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Replication of Colicin E1 Plasmid DNA in Minicells from a Unique Replication Initiation Site

(*Eco*R1 restriction endonuclease/electron microscopy/initiation and direction of replication)

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ABSTRACT **Replicating DNA molecules of the colicin E1 plasmid isolated from minicells are cleaved at a single site by R1 restriction endonuclease (*Eco*R1). Electron microscopic measurements of the replicating molecules treated with the endonuclease indicate that (*a*) replication is initiated at a site between 14% and 20% of the distance from the *Eco*R1 endonuclease cleavage site; and (*b*) extensive replication of most molecules occurs in one direction from the initiation site, although a limited amount of replication in the opposite direction may occur. Single-stranded regions at one or both replication forks, involving one or both DNA strands, can be frequently found in replicating molecules.**

DNA of the colicin E1 plasmid (Col E1), can segregate into and replicate in minicells produced by the colicinogenic strain of *Escherichia coli* P678-54 (Col E1) (1). Replicating Col E1 DNA molecules have previously been isolated from the minicells and identified as replicating twisted and open circular structures (2–4). A determination of both the position of a unique replication initiation site and the direction of replication from such a site on Col E1 DNA may be achieved by establishing an electron microscopically determinable internal molecular marker (5–7). The production of a single unique cleavage site, caused by the *Eco*R1 restriction endonuclease, in DNA of simian virus 40 has been used to establish an internal marker in that molecule for mapping the location of that virus' replication initiation site (8, 9). J. Tomizawa and Y. Sakakibara found that the *Eco*R1 restriction endonuclease caused a single break in Col E1 DNA (personal communication). On the basis of the size of Col E1 DNA (4.2×10^6 daltons) and character of the site sensitive to *Eco*R1 restriction endonuclease, this is the number of sites sensitive to *Eco*R1 restriction endonuclease that would be expected in that molecule (10, 11).

In the present work, replicating Col E1 DNA obtained from minicells (3) was treated with the *Eco*R1 restriction endonuclease, and a determination of the location of the *in vivo* replication initiation site of the Col E1 DNA in relation to the cleavage site of *Eco*R1 restriction endonuclease was determined. The direction of replication from that replication initiation site and the structure of the replication forks in the replicating molecules was also examined.

MATERIALS AND METHODS

Reported procedures for isolating replicating Col E1 DNA from minicells produced by *E. coli* strain P678-54 (Col E1) have been used (3). The Col E1 DNA in the minicells was labeled with tritiated thymidine, released by a lysozyme–Sarkosyl lysis procedure, and banded in a sucrose density gradient (3). Selected fractions of the gradient known to contain replicating DNA were pooled (3). The DNA was dialyzed in 50 mM Tris (pH 8.0)–10 mM EDTA, concentrated, dialyzed into 0.1 M Tris (pH 7.5)–50 mM NaCl–6 mM $MgCl_2$, and treated with *Eco*R1 restriction endonuclease. The activity of *Eco*R1 restriction endonuclease (a generous gift of Dr. Daniel Nathans) was measured by the method of Dr. D. Nathans (personal communication). *Eco*R1 restriction endonuclease (0.002 ml) was added to 0.03 ml of the Col E1 DNA, and the mixture was incubated at 37° for 10 min. The amount of enzyme and incubation time used was determined empirically for the particular enzyme and Col E1 DNA preparations to achieve a maximum amount of DNA cleavage in a short period of time. (More than 95% of circular molecules were converted to linear molecules.) The DNA was diluted into 0.1 M Tris (pH 8.5)–10 mM EDTA; appropriate amounts of cytochrome *c* and formamide were added and the DNA was spread for electron microscope examination by the Kleinschmidt method (12) using the formamide technique (13, 14). DNA picked up on parlodian grids was stained with uranyl acetate, rotary shadowed with Pt-Pd (80:20), and examined with a Siemens 101 electron microscope as described (14). Approximately 4% of molecules examined were clearly identified as replicating structures.

RESULTS AND DISCUSSION

The effect of treating Col E1 DNA molecules isolated from P678-54 (Col E1) minicells with *Eco*R1 restriction endonuclease is shown in electron micrographs in Fig. 1*a* and *b*. These electron micrographs show the conversion of the twisted circular to linear structures after *Eco*R1 endonuclease treatment. The lengths of 250 consecutive, randomly selected, linear Col E1 DNA fragments in the DNA preparation shown in Fig. 1*b* were measured electronmicroscopically and are shown in Fig. 2. The results indicate that one double-strand break was introduced into each Col E1 DNA molecule.

Replicating circular Col E1 DNA molecules were isolated from minicells, exactly as described (3), treated with the *Eco*R1 restriction endonuclease, and the linear structures generated by the treatment were examined electron microscopically. Fig. 3 shows electron micrographs of replicating Col E1 DNA molecules, treated with *Eco*R1 restriction endonuclease, that have replicated to various extents in minicells. The replicated DNA is that DNA between the two Y-shaped forks in each molecule. The forks will be referred to as "replication forks," although no direct evidence indicates whether active replication is occurring at one or both forks.

Abbreviation: Col E1, colicin E1 plasmid.

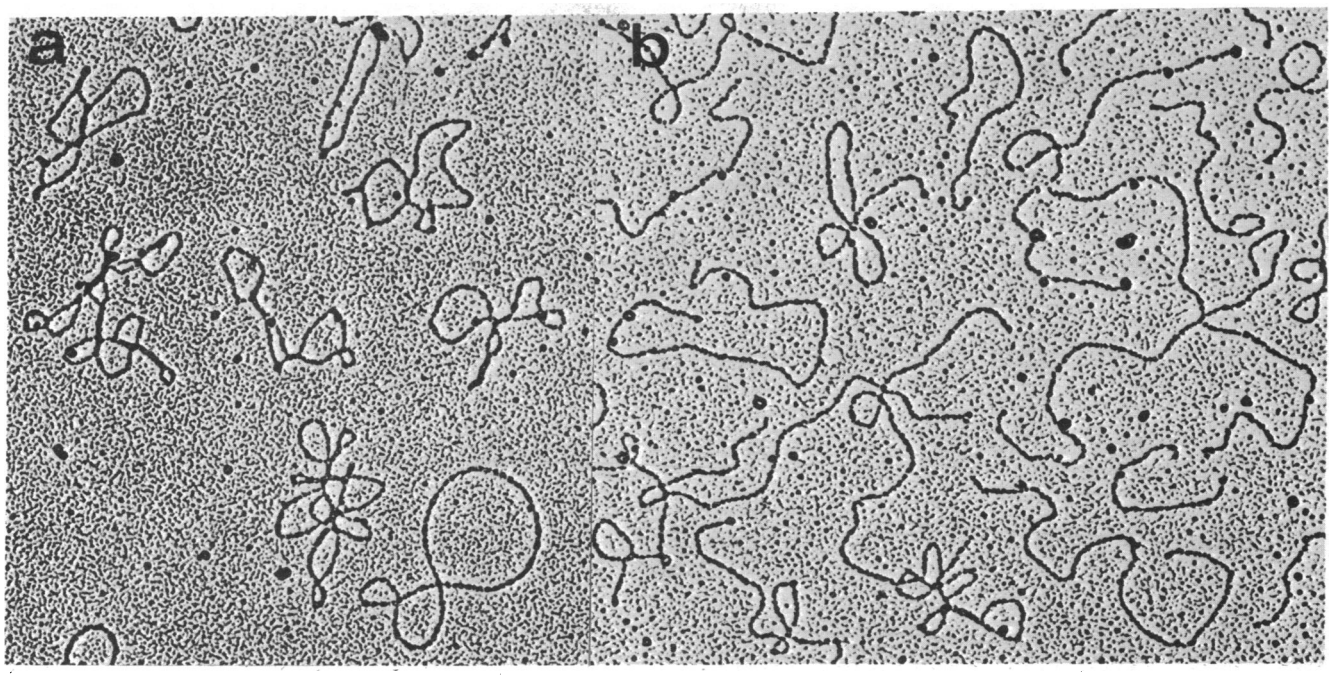

FIG. 1. The effect of the *Eco*R1 restriction endonuclease on Col E1 DNA. Col E1 DNA isolated from minicells of *E. coli* strain P678-54 (Col E1) was (*a*) untreated or (*b*) treated with *Eco*R1 restriction endonuclease and spread by the formamide technique (13, 14). The electron micrographs of both preparations are at the same magnification.

Measurements of 53 randomly selected replicating molecules are shown in Fig. 4. The overall length of these replicating molecules was 1.97 ± 0.12 μm, as compared to 1.99 ± 0.10 μm for the unreplicated linear Col E1 DNA shown in Fig. 2. The average length of the short unreplicated DNA strand to the left of the replicated region (Fig. 4) in the endonuclease-treated replicating molecules does not exceed 20% of the Col E1 DNA length, and has an average length of 0.33 ± 0.06 μm (16.8 ± 3.0% of the overall molecular length). The short unreplicated region varies from 20% to 14% of the Col E1 DNA molecular length, although in three cases (molecules 1, 2, and 3, Fig. 4), the unreplicated lengths are less than 14% of that length. The unreplicated DNA to the right of the replicated region (Fig. 4) shows much greater variability in its size, approaching a maximum length of 80% of the Col E1 DNA length (molecules 6 and 32, Fig. 4).

It was also observed that replicating molecules treated with *Eco*R1 restriction endonuclease did not contain branched ends, indicating that frequent replication through an endonuclease-sensitive cleavage site in those molecules did not occur, though replication of the DNA exceeding 20% and even 50% of a molecule's total length often did occur (Fig. 4).

The relatively constant relationship between the single cleavage site of *Eco*R1 restriction endonuclease on each molecule and the narrowly localized region in which replication initiation occurs (14–20% of the distance from the cleavage site) strongly suggests, though does not prove, that Col E1 DNA contains a unique *Eco*R1 restriction endonuclease cleavage site and unique replication initiation site. This constant relation will be discussed later.

Two interpretations of the data, which may clarify the location of the replication initiation site, seem reasonable and will be presented. One interpretation locates the replication initiation site at the average distance of the left replication fork (Fig. 4) from the left end of the replicating molecules (16.8 ± 3.0% of the total molecular length). It assumes that the variation in length of the short unreplicated strand of DNA (Fig. 4, *left*) simply reflects a measurement variation. Having made that assumption, it can be seen (Fig. 4) that replication then proceeds unidirectionally to the right of that point. As replicated portions of the molecules examined frequently exceed the shorter mean distance (16.8%) to the

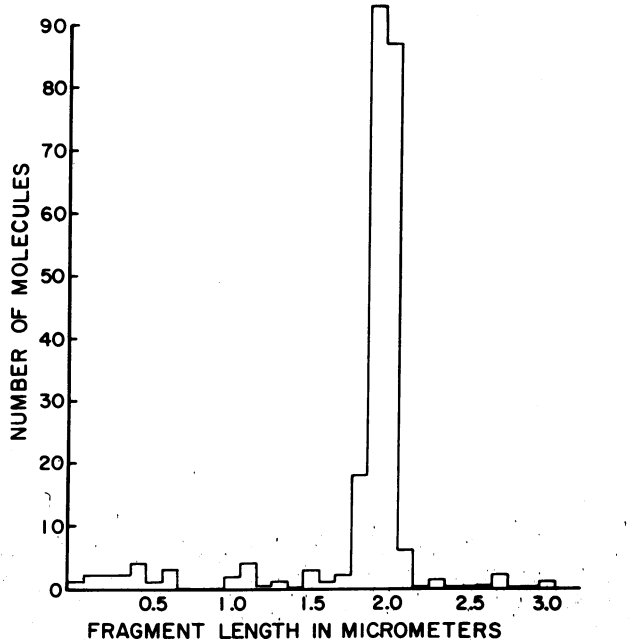

FIG. 2. Two hundred and fifty consecutively observed linear fragments of Col E1 DNA from the DNA preparation treated with *Eco*R1 restriction endonuclease shown in Fig. 1*b* were measured electron microscopically. The images from electron micrographs were projected on a screen and their lengths were measured with a Graf/Pen (Science Accessories Corp.). The measurements were calibrated with a carbon grating replica, 54,864 lines per inch (21,600 lines per cm). The average length of the molecules was 1.99 ± 0.10 μm.

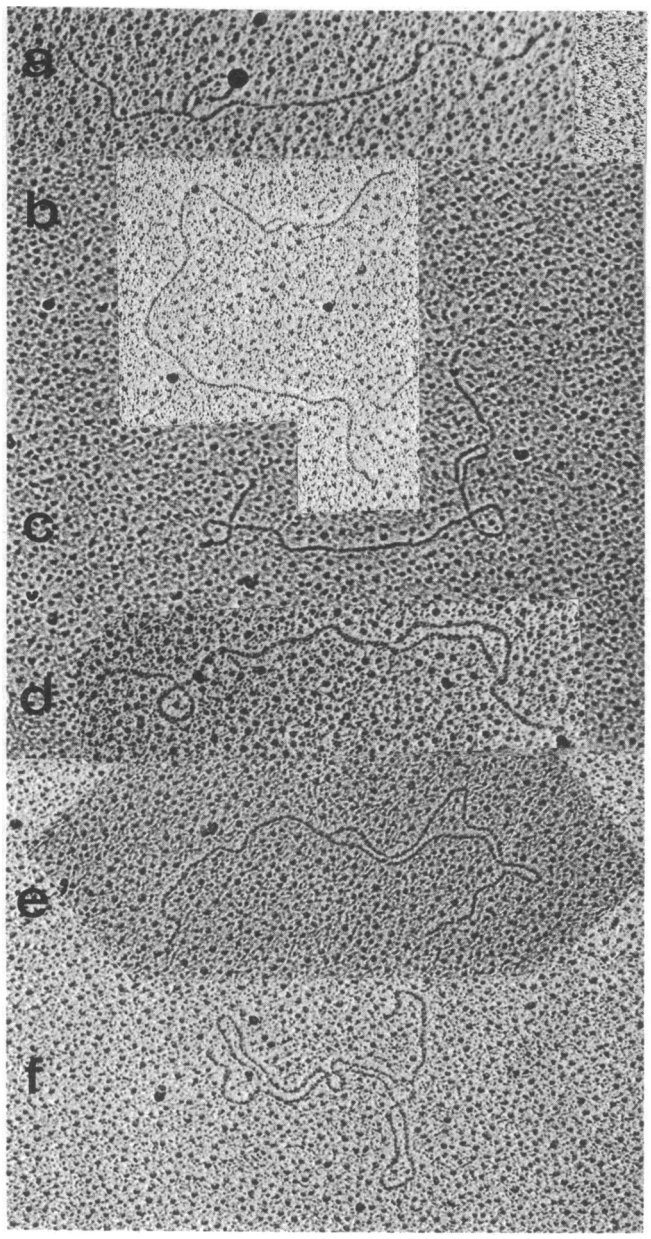

FIG. 3. Electron micrographs of replicating Col E1 DNA treated with *Eco*R1 restriction endonuclease. Replicating Col E1 DNA was obtained from minicells derived from P678-54 (Col E1) exactly as described (3) and treated with *Eco*R1 restriction endonuclease. Six replicating molecules (*a–f*), exhibiting different degrees of replication, are shown.

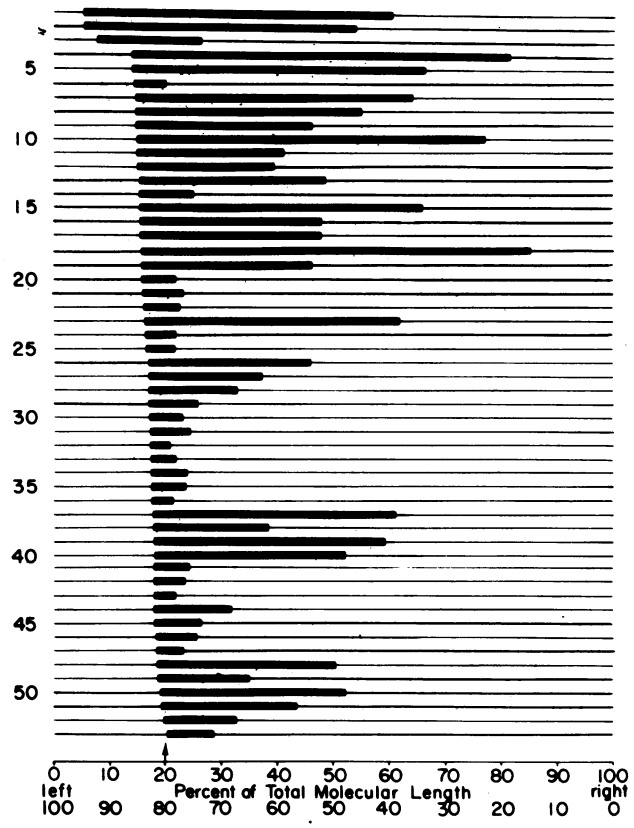

FIG. 4. Measurements of replicating molecules of Col E1 DNA treated with *Eco*R1 restriction endonuclease. Replicating Col E1 DNA molecules obtained, treated, and prepared for electron microscopy as described in Fig. 3 were photographed and measured as described in Fig. 2. The linear replicating structures generated by *Eco*R1 restriction endonuclease treatment of circular replicating Col E1 DNA are aligned with the short unreplicated length of DNA to the left. The replicated region between the forks is represented by a *heavy line*. The average total length of the replicating molecules was 1.97 ± 0.12 μm (——, unreplicated region) (▬, region between forks). Measurements for each molecule are presented in terms of percent of the total molecular length.

*Eco*R1 cleavage site from the region containing the replication initiation site without crossing the cleavage site to generate molecules with split ends, it may be concluded that replication rarely proceeds through the cleavage site to the left of the replication initiation site. This observation further supports the interpretation of a unidirectional replication from a unique initiation site.

A second interpretation of the data is that the variation in length of the short unreplicated strand of DNA is not due to measurement variation but represents a limited degree of replication to the left of a replication initiation site (Fig. 4). Three observations may support this interpretation. (*1*) The variation in length of the short unreplicated DNA is three times greater than the variation in total length of the replicating molecule (0.33 ± 0.06 μm compared to 1.97 ± 0.12 μm). (*2*) The maximum length of the short unreplicated section of DNA measured from the left end of the molecule (Fig. 4) approaches 20% of the total molecular length. (*3*) The maximum length of the long unreplicated section of DNA measured from the right end of the molecule (Fig. 4) approaches 80% of the total molecular length.

The convergence of the measurements of the short and long lengths of unreplicated DNA at a point about 20% of the overall molecular length from the left end of the molecular (Fig. 4) suggests that the convergence point represents the replication initiation site. In such a case replication to both the left and right of the replication initiation site would occur in most molecules; however, extensive replication would preferentially be to the right (Fig. 4). In this case replication is asymmetric and bidirectional.

The relatively constant relationship between the *Eco*R1 restriction endonuclease site and the narrow replication initiation region (Fig. 4), while suggesting a constant relationship between a unique *Eco*R1 restriction endonuclease site and a unique replication initiation site, may also reflect more complex relations. The presence of two replication initiation sites

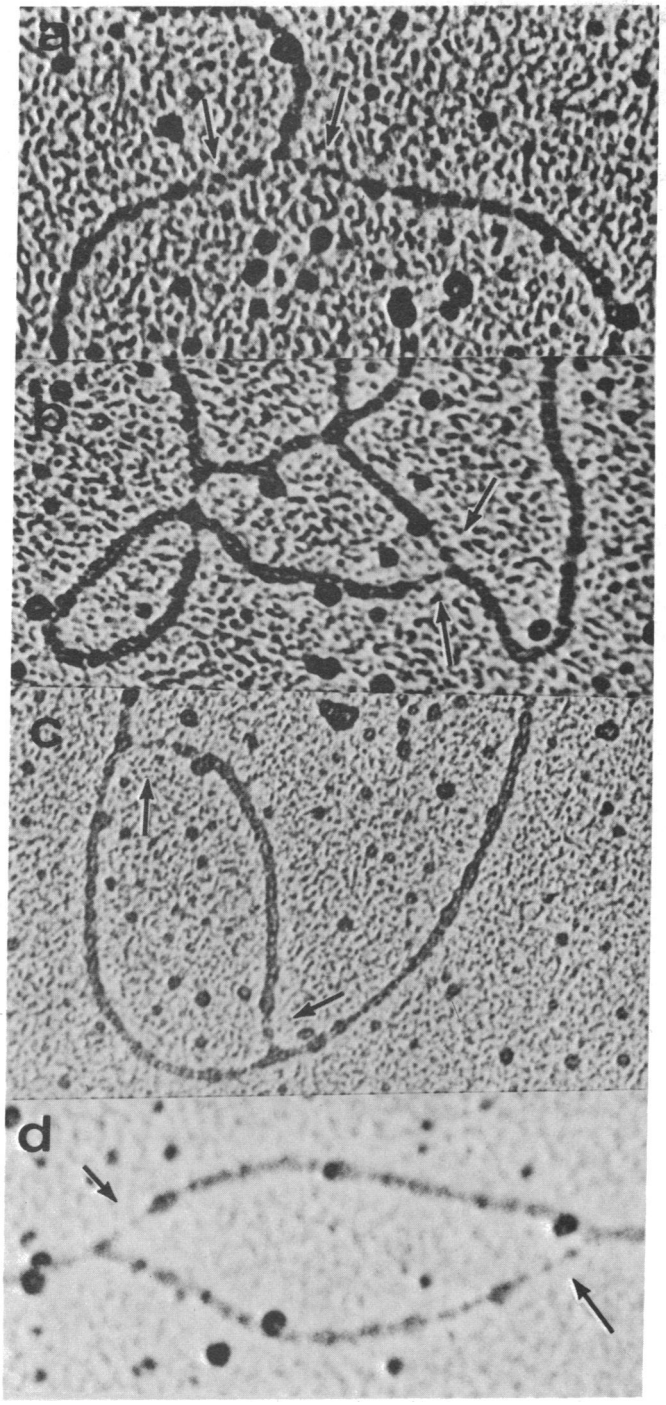

FIG. 5. Electron micrographs of replicating Col E1 DNA showing single-stranded DNA at the forks. The DNA preparation examined was that used in Fig. 3. The *arrows* designate single-stranded DNA. The electron micrographs show single-stranded DNA at the forks associated with the (*a*) short, (*b*) long; (*c* and *d*) short and long unreplicated portion of the molecule. As the crossing of two strands of DNA may produce an appearance of single strandedness in one strand, the single-stranded DNA at only one fork of *b* is noted.

equidistant from a single *Eco*R1 restriction endonuclease site could generate the same results. In that case, however, replication from only one site on a molecule would be permitted and the predominant replication from both sites would have to be in opposite directions, and away from the restriction sensitive site.

Other models involving either multiple or variable located *Eco*R1 restriction endonuclease-sensitive sites and replication initiation sites are possible; however, they also entail a variety of qualifying assumptions about either the occurrence and variable sensitivity of the *Eco*R1 restriction endonuclease-sensitive sites, which do not seem warranted, or about the nature of replication from different initiation sites. The presence of a unique *Eco*R1 restriction endonuclease-sensitive site and unique replication initiation site presently best explains these observations.

An examination of the structure of the replication forks of replicating molecules prepared for electron microscopic examination of single-stranded DNA (13, 14) indicates that single-stranded regions can be found at either or both forks and may involve one or both strands at a fork (Fig. 5). Approximately 50% of replicating molecules observed before and after endonuclease treatment had an identifiable single-stranded region associated with the replicated portion of the molecule. A similar finding has been made in studies of replicating λ bacteriophage DNA (5, 6). As the presence of these single-stranded regions at the replication forks may represent the occurrence of discontinuous DNA synthesis first reported by Okazaki *et al.* (15), a distinction between the unidirectional and asymmetric bidirectional synthesis would complement our understanding of these single-stranded structures in replicating Col E1 DNA.

While the definitive evidence to clearly distinguish between unidirectional and asymmetric bidirectional replication is not now available, it now appears that the observations tend to suggest the latter interpretation.

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