose gradient centrifugation without further treatment (a, b, c, and d for pools 1, 2, 3, and 4, respectively) or after it was heated at 90° for 3 min (e, f, g, and h for pools 1, 2, 3, and 4, respectively), or after it was heated in a boiling-water bath for 5 min (i, j, k, and l for pools 1, 2, 3, and 4, respectively). The left, middle, and right arrows in each panel represent the sedimentation positions of 23S, 12S, and 5S molecules corresponding to 100%, 27%, and 4% of the length of single-stranded monomer DNA, respectively. Total ^{3}H cpm in (a)–(l) were 1725, 1774, 1690, 2626, 1985, 1786, 1632, 2209, 1964, 1701, 1900, and 2416, respectively.

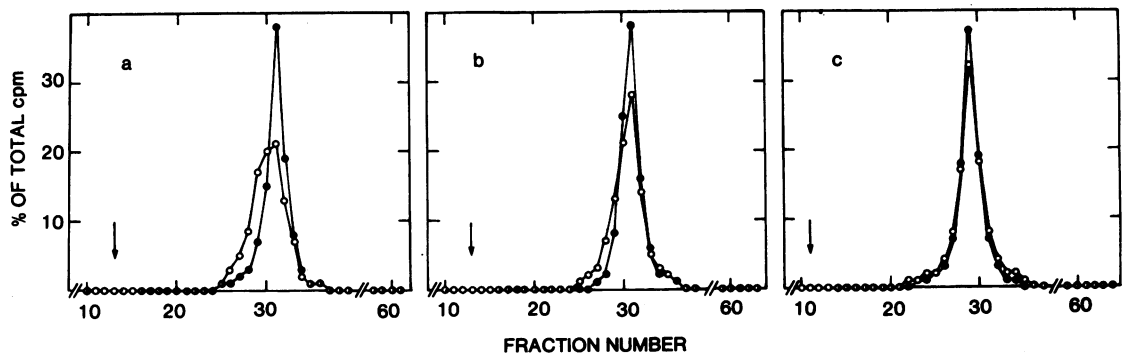

FIG. 7. Cs_2SO_4 equilibrium centrifugation of newly synthesized single-stranded DNA released from replicating Col E1 DNA molecules by heat treatment. Pulse-labeled Col E1 DNA was banded in a CsCl density gradient after heat treatment at 90° for 3 min, and the fractions containing the newly synthesized single-stranded fragments from a preparation similar to that shown in Fig. 3b were pooled, dialyzed, and centrifuged with fragmented denatured Col E1 [^{32}P]DNA and [^{32}P]RNA in a Cs_2SO_4 gradient without further treatment (a), after treatment with a mixture of 150 μg/ml of RNase A and 50 μg/ml of RNase T1 for 30 min at 37° in 10 mM Tris·HCl (pH 7.5)/20 mM NaCl/2.5 mM EDTA (b), or after heat treatment with 0.3 M NaOH in a boiling-water bath for 5 min followed by neutralization with 1 M Tris·HCl (pH 7.5) (c). Total cpm of ^{3}H (O) and ^{32}P (●), respectively, were 2146 and 1811 in (a), 1769 and 1925 in (b), and 1683 and 1715 in (c). ^{32}P radioactivity in the RNA marker, which was sharply localized about the peak fraction shown by an arrow, is not included in total ^{32}P radioactivity.

was divided into four fractions, as shown in Fig. 5. Parts of each fraction were examined by neutral sucrose gradient centrifugation without further treatment or after heating at either 90° for 3 min or at 100° for 5 min. No nicking or fragmentation of ccc Col E1 DNA occurred during heat treatment (unpublished data). After the DNA in fractions 1 and 2 was heated at 90° (Fig. 6e and f) or 100° (Fig. 6i and j), most of the rapidly sedimenting molecules were converted to 23S and 5–13S materials. The remaining fast sedimenting materials may be catenated molecules (3). The 23S peak produced by heat treatment of fractions 1 and 2 was found to principally contain ccc monomers, and less than 8% of the 23S peaks in either fraction contained single-stranded linear or oc monomers, as determined by alkaline sucrose gradient centrifugation (data not shown). Almost no fragments with sedimentation coefficients larger than 13 S were releasable from these pulse-labeled molecules. A similar release of 5–13S material was observed when DNA in fractions 3 and 4 was heated at 90°, although less 5–13S materials were observed in fraction 4 (Fig. 6g and h). After fractions 3 and 4 were heated at 100°, the rapidly sedimenting materials were converted to materials with sedimentation coefficients between 5 and 23 S (Fig. 6k and l). The replicating molecules in fractions 3 and 4, therefore, not only contained 5–13S fragments, but also much larger fragments. The ratio of ccc double-stranded monomers to single-stranded monomers in the 23S DNA produced by heating fractions 3 and 4, were 1:4 and 1:10, respectively, as determined by alkaline sucrose gradient centrifugation. Some of the 23S fragments might be derived from completely replicated oc Col E1 DNA.

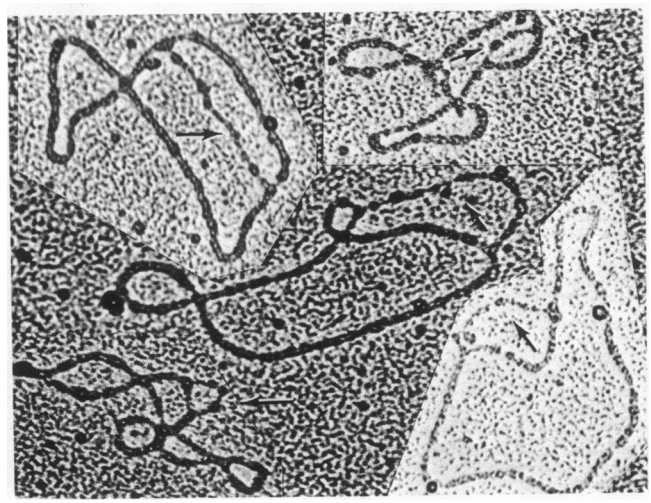

FIG. 8. Electron micrographs of D-loop structures in replicating Col E1 DNA. The replicating Col E1 DNA was isolated from fractions of rapidly sedimenting materials in a neutral sucrose gradient. Single-stranded DNA was made visible by the formamide spreading technique (15, 16). The final magnifications for each molecule in this figure are not the same. Arrows indicate the single-stranded branch of the replication loop.

RNA has been found to be covalently linked to newly synthesized DNA fragments in various experimental systems (7, 17–21). In order to determine if the newly synthesized single-stranded Col E1 DNA contains RNA, the pulse-labeled DNA was released from parental molecules by heating at 90° and pooled from a CsCl density gradient similar to that shown in Fig. 3b. The pooled DNA was then banded together with fragmented, denatured Col E1 [^{32}P]DNA in a Cs_2SO_4 density gradient. Some ^{3}H-labeled material was denser than the reference single-stranded Col E1 DNA, as seen by the skewing of the band in the gradient (Fig. 7a). Treatment of the materials with a mixture of RNase A and T1 or by heating in alkaline solution before centrifugation led to a reduction and elimination of the density difference (Fig. 7b and c). These results are consistent with the interpretation that RNA is linked to some of the newly synthesized fragments in the replicating molecules, but does not establish the nature of the linkage.

Replicating Col E1 DNA isolated from minicells was reported to contain short single-stranded regions localized at either or both replicating forks (11). Upon subsequent exam-

ination of replicative intermediates, approximately 2% of them were found to contain a D-loop structure originally observed in replicating mitochondrial DNA (8) and recently reported in replication of Col E1 DNA *in vitro* (12). Examples of these D-loop structures are shown in Fig. 8. While it may be argued that these D-loop structures represent an artifact caused by the release of the newly replicated single-stranded DNA from the ccc replicative intermediate structures, it may also be considered that the potential exists in the Col E1 replication process for a choice between the process that leads to the formation of D-loops or double-stranded replication loops in replicating molecules.

DISCUSSION

Rapidly sedimenting pulse-labeled Col E1 DNA has been found to be ccc replicating molecules containing newly synthesized DNA fragments of various sizes (4–100% of a unit length), some of which were shown to contain an RNA component. While the RNA component's size and the nature of its association with DNA remains to be elucidated, the small density shift it causes in the short heat-releasable fragments and the stability of the association at 90° suggests, though it does not prove, that the RNA could be covalently linked to DNA fragments. The banding position of a replicating Col E1 DNA molecule in a CsCl/EthBr density gradient reflected the size of newly synthesized DNA fragment releasable from it. This finding is consistent with earlier work (5).

The sizes of the replication loops of Col E1 DNA replicative intermediates we observed electron microscopically and the releasable newly synthesized single-stranded DNAs released from them appears to be more heterogeneous than those found *in vitro* (6, 7). While these differences may reflect the selective isolation o some replicative intermediates or be related to differences in the labeling procedure, they may also reflect a difference in detecting rate-limiting steps in the two systems. The small fragments observed *in vivo* may represent both initiation fragments and Okazaki pieces (19). The presence of Okazaki pieces in replicating Col E1 DNA is suggested by the fact that the short single-stranded DNA (5–13 S) was not only obtained by heat treatment of fractions near ccc Col E1 DNA but also from fractions banding near oc DNA in the CsCl/EthBr density gradient (Figs. 5 and 6); however, the possible tailing of molecules fram fractions 1 and 2 into fractions 3 and 4 during the fractionation to give this result cannot be ruled out. A determination of the origin of the 5–13S single-stranded fragments would resolve the problem of whether they are initiation fragments or Okazaki pieces.

Note Added in Proof. The 5–13S single-stranded DNA fragments are derived from all parts of the Col E1 DNA molecule (to be published elsewhere), as expected for Okazaki pieces.

We thank Dr. T. Maniatis for *Eco*R1 endonuclease and Dr. J. Tomizawa for a critical reading of the manuscript. This work was supported by Grants 5RO1 AI 08937-06 and 5KO4 AI 28818-03 to J.I. from the National Institutes of Health.

1. Inselburg, J. (1970) *J. Bacteriol.* **102**, 642–647.
2. Inselburg, J. & Fuke, M. (1970) *Science* **169**, 590–592.
3. Inselburg, J. & Fuke, M. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2839–2842.
4. Fuke, M. & Inselburg, J. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 89–92.
5. Jaenisch, R., Mayer, A. & Levine, A. (1971) *Nature New Biol.* **233**, 72–75.
6. Sakakibara, Y. & Tomizawa, J. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 802–806.
7. Sakakibara, Y. & Tomizawa, J. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 1403–1407.
8. Kasamatsu, H., Robberson, D. L. & Vinograd, J. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2252–2257.
9. Adler, H. I., Fisher, W. D., Cohen, A. & Hardigree, A. A. (1967) *Proc. Nat. Acad. Sci. USA* **57**, 321–326.
10. Blair, D. G., Sherratt, D. J., Clewell, D. B. & Helinski, D. R. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2518–2522.
11. Inselburg, J. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 2256–2259.
12. Tomizawa, J., Sakakibara, Y. & Kakefuda, T. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 2260–2264.
13. Bazaral, M. & Helinski, D. R. (1968) *Biochemistry* **7**, 3513–3519.
14. Studier, F. W. (1965) *J. Mol. Biol.* **11**, 373–390.
15. Inselburg, J. (1973) *Nature New Biol.* **241**, 234–237.
16. Davis, R. W. & Davidson, N. (1972) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 21, part D, pp. 413–428.
17. Sato, S., Ariake, S., Saito, M. & Sugiura, T. (1972) *Biochem. Biophys. Res. Commun.* **49**, 827–834.
18. Schekman, R., Wicker, W., Westergard, O., Brutlag, D., Geider, K., Bertsch, L. L. & Kornberg, A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2691–2695.
19. Sugino, A., Hirose, S. & Okazaki, R. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1863–1867.
20. Fox, R. M., Mendelsohn, K., Barbosa, E. & Goulian, M. (1973) *Nature New Biol.* **245**, 234–237.
21. Wagar, M. A. & Huberman, J. A. (1973) *Biochem. Biophys. Res. Commun.* **51**, 174–180.