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Replication of Colicin E1 Plasmid DNA in Cell Extracts*. Origin and Direction of Replication

(replicative intermediates/restriction endonuclease *Eco*R1/electron microscopy)

JUN-ICHI TOMIZAWA, YOSHIMASA SAKAKIBARA†, AND TSUYOSHI KAKEFUDA

Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism and Digestive Diseases, and Chemistry Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT **The structures of molecules of colicin E1 plasmid DNA that were in the process of being replicated *in vitro* were examined electron microscopically. Circular molecules containing a loop of approximately 7% of the length of the molecules were the major class of replicating molecules. The location of the loop was determined by treating the molecules with restriction endonuclease *Eco*R1, which introduced one unique double-strand break in the colicin E1 plasmid DNA molecule. The loops had a specific location with two branch points at approximately 17 and 24% of the molecular length from the endonuclease-sensitive site. Molecules with a larger loop were observed with a preparation labeled with 5-bromodeoxyuridine and enriched for these molecules. One of the branch points in these molecules was located at approximately 17% of the molecular length from the endonuclease-sensitive site independent of the size of the loops. These results indicate that the origin of replication of the plasmid DNA is located within the small loop and the replication proceeds unidirectionally. The molecules that had completed a round of replication had the monomeric twisted circular structure.**

A soluble system that is capable of replicating colicin E1 plasmid (Col E1) DNA was described in a previous paper (1). In this system, the closed-circular Col E1 DNA molecules in cell extracts prepared from *Escherichia coli* carrying Col E1 can initiate semiconservative replication. The major products of Col E1 DNA synthesis are completely replicated molecules and a class of replicative intermediates carrying DNA fragments with an average length of approximately 7% of Col E1 DNA (1). The molecules carrying small DNA fragments are selectively synthesized in the presence of 10% (v/v) glycerol and 2 mM spermidine (2).

The structures of early replicative intermediates, as well as the molecules replicated more extensively, were examined electron microscopically. A necessary internal marker for locating the replication loop in each molecule is provided by treatment of the DNA with restriction endonuclease *Eco*R1 (3), which was found to introduce one unique double-strand break in the Col E1 DNA molecule. The endonuclease has been used to show bidirectional replication from a fixed origin in simian virus 40 DNA (4).

MATERIALS AND METHODS

Materials. Most of the materials used have been described in the previous papers (1, 2). Restriction endonuclease *Eco*R1 was supplied by Drs. D. Nathans and T. J. Kelley, Jr. Cytochrome *c* was obtained from Calbiochem.

Preparation of Replicative Intermediates and Completely Replicated Molecules. Cell extracts prepared by lysis of *E. coli* YS10 (Col E1) (1) were incubated for 60 min in the standard reaction mixture with [α-^{32}P]dTTP in the presence or absence of 10% (v/v) glycerol and 2 mM spermidine (2). DNA was extracted and purified by CsCl density gradient centrifugation. The DNA, which formed a single band, was dialyzed against 0.1 M Tris·HCl (pH 8.0)–1 mM EDTA. With these preparations, structures of early replicative intermediates were examined.

To study structures of DNA molecules that had replicated more extensively or completed a round of replication, a DNA preparation that was labeled with 5-bromodeoxyuridine (BrdU) was fractionated by CsCl density gradient centrifugation. The fractions halfway between light DNA and the half-heavy DNA (fractions 26–28 of Fig. 5*a*, ref. 2) were used after dialysis for examination of molecules replicated to variable extents; the fraction containing half-heavy DNA (fraction 24 of Fig. 5*a*, ref. 2) was used for examination of completely replicated molecules.

Enzyme Treatments. Closed-circular Col E1 DNA molecules were converted to open circular structures by treatment with pancreatic DNase I. The reaction mixture (20 μl) contained approximately 20 ng of DNA, 100 pg of DNase I, 0.1 M Tris·HCl (pH 8.0), 5 mM $MgCl_2$, and 0.5 mM EDTA. The mixture was incubated at 30° for 10 min before addition of 0.2 M EDTA to 10 mM to terminate the reaction.

Circular Col E1 DNA molecules were converted to linear structures by treatment with restriction endonuclease *Eco*R1. The reaction mixture (20 μl) contained approximately 20 ng of DNA, an appropriate amount of endonuclease *Eco*R1, 50 mM Tris·HCl (pH 7.5), 50 mM NaCl, and 6 mM $MgCl_2$. One microliter of an enzyme solution was added to the reaction mixture, which was incubated at 37° for 60 min before addition of 0.2 M EDTA to 10 mM. The concentration of enzyme that was sufficient to introduce a double-strand break in more than 95% of the molecules had been previously determined by measuring the rate of conversion of ^{32}P-labeled closed-circular Col E1 DNA to linear molecules.

Electron Microscopic Observation. The basic protein film technique (5) and its modification with formamide (6, 7) were used. The solution of DNA in 2 M ammonium acetate, 0.1 M Tris·HCl (pH 8.5), 10 mM EDTA, and 40 μg/ml of cytochrome *c* was spread over the surface of 10 mM Tris·HCl

Abbreviations: Col E1, colicin E1 plasmid.

* This paper is the third of a series. The second is ref. 2.

† On leave from the National Institute of Health, Tokyo, Japan (present address).

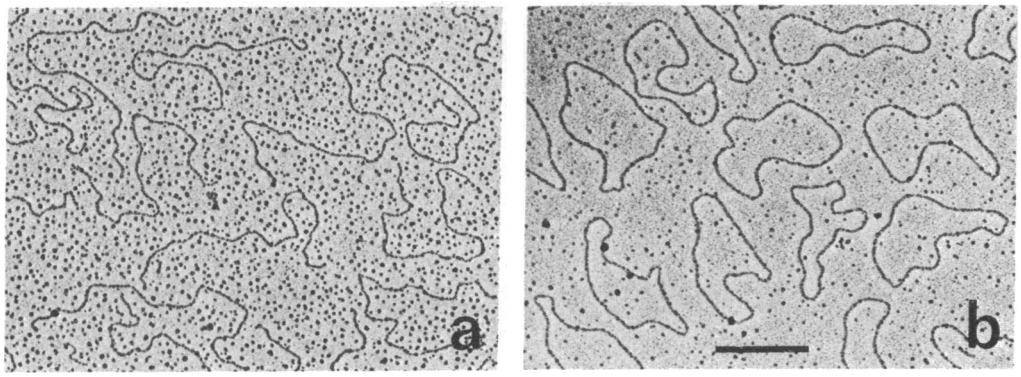

FIG. 1. Electron micrographs of Col E1 DNA molecules treated with enzymes. Col E1 DNA from a chloramphenicol-treated culture of *E. coli* A745(Col E1) (1) was treated with restriction endonuclease *Eco*R1 (*a*) or DNase I (*b*). The *scale* represents 0.5 μm.

(pH 8.5) and 1 mM EDTA. Samples were picked up on copper grids coated with nitrocellulose film. In the formamide technique, the solution of DNA in 0.1 M Tris·HCl (pH 8.5), 10 mM EDTA, 50% (v/v) formamide, and 40 μg/ml of cytochrome *c* was spread over the surface of the solution of 10 mM Tris·HCl (pH 8.5), 1 mM EDTA, and 17% (v/v) formamide. With the exception of Fig. 9, the formamide technique was used throughout. The samples stained with uranyl acetate and shadowed with platinum–palladium were observed with a Hitachi HU12 or Philips 200 electron microscope. Length of the molecules was measured on 10- to 20-fold enlarged images. Circular single- and double-stranded DNAs of phage φX174 were added to the DNA samples as the internal references. The length measurements were further calibrated with a carbon grating replica.

RESULTS

A Single Cleavage of Col E1 DNA by Restriction Endonuclease EcoR1. Considering the number of nucleotides constituting the specific base sequence of the endonuclease-sensitive site (8, 9) and the actual frequency of occurrence of the sensitive sites in various DNA species (8), it would be reasonable to expect that few, if any, sensitive sites would exist in a Col E1 DNA molecule. By treatment with the enzyme, ^{32}P-labeled, closed-circular Col E1 DNA molecules were converted to molecules with a sedimentation rate indistinguishable from that of linear molecules of a unit length in sucrose gradient centrifugation (data not shown). Electron micrographs of molecules treated with either restriction endonuclease *Eco*R1 or DNase I are presented in Fig. 1. The mean length of 50 linear molecules produced by treatment with restriction endonuclease *Eco*R1 was 2.01 ± 0.10 μm, and the mean contour length of 50 open-circular molecules formed by treatment with DNase I was 2.04 ± 0.07 μm. Less than 2% of endonuclease *Eco*R1-treated molecules were distinctly shorter than the mean length and excluded in the sampling. Otherwise, the molecules for the length measurement were selected randomly. The result indicates that endonuclease *Eco*R1 introduces one or multiple cleavages in a Col E1 DNA molecule within a very short segment. The multiple cleavages are unlikely in view of the specificity of the sensitive site.

Structure of Early Replicative Intermediates. When a DNA sample obtained from a reaction mixture incubated in the presence of glycerol and spermidine was examined, most Col E1 DNA molecules showed twisted circular structures. Among them molecules with a small loop were observed. These molecules were converted to open-circular structures to facilitate examination of the loops. Approximately 30% of the molecules in the sample contain a small loop. The loops were heterogeneous with respect to size and strandedness. Electron micrographs of loops of different types are presented in Fig. 2. Among 121 molecules with a loop that were randomly selected, 67 loops had two double-stranded branches and 40 loops had one each of single- and double-stranded branches. Identification of the strandedness of the branches of the rest of the molecules was ambiguous. Most double-stranded branches were double-stranded from one end to the other, but some had thin regions at one or both ends.

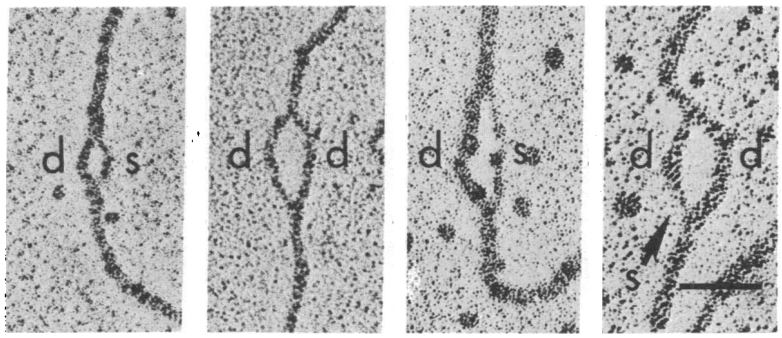

FIG. 2. Electron micrographs of representatives of small loops. A DNA sample from a standard reaction mixture that had been incubated for 60 min in the presence of glycerol and spermidine was examined after treatment with DNase I. The symbol, *s* or *d*, shows a single- or double-stranded region, respectively. The *scale* represents 0.1 μm.

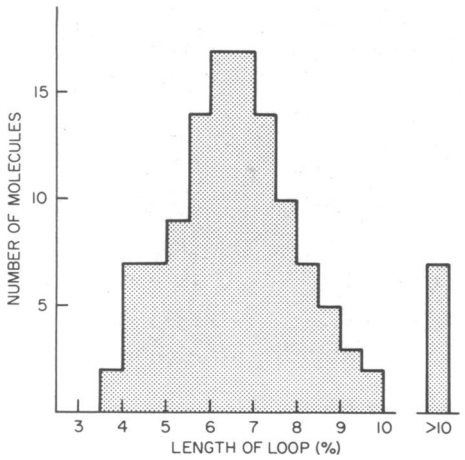

FIG. 3. Histogram showing distribution of the fractional length of loops carried by 121 molecules in a sample described in the legend to Fig. 2. For a loop with two double-stranded branches, the mean length of these branches is taken as the length of the loop. For a loop with one each of single- and double-stranded branches, the length of the double-stranded branch is used as the length of the loop. The total length of a molecule is the sum of the length of a loop and that of the unreplicated region. The mean total length of these molecules was 2.12 ± 0.14 μm.

A histogram of the distribution of the fractional length of the loops in 121 randomly selected molecules is presented in Fig. 3. The mean length of 114 loops that were shorter than 10% of the total length was 6.6 ± 1.7% (0.14 ± 0.04 μm). While the lengths of the loops were relatively uniform, they were distinctly heterogeneous.

In a sample of DNA obtained from the reaction mixture incubated without glycerol or spermidine, several percent of the molecules contained loops. Among 34 molecules examined, 16 had two double-stranded branches and 10 had one single- and one double-stranded branch. Identification of strandedness of 8 loops was ambiguous. The mean length of the loops was 7.4 ± 1.0% (0.15 ± 0.02 μm).

In a DNA sample that was prepared from an unincubated cell extract and treated with DNase I, most molecules had the open-circular structure and no molecules with a loop or a branch were found among more than 1000 molecules in the micrographs.

Location of Small Loops in Early Replicative Intermediates. When a DNA sample from a reaction mixture incubated in the presence of glycerol and spermidine was examined after treatment with restriction endonuclease *Eco*R1, linear molecules with a loop were observed. Electron micrographs of some of these molecules are presented in Fig. 4. Forty molecules were randomly selected and the lengths of the loops and the two linear unreplicated regions were measured. Fig. 5 shows the line diagram of these molecules normalized to a scale of 100 units and aligned on the assumption that the shorter unreplicated regions are derived from a common region of the molecule. The external end of a shorter unreplicated region will be referred to as the left end of the molecule. The left branch points of the loops were located at 16.8 ± 1.7% (0.35 ± 0.04 μm) of the total length from the left end of the molecule. The position of the right branch points of the loops varied more widely, giving a value of 24.8 ± 3.2% (0.51 ± 0.07 μm). The mean length and its standard deviation for shorter unreplicated regions adjacent to loops with a single-stranded branch and those for the regions adjacent to loops with only double-stranded branches were not significantly different.

A DNA sample from a reaction mixture incubated in the absence of glycerol or spermidine was similarly treated with

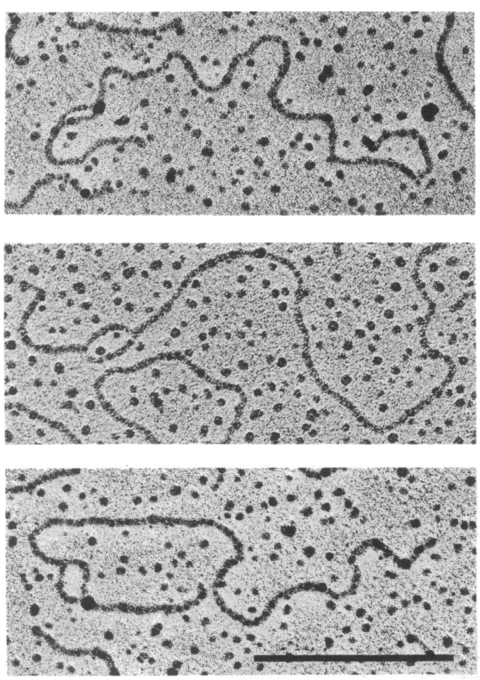

FIG. 4. Electron micrographs of Col E1 DNA molecules with a small loop after treatment with restriction endonuclease *Eco*R1. The same DNA sample described in the legend to Fig. 2 was treated with endonuclease *Eco*R1 instead of DNase I. The *scale* represents 0.5 μm.

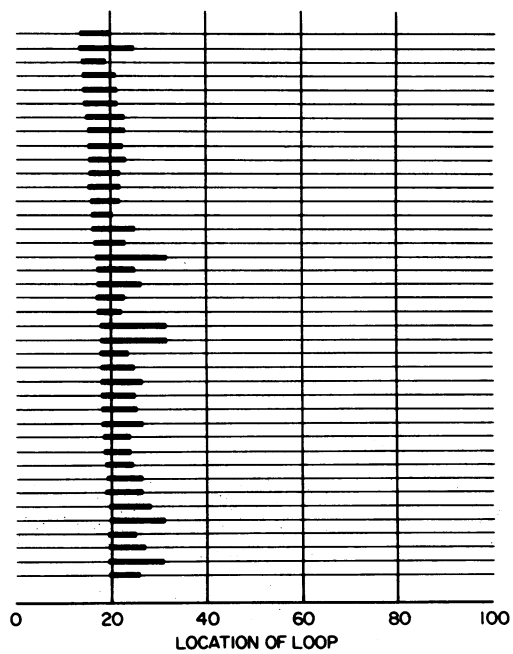

FIG. 5. Line diagram showing the location of small loops (*thick lines*) in the molecules treated with endonuclease *Eco*R1. The molecules observed in the sample described in the legend to Fig. 4 were examined. The principle of alignment of the molecules is described in the *text*. The mean total length of these molecules was 2.06 ± 0.10 μm.

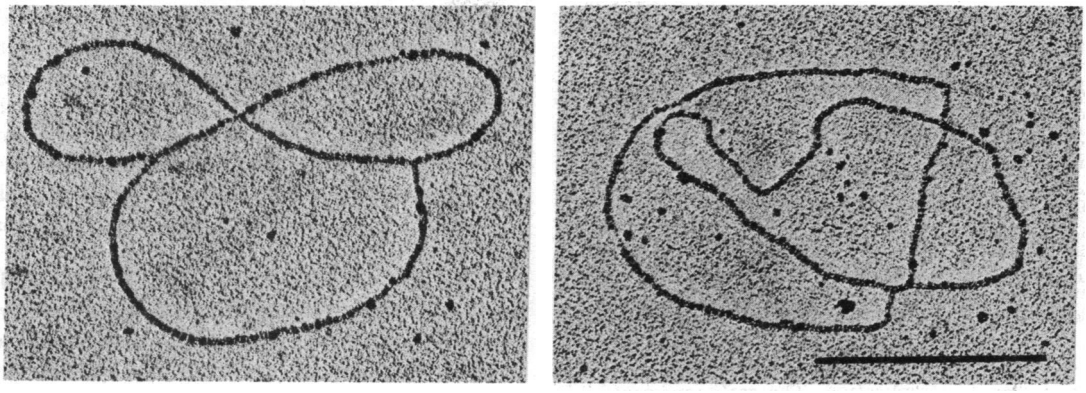

FIG. 6. Electron micrographs of DNase I-treated molecules that had replicated extensively. The *scale* represents 0.5 μm.

restriction endonuclease *Eco*R1 and examined. The left and the right branch points in 34 molecules with a loop were located at 16.6 ± 2.7% (0.35 ± 0.06 μm) and 24.1 ± 3.0% (0.50 ± 0.06 μm), respectively.

Structures of More Extensively Replicated Molecules. When an *in vitro* BrdU-labeled preparation in which replicating molecules were enriched was examined, circular molecules containing a loop with structures similar to those described for replicating Col E1 DNA *in vivo* (10–12) were observed. The preparation was treated with DNase I or endonuclease *Eco*R1 for further examination. Among 120 enzyme-treated replicating molecules, 40 had loops shorter than 10% of the total length. They are the class of molecules described before. The rest of the molecules had a loop of various sizes. Only two longer loops had a single-stranded branch in contrast to the shorter loops of which more than 30% had a single-stranded branch. Electron micrographs of extensively replicated molecules treated with DNase I are presented in Fig. 6.

Molecules with branches were searched for with the DNA sample treated with endonuclease *Eco*R1. While about 100 linear molecules with loops were observed, no molecules in which the cleavage site had been replicated were detected. Electron micrographs of replicating molecules treated with *Eco*R1 are presented in Fig. 7. With replicating molecules treated with the endonuclease, the lengths of the branches of the loops as well as those of unreplicated regions of the molecules were measured. The length of the shorter unreplicated region was 16.5 ± 1.8% (0.34 ± 0.04 μm) of the total length independent of the extent of replication (Fig. 8). This result indicates that extensive replication proceeds only to the right of the loops.

Structure of Molecules That Had Completed a Round of Replication. The BrdU-labeled DNA molecules at the half-heavy peak showed the typical twisted structures when examined by electron microscopy (Fig. 9). The structure of the molecules treated with DNase I was indistinguishable from that of open-circular monomeric molecules. The result shows that upon completion of replication most of the molecules return to the monomeric twisted-circular structure from which the Col E1 DNA molecules start to replicate (1).

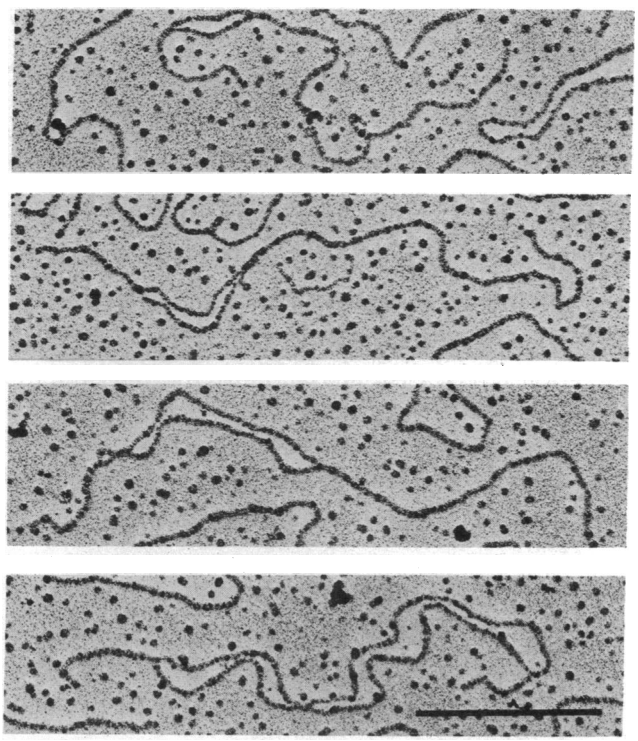

FIG. 7. Electron micrographs of molecules treated with endonuclease *Eco*R1 that had replicated to different extents. The *scale* represents 0.5 μm.

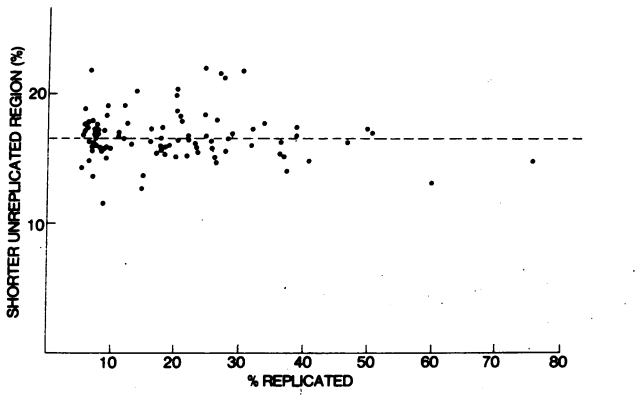

FIG. 8. Relation between the length of shorter unreplicated regions of replicating molecules treated with endonuclease *Eco*R1 and the extents of replication. The length of various regions of 96 endonuclease *Eco*R1-treated molecules that had replicated to variable extents were measured. For the molecule showing the most replication, the length of the longer unreplicated region is arbitrarily plotted. The length of the shorter unreplicated region was 9.1%. The mean length of the molecules was 2.08 ± 0.13 μm.

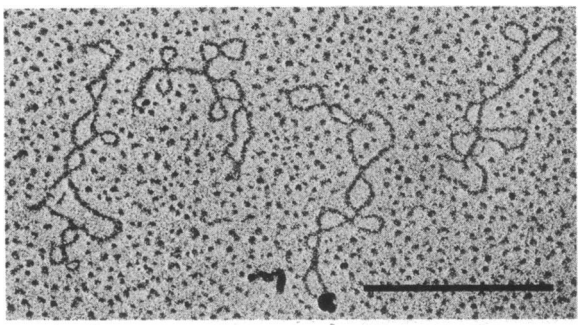

FIG. 9. Electron micrograph of Col E1 DNA molecules that had completed a round of replication. The *scale* represents 0.5 μm.

DISCUSSION

Col E1 DNA molecules observed with a DNA preparation from an unincubated cell extract had circular structures with neither loop nor branch, indicating infrequent presence of replicating molecules in cells from which the extract was made and/or inefficient extraction of replicating molecules during preparation of the extract. Molecules with a loop were found only in extracts incubated in the reaction mixtures. Incubation in the presence of glycerol and spermidine, which is known to stimulate the synthesis of early replicative intermediates (2), stimulated the synthesis of molecules with a small loop. In addition, the average length of the loops corresponded to that of DNA fragments carried by the replicative intermediates (1, 2). These results indicate that small loops are formed, *in vitro*, on circular molecules and that the molecules with a small loop represent the early replicative intermediates that were found by previous biochemical experiments (1, 2).

The study of the position of the loops in early replicative intermediates treated with restriction endonuclease *Eco*R1 shows that the loops are located in a unique region of the molecule. The value of coefficient of variation (standard deviation divided by mean) for the measured length of the shorter unreplicated regions is significantly larger than the value for linear whole molecules. This may be due to significant errors arising in the assignment of the ends of molecules or segments. The left branch points, therefore, appear to be located at a physically fixed point in the molecule. The difference of the position of the right branch points results from the variation of the length of shorter unreplicated regions and the difference of the length of loops. Given that these molecules are early replicative intermediates, the origin of DNA replication must thus be within the loop in a fixed region and no efficient secondary origin of replication can exist.

The presence of early replicative intermediates that are different in size and in strandedness seems to show the presence of more than one slow step in early replication or one slow key reaction that affects various steps. The similarity of constituents of early replicative intermediates formed in the presence and in the absence of glycerol or spermidine suggests the presence of a slow key reaction that is sensitive to these chemicals. The fact that more extensively replicated molecules did not accumulate in the reaction mixture indicates that the further replication of early replicative intermediates, once started, proceeds much faster than early replication.

Circular molecules that contained a larger loop were observed with a preparation in which these molecules were enriched. Branches of these longer loops were almost always double-stranded in contrast to the high incidence of single-stranded branches in smaller loops. In replication of mitochondrial DNA, small loops with a single-stranded branch (13) continue to expand by displacement-synthesis (7).

The location of loops in the molecules replicated to variable extents (Fig. 8) indicates that extensive replication proceeds, without exception, to the right of the loops.

Complementary data on Col E1 DNA replicated *in vivo* are presented in the accompanying paper (14). The excellent agreement between the origin and direction of replication of Col E1 DNA *in vitro* and those *in vivo* provides additional evidence that Col E1 DNA replicates *in vitro* as it does *in vivo*.

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