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Genetics

Bidirection replication from a unique origin in a mini-F plasmid

(circular DNA/replicative intermediates/plasmid incompatibility/restriction endonucleases/electron microscopy)

RUDOLF EICHENLAUB, DAVID FIGURSKI, AND DONALD R. HELINSKI

Department of Biology, University of California, San Diego, La Jolla, Calif. 92093

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ABSTRACT **Replicating molecules of the mini F-kanamycin resistance plasmid, pML31, derived from F'*lac*, have been isolated from *Escherichia coli* as covalently-closed circular DNA molecules. These molecules were examined in the electron microscope after digestion with either *Eco*RI or *Bam*HI restriction endonuclease. The structure of the majority of the replicating molecules was consistent with a bidirectional mode of replication starting at a unique origin on the F-fragment. This origin is located approximately 2.3 kilobases from one of the *Eco*RI sites. Orientation of the F-fragment relative to the physical map of F showed the position of the origin to be at 42.6 kilobases. A small proportion of molecules appeared to be replicating unidirectionally in either direction from this origin. Termination of replication of pML31 apparently occurs in the fragment containing the locus for kanamycin resistance in a unique region opposite the origin in the circular DNA molecule.**

Mini F-kanamycin resistance (Km), designated pML31, is a hybrid plasmid containing the *Eco*RI endonuclease-generated fragment V (molecular weight of 6×10^6) of the F plasmid, which was selected for its ability to replicate autonomously by linkage to the *Eco*RI-generated, nonreplicating, Km fragment of plasmid pSC105 (1). pML31 contains the replication region of F [coordinates 40.3 to 49.3 kilobases (kb) on the physical map of F (2)] and is indistinguishable from F with respect to its low copy number, incompatibility with F, and curing by acridine orange (1). pML31, therefore, is useful in studying certain replication properties of the much larger parental F plasmid (molecular weight of 62×10^6) (3). In this report, we have located a unique origin of replication of the F-fragment of pML31 and demonstrated predominantly bidirectional replication from this origin with termination most likely in a region of the Km fragment that is located opposite the origin. The position of this origin of replication, located at 42.6 kb on the F map, is substantially different from the 46 to 48 kb region of F that has been shown to be required for F incompatibility (4).

MATERIALS AND METHODS

Supercoiled pML31 DNA was isolated from *Escherichia coli* CR34 (pML31) (2) and introduced by transformation (5) into the mini-cell producing *E. coli* strain P678-54 Thy$^-$, which was used previously in the replication study of the plasmid R6K (6). Replicating molecules of pML31 were labeled with a radioisotope and isolated by methods similar to those used in studies of the replicating molecules of ColE1 and R6K (6, 7). A 500-ml culture of P678-54 Thy$^-$ (pML31) in M9 medium supplemented with 0.5% casamino acids, 5 μg/ml of thiamine, 200 μCi of [2-^{14}C]thymine (48 Ci/mmol, New England Nuclear Corp., Boston, Mass.), and 2.5 μg/ml of unlabeled thymine was grown at 37° to a turbidity of 120 Klett units (no. 54 filter). Cells were harvested by centrifugation at room temperature and resuspended into 200 ml of warmed, supplemented M9 medium lacking thymine and containing 3 mM adenosine 3':5'-cyclic monophosphate (Sigma Chemicals, St. Louis, Mo.). After thymine starvation of the cells for 35 min at 37°, the culture was shifted to 25° and the cells were pulse-labeled for 1.5 min by the addition of 7.5 mCi of [^{3}H]thymine (50 Ci/mmol, New England Nuclear Corp., Boston, Mass.). Sodium azide was then added to a final concentration of 0.1 M, and the culture was rapidly frozen in a Dry Ice-ethanol bath. After thawing, the cells were harvested by centrifugation and lysed by the sodium dodecyl sulfate-salt method (8). Replicating molecules of pML31 were purified by centrifugation in an ethidium bromide–CsCl gradient followed by sedimentation in a neutral 5–20% sucrose gradient in a Beckman SW27 rotor for 4 hr at 25,000 rpm.

Replicating DNA molecules were cleaved with restriction endonucleases *Eco*RI or *Bam*HI using reaction conditions previously reported (9, 10) and then examined in the electron microscope as described (6). As an internal length standard, open circular ColE1 DNA (2.15 μm) (11) was added to the spreading mixture. Electron micrographs were taken on 35 mm film and projected on a Hewlett-Packard digitizer board (model 9864A) connected with a Hewlett-Packard calculator (model 9821A).

For the pulse-chase experiment, a 500-ml culture of P678-54 Thy$^-$ (pML31) was grown and labeled with [^{14}C]thymine as described above. After thymine starvation the culture was divided into four portions of 50 ml each. DNA was pulse-labeled for 1.5 min with 2 mCi of [^{3}H]thymine followed by a chase for 0, 1, 3, and 10 min by the addition of 5 ml of a mixture of thymine (200 μg/ml) and thymidine (2 mg/ml). Sodium azide was then added and the cells were rapidly frozen. Cells were lysed and the DNA was centrifuged to equilibrium in an ethidium bromide–CsCl gradient as described above.

RESULTS

Isolation of replicating intermediates of plasmid pML31

A majority of plasmid DNA, labeled with a [^{3}H]thymine pulse, bands at a position between supercoiled and open circular DNA (Fig. 1a). To separate further the replicating molecules from supercoiled, open circular, and linear pML31, the pooled fractions containing the pulse-labeled DNA were centrifuged on a neutral 5–20% sucrose gradient (Fig. 1b). Examination of the fast-sedimenting DNA from the sucrose gradients by electron microscopy showed partially supercoiled and partially open circular DNA molecules that were similar in appearance to the replicating covalently closed DNA of simian virus 40 (12, 13). The nature of the slowly sedimenting peak in the sucrose gradient (Fig. 1b) is not known. It is conceivable that it represents, at least in part, Okazaki fragments that are released from replicative forms during purification. To test whether the pulse-labeled material between the supercoiled and open circular bands of DNA represents true intermediates of replication of the plasmid, we performed a pulse-chase experiment. Fig. 2b shows that after a pulse of 1.5 min with [^{3}H]thymine, followed by a 1-min chase with unlabeled thymine, most of the

Abbreviations: Km, kanamycin resistance; kb, kilobase.

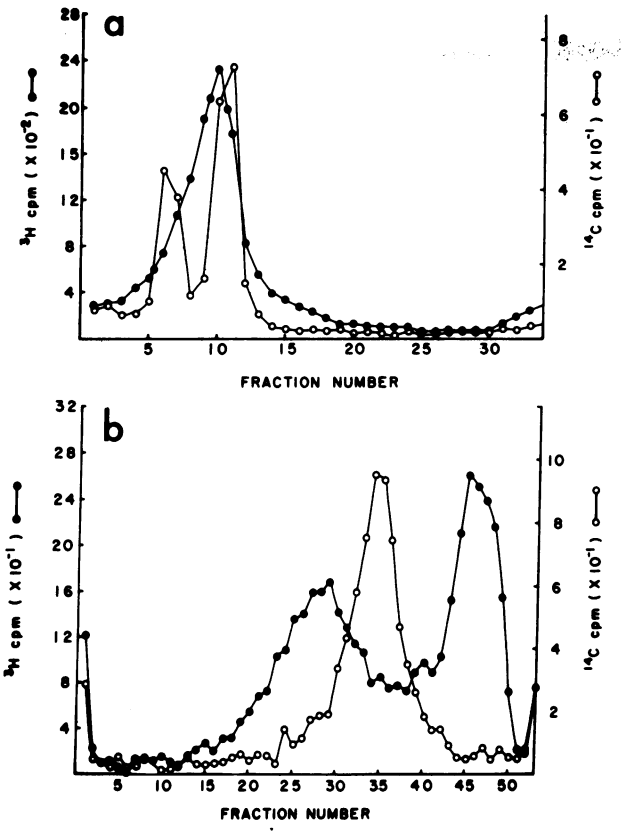

FIG. 1. Purification of replicating molecules of plasmid pML31. (a) A lysate of P678-54 Thy⁻ (pML31) first labeled with [^{14}C]thymine and then pulse-labeled with [^{3}H]thymine was prepared as described in *Materials and Methods* and centrifuged to equilibrium in a cesium chloride-ethidium bromide density gradient in a Beckman 60Ti rotor at 38,000 rpm for 40 hr at 15°. Fractions of 0.5 ml were collected from the bottom of the gradient. (b) Fractions 8–10 of the cesium chloride–ethidium bromide gradient were pooled and centrifuged on a neutral 5–20% sucrose gradient as described in *Materials and Methods*. Fractions of 1 ml were collected from the bottom of the gradient.

pulse label is already chased into supercoiled and open circular DNA. After a 3-min chase the DNA banding at the intermediate density has disappeared and more than 60% of the pulse-labeled DNA bands at the position of supercoiled DNA.

Examination of replicating molecules of pML31 after cleavage with *Eco*RI

Cleavage of pML31 with *Eco*RI generates two DNA fragments, RI-fragment A, corresponding to the F replicon with a molecular weight of 6×10^6, and RI-fragment B, corresponding to the Km fragment with a molecular weight of 4.5×10^6 (1). These two *Eco*RI fragments are easily distinguished by electron microscopy on the basis of their size. Replicating molecules were digested with restriction endonuclease *Eco*RI and examined in the electron microscope. Representative forked molecules of RI-fragments A and B are shown in Fig. 3. An analysis of the position of the replication fork in these molecules indicates that replication starts at a unique origin approximately 2.3 kb from one of the *Eco*RI sites in the F-fragment and proceeds bidirectionally (Fig. 4). Approximately 20% of the molecules replicate unidirectionally in either direction from the same origin (Fig. 4b). Of 112 eye-structures, only 5 were found to have a length corresponding to that of fragment B. These molecules may represent replication from a cryptic origin in the Km-fragment.

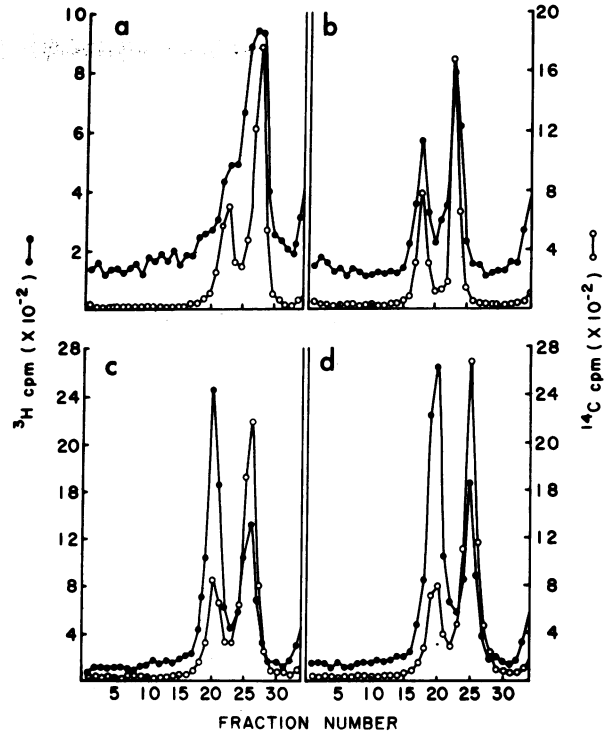

FIG. 2. Cesium chloride–ethidium bromide gradients of pulse-chased replicating intermediates of plasmid pML31. Cells of P678-54 Thy⁻ (pML31) were grown as described in *Materials and Methods*. After the culture was starved for thymine over a period of 35 min, it was divided into four equal portions of 50 ml. The DNA was then pulse-labeled for 1.5 min by the addition of 2 mCi of [^{3}H]thymine followed by a chase for (a) 0 min, (b) 1 min, (c) 3 min, and (d) 10 min by the addition of a mixture of thymine (200 μg/ml) and thymidine (2 mg/ml). Incorporation was stopped by the addition of sodium azide. Sodium dodecyl sulfate-salt lysates of the four cultures were centrifuged to equilibrium in a cesium chloride–ethidium bromide gradient in a Beckman 50Ti rotor at 38,000 rpm for 36 hr at 15°.

Examination of replicating molecules of pML31 after cleavage with *Bam*HI

Cleavage of pML31 with restriction endonuclease *Bam*HI generates two fragments, *Bam*-fragment A (molecular weight 1.6×10^6) and *Bam*-fragment B (molecular weight 9×10^6). Both *Bam*HI sites are in the F-fragment (D. Figurski, unpublished observations). Examination of *Bam*HI-restricted replicating molecules of pML31 in the electron microscope showed that the origin of replication is located very near *bam*HI site 2 and is most likely within *Bam*-fragment A since replication loops have been observed only in this *Bam*-fragment. Consequently, replication forks in *Bam*-fragment B move into the molecule from the *Bam*HI-generated ends of the fragment (Fig. 3c and d). In Fig. 5 the position of the branch points in *Bam*-fragment B is plotted against percent replication. The regression lines cross at 0.6 (60% from one end), which represents the site of termination. The scattering of the points may be caused by different rates of synthesis of the two arms or nonsynchronous initiation of the two branches. It is possible, therefore, that termination occurs in a region around 0.6 rather than at a precise site.

Mapping of the F-fragment origin relative to F-factor

Heteroduplex studies of plasmids pML31 and F8-33 have shown that pML31 forms a heteroduplex with F between 40.3 and 49.3 kb on F (2). Assuming that this 9.0 kb region of homology to F8-33 corresponds to the 9.0 kb *Eco*RI restriction fragment derived from F'*lac*, then the origin of replication

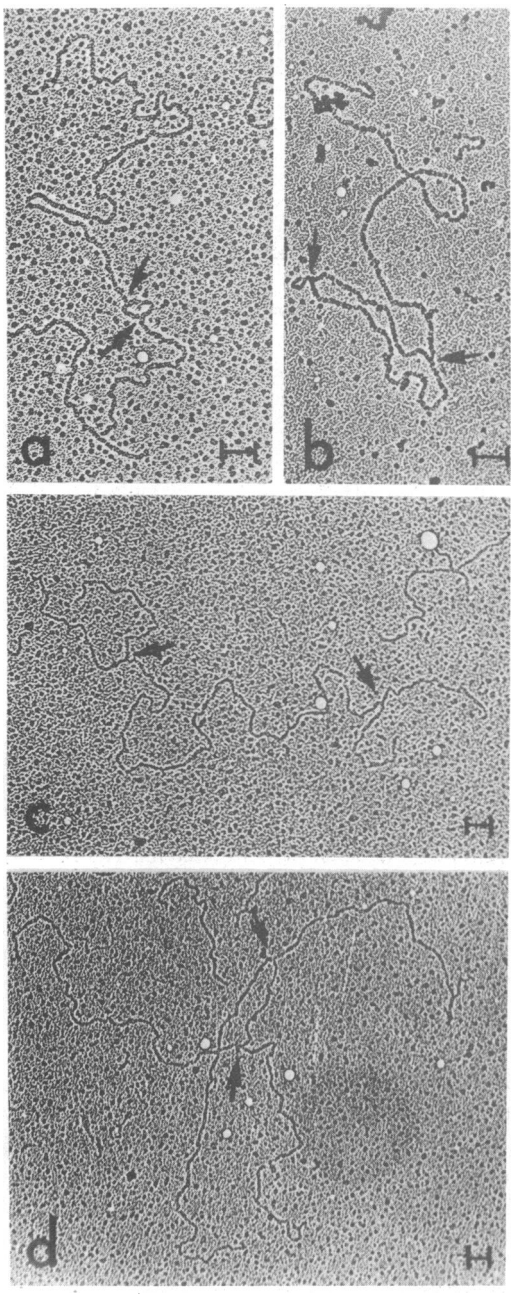

FIG. 3. Electron micrograph of replicating molecules of pML31 restricted with endonucleases *Eco*RI and *Bam*HI. (a and b) Replication loop in the *Eco*RI-generated F-fragment. The arrows indicate the location of the branch points. (c and d) Replication forks (indicated by arrows) in the large *Bam*-fragment (see insert in Fig. 5) obtained by *Bam*HI cleavage of replicating molecules. Bars represent 0.1 μm.

would be either at 42.6 kb or at 47 kb on the physical map of F, since the origin is located 2.3 kb from one of the *Eco*RI sites of pML31. In addition, it has been observed that the nonhomologous region of pML31 contains an inverted repeat that is located asymmetrically from the ends of the region of homology (2). The distance from the 40.3 kb coordinate to the stem of the inverted repeat is 2.3 ± 0.2 kb, whereas the distance from the stem to the 49.3 kb coordinate is 1.5 ± 0.1 kb (2). Since the position of the origin is known relative to the *Bam*HI sites of pML31, mapping of the inverted repeat relative to the *Bam*HI sites will locate the position of the origin with respect to the inverted repeat. Consequently, the coordinate of the replication origin on the F-factor map can be identified.

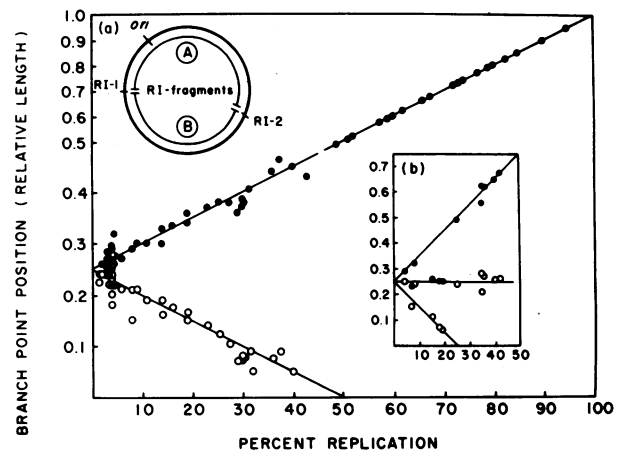

FIG. 4. Plot of branch point position against percent replication of replicating molecules of RI-fragment A. Replicating molecules of pML31 were digested with endonuclease *Eco*RI, and branched molecules of RI-fragment A were measured with open circular ColE1 DNA as an internal standard. The lengths of the short and long unreplicated arms to the branch point relative to the length of RI-fragment A are depicted by open and filled circles, respectively. (a) Plot of branch point position against percent replication of bidirectionally replicating molecules. Since the origin is at a position 25% from an *Eco*RI end of RI fragment A, only one branch point is visible when 50% of this fragment is replicated because the other branch has moved into RI-fragment B. This causes the slope of the regression line to double as if replication were unidirectional, with only one migrating replication fork. (b) Plot of branch point position against percent replication of unidirectionally replicating molecules. Inset shows the relative positions of the *Eco*RI sites on pML31.

Since the distance between *Bam*HI site 1 and *Eco*RI site 1 (Fig. 4) has been shown to be 0.14 kb (R. Kolter, unpublished observation), the shortest distance between *Bam*HI site 1 and the stem of the inverted repeat would be 2.44 kb if the origin were located at 42.6 kb. If the origin were at 47 kb, the shortest single-strand arm between *Bam*HI site 1 and the stem would

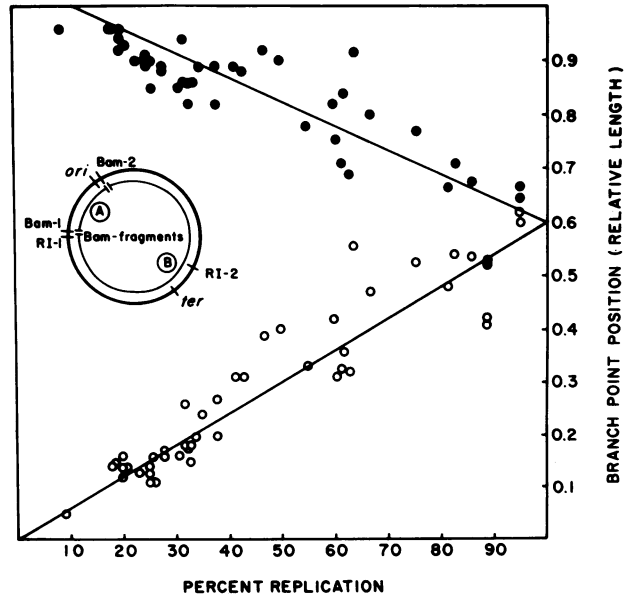

FIG. 5. Plot of branch point position against percent replication on the large *Bam*-fragment B. Replicating molecules of pML31 were digested with endonuclease *Bam*HI. The lengths of the short and long replicated arms to the branch point relative to the length of *Bam*-fragment B are depicted by open and filled circles, respectively. Inset shows the fragments of pML31 generated by digestion with endonuclease *Bam*HI.

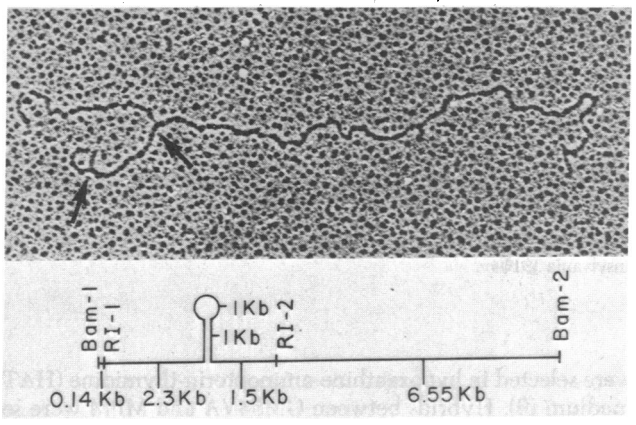

FIG. 6. Electron micrograph of the denatured large *Bam*HI fragment. After restriction of pML31 DNA with *Bam*HI, the DNA was denatured and mounted for electron microscopy by the formamide technique (14). Single-stranded ϕX174 DNA (5.1 kb) (2) was added as an internal standard. The arrows indicate the ends of the double-stranded region of the inverted repeat in the Km-fragment.

be 1.64 kb long. Supercoiled DNA of pML31 was digested with restriction endonuclease *Bam*HI, denatured, mounted by the formamide technique, and examined in the electron microscope as described in Fig. 6. This figure shows an electron micrograph of a *Bam*HI-restricted and denatured molecule of pML31 with a clearly defined inverted repeat. Analysis of 46 such molecules showed that the length of the short single-stranded region is 2.3 $\pm$ 0.2 kb. This is in agreement with an origin located at 42.6 kb on the F-factor.

DISCUSSION

The data show that pML31 replicates in a majority of the molecules bidirectionally from a unique origin on the F-fragment which maps at 42.6 kb on F. Bidirectional replication has also been described for phage λ DNA (15), the chromosomes of *E. coli* (16), *Bacillus subtilis* (17), *Salmonella typhimurium* (18), the genomes of the animal viruses simian virus 40 (19) and polyoma (20), and the plasmid R6K (5). The finding of some unidirectional replication (about 20% of the molecules in the case of pML31) in a predominantly bidirectional replication system is similar to that observed during replication of phage λ (15).

Recently, it has been found that the locus responsible for F-incompatibility maps between 46 and 48 kb on F (4). This indicates that the origin itself is not responsible for the expression of F-incompatibility. Additional support for a secondary role of the origin in the phenomenon of incompatibility comes from the studies of Cabello *et al.* (21), demonstrating the expression of incompatibility by a plasmid element under conditions where the origin of replication is not functioning. These observations, of course, do not rule out the possibility that the product(s) of the incompatibility gene(s) exerts its effect at the origin of plasmid replication.

The data are consistent with the terminus of pML31 replication being located in a region of the Km fragment that is opposite the origin. Assuming that initiation of replication in both directions is simultaneous and that the growing forks move at the same rate, a predicted terminus would be at 0.9×10^6 daltons from *Eco*RI site 2 in the Km fragment. This would correspond to a position 57% from one end of the large *Bam*HI fragment, which agrees closely with the measured value of 60%. However, since the ends of the *Bam*HI fragment cannot be distinguished from each other, it is also possible that the terminus is located in the F segment of pML31 approximately 0.8×10^6 daltons from *Eco*RI site 2. This would be analogous to the asymmetric terminus of replication identified on the plasmid R6K (6). However, this would require a mechanism where the rate of replication of the Km segment of pML31 proceeds faster than replication through the F fragment, since no difference in rates of movement of the two growing forks can be detected during replication of the first 50% of the F fragment (Fig. 4). The simplest model remains that termination occurs in a region of the Km segment of DNA that is opposite the origin. Assuming that the mechanism of replication of the parental F plasmid is identical to pML31, the terminus of F should map at approximately 90 kb. However, it is of course entirely possible that the F-factor may have a termination site at another location that is not present in the region of F cloned on pML31.

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