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Bidirectional replication of plasmid R6K DNA in *Escherichia coli*; correspondence between origin of replication and position of single-strand break in relaxed complex

(supercoiled DNA/antibiotic resistance/restriction enzyme/endonuclease/electron microscopy)

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ABSTRACT **Replicating molecules of plasmid R6K DNA have been purified as covalently closed circular DNA forms and analyzed in the electron microscope after cleavage with the *Eco*RI restriction endonuclease. It has been determined that in most cases replication proceeds bidirectionally from an origin whose position is indistinguishable from the site of the single-strand break (nick) in the open circular DNA form of the relaxation complex of R6K DNA and protein. Evidence is presented for the existence of a unique replication terminus asymmetrically placed approximately 20% of genome size from the origin. The positions of the replication forks in a majority of the molecules indicate that replication proceeds sequentially from the fixed origin first in one direction to the terminus and then progresses from the origin in the other direction.**

Recently it has been reported that the replication of colicinogenic plasmid E1 (ColE1) DNA in cells of *Escherichia coli* is unidirectional from an origin whose position is the same distance from the single *Eco*RI restriction endonuclease cleavage site in the ColE1 DNA molecule as the site of the single-strand break (nick) found in the open circular DNA of the relaxed ColE1 DNA–protein complex (1, 2). Relaxation complexes of supercoiled plasmid DNA and protein have been isolated for a variety of plasmids in *E. coli* (see ref. 3). The characteristic property of these complexes is the conversion of the supercoiled DNA of the complex to the open circular DNA form after treatment with certain agents that alter protein structure, such as ionic detergents. It has been found that the open circular DNA products of induced relaxation of the ColE1 (2), ColE2 (2), and R6K (5) complexes each contain a single, strand-specific and site-specific nick. The ubiquity of plasmid DNA–protein relaxation complexes and the relation of the ColE1 relaxation complex to the origin-terminus for unidirectional replication led to the suggestion that relaxation complex is an essential part of the organization of *E. coli* plasmids as replicons (2), reflecting in the case of ColE1 a need for the nicking activity of the relaxation complex at the origin of DNA replication or at the adjacent termination point.

The purpose of this report is to identify the position of the origin and the direction of replication of the antibiotic resistance plasmid R6K in *E. coli*. R6K is a self-transmissible plasmid of molecular weight 25×10^6 that determines resistance to the antibiotics ampicillin and streptomycin (4). Cleavage of R6K DNA with the *Eco*RI restriction endonuclease generates two fragments whose sizes are approximately 15×10^6 daltons and 10×10^6 daltons (5). The nicked site in the DNA of the relaxed R6K DNA–protein complex is located in the smaller of the two fragments (5). In this report it is shown that replicative intermediates of R6K can be isolated as covalently closed circular DNA forms. Replication in the majority of these molecules proceeds bidirectionally from an origin on the smaller of the two *Eco*RI fragments whose position is approximately the same distance from one end of the fragment as the site of the relaxation complex nick. The structures of the majority of replicating molecules of R6K also indicate that replication proceeds sequentially from the origin first in one direction to the terminus and then from the origin in the other direction.

Abbreviation: ColE1, colicinogenic plasmid E1.

MATERIALS AND METHODS

Supercoiled R6K DNA, isolated from *E. coli* JC 7200 (R6K) (6) was introduced into the mini-cell producing *E. coli* strain P678-54 Thy$^-$ (requires thymine for growth) by transformation (7). R6K replicating molecules were labeled and isolated by methods similar to those employed for the isolation of replicating ColE1 DNA molecules (1). A 200 ml culture of *E. coli* P678-54 Thy$^-$ (R6K) was grown at 37° to a turbidity of 100 Klett units (no. 54 filter)-in M9 medium containing 0.5% casamino acids, 5 μg/ml of thiamine, 13.3 μCi of [2-^{14}C]thymine (46 Ci/mol, New England Nuclear Corp., Boston, Mass.) and 360 μg of unlabeled thymine. Cells were harvested at room temperature and were then resuspended in M9 casamino acids medium lacking thymine and containing 3.0 mM adenosine 3′:5′-cyclic monophosphate (Sigma Chemicals, St. Louis, Mo.). Growth was continued for 25 min at 37°. The culture was then cooled at 25° by immersion in a room temperature water bath. The cells were pulse labeled for 1 min, 45 sec by the addition of 5 mCi of [^{3}H]thymidine (50 Ci/mmol, New England Nuclear Corp., Boston, Mass.) in 25 ml of H_2O and sufficient unlabeled thymidine to give a final concentration of 0.4 μg/ml of thymidine. Sodium azide was then added to a final concentration of 0.1 M and the culture was quickly frozen by immersion in a dry ice-ethanol bath.

The culture was then allowed to thaw and the cells were lysed by the sodium dodecyl sulfate–salt method (8, 9). The supernatant, containing plasmid DNA, was centrifuged to equilibrium in a cesium chloride–ethidium bromide density gradient in either a Beckman Ti50 or Ti60 rotor at 38,000 rpm for 36–40 hr at 15°. The gradients were fractionated by puncturing the bottom of the tube with a large bore needle. Radioactivity was assayed and ethidium bromide was removed by isopropanol extraction as reported elsewhere (2). Fractions containing the pulse-labeled DNA were dialyzed against TESX buffer [100 mM Tris·HCl, 50 mM NaCl, 0.1 mM EDTA (pH 7.5)] and then centrifuged preparatively on 5–20% neutral sucrose density gradients as described in Fig.

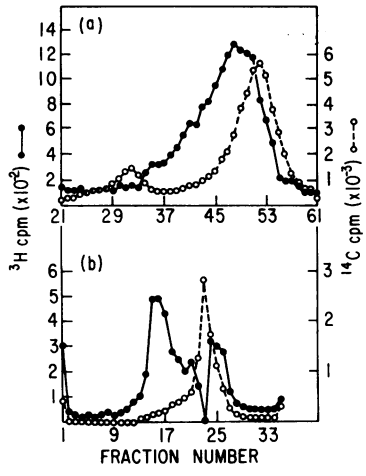

FIG. 1. Purification of replicating R6K DNA molecules. (a) A sodium dodecyl sulfate–salt lysate of P678-54 Thy$^-$ (R6K), labeled with [^{3}H]thymidine and [^{14}C]thymine and prepared as described in *Materials and Methods*, was centrifuged in a cesium chloride–ethidium bromide density gradient in the Spinco Ti60 rotor at 38,000 rpm for 36 hr at 15°. Fractions (20 drop) were collected from the bottom of the gradients. (b) Fractions 35–50 of the cesium chloride–ethidium bromide density gradient shown in (a) were pooled and treated as described in *Materials and Methods*. The pooled DNA in TES [100 mM Tris·HCl, 50 mM NaCl, 5 mM EDTA (pH 7.5)] was centrifuged on a 5–20% neutral sucrose density gradient in the Spinco SW27 rotor at 25,000 rpm for 4 hr at 15°. Fractions (40 drop) were collected from the bottom of the gradients.

1. Fractions containing the pulse-labeled DNA were precipitated with ethanol and were dialyzed against TESX buffer. $MgCl_2$ was added to a final concentration of 5–10 mM to DNA samples in TESX prior to digestion with an appropriate amount of *Eco*RI endonuclease at 25° for 30 min. *Eco*RI endonuclease was the generous gift of Herbert Boyer.

RESULTS

Purification of replicating R6K DNA molecules

The supernatant obtained from a sodium dodecyl sulfate–salt lysate of cells of *E. coli* P678-54 Thy$^-$ (R6K), prelabeled with [^{14}C]thymine and pulse-labeled for 1 min, 45 sec with [^{3}H]thymidine at 25° was centrifuged to equilibrium in a cesium chloride–ethidium bromide density gradient (Fig. 1a). The pulse-labeled DNA exhibited a broad density distribution between the positions of supercoiled and nonsupercoiled DNA. This intermediate density of replicating circular DNA previously has been observed for simian virus 40 (10) and polyoma (11) virus DNA and for the ColE1 plasmid (ref. 2 and L. Katz, P. Williams, S. Sato, R. Leavitt, and D. Helinski, manuscript in preparation). The pulse-labeled DNA was further separated from open circular DNA and linear R6K DNA by preparative centrifugation on a 5–20% neutral sucrose density gradient (Fig. 1b). The pulse-labeled DNA sedimented as a broad 70S peak and as a 35S peak that probably represents either open circular R6K molecules (5) or replicating molecules with broken parental strands. Most of the [^{14}C]thymine prelabeled DNA sedimented at the 39S position characteristic of open circular R6K molecules (5). The DNA of the 70S peak was recovered by precipitation with ethanol and dialyzed against TESX. Upon examination in the electron microscope it was found to consist of molecules with the partially supercoiled, partially open configuration that is characteristic of replicating molecules with covalently closed parental strands (10, 11, 2). This property of replicating R6K DNA was consistent with its intermediate density in cesium chloride–ethidium bromide density gradients and with its rapid sedimentation in neutral sucrose density gradients. At least two-thirds of the pulsed radioactive counts in R6K DNA are recovered in the covalently closed replicating form of this plasmid.

Cleavage of replicating R6K DNA with the *Eco*RI endonuclease

The 70S peak of pulse-labeled R6K DNA was treated with the *Eco*RI endonuclease as described in *Materials and Methods*, and examined by electron microscopy. Selected replicating R6K molecules, cleaved with *Eco*RI, are shown in Fig. 2. Two kinds of structures, representing replicating DNA, were observed on the smaller of the two *Eco*RI fragments (designated fragment B) (Fig. 2a); molecules with two linear segments joining a symmetrical bubble of replicated DNA, designated "eye" structures, and molecules with a single unreplicated segment joined by two replicated segments of equal length, termed "Y" structures. Y structures are generated when replication has proceeded past an *Eco*RI cleavage site (12, 2). Only Y structures were found for the larger fragment (designated fragment A) of replicating molecules. No molecules with a double Y structure were observed. The average length of 148 molecules of fragment B, determined by comparison to the known length of ColE1 DNA (2.14 ± 0.08 μm) spread on the same grid was 4.7 ± 0.3 μm. This corresponds to a molecular weight of 9.3×16^6 for fragment B. The average length of 35 molecules of fragment A was 7.6 ± 0.3 μm, corresponding to a molecular weight of 15.0×10^6.

Eye structures of fragment B

An analysis of the eye structures of fragment B is shown in Fig. 3a and Fig. 4. Fig. 3a represents the eye structures in order of increasing extent of replication. These molecules range from 9.8% to 88.3% replicated, representing from 4% to 35% of the R6K genome. It is possible to represent each molecule in two different orientations on this bar diagram, depending on which of the two unreplicated segments is arbitrarily designated U_1 or U_2. The designation of an unreplicated segment as U_1 or U_2 was based on the assumption that there is a single unique origin of DNA replication for the R6K plasmid in fragment B. The arrangement of molecules in Fig. 3a is consistent with the existence of a replication origin located approximately 40% of the length of fragment B from one *Eco*RI end. Most molecules conform to a single general pattern of replication. Replication proceeds from the origin in one direction to a unique termination point located about 7.5% of the length of fragment B from an *Eco*RI end. Molecules replicated to a greater extent exhibit a bubble that extends away from the origin in the opposite direction. Six of the 97 eye structures (molecules numbered 2, 6, 9, 11, 16, and 21 in Fig. 3a) probably represent cases where replication has progressed in one direction away from the terminus, i.e., not in the usual initial direction of replication. Reversing the designations of U_1 and U_2 for these molecules, while orienting them in the other direction of replication, would in each case require the existence of an unusual origin of replication. Fig. 4 shows the change in length of the unreplicated segments U_1 and U_2 as a function of the extent of replication for the 91 eye structures of fragment B. Replication progresses unidirectionally until fragment B is about 55% replicated (corresponding to 22% of the R6K genome), reaching a terminus approximately 7.5%

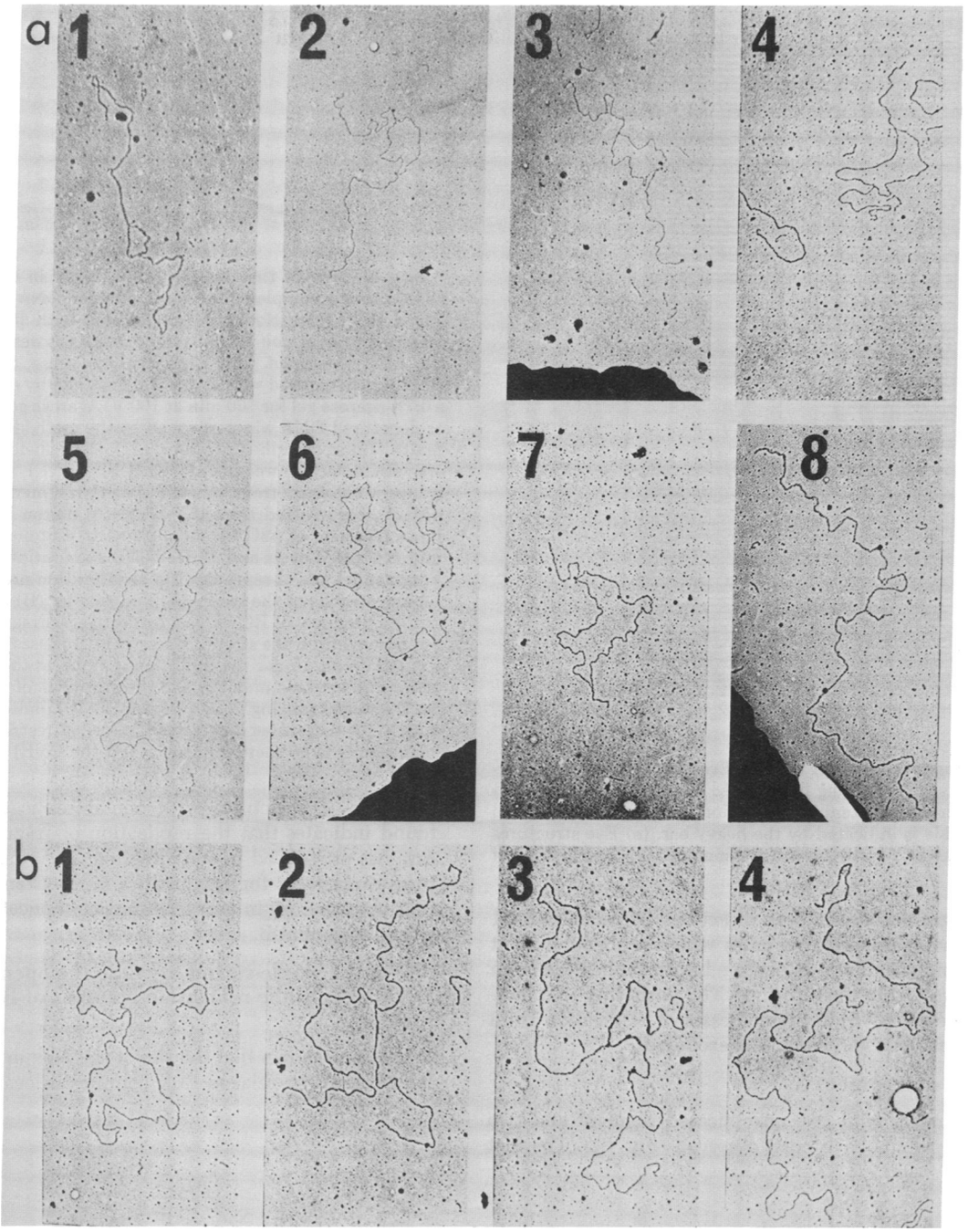

FIG. 2. Replicating R6K DNA molecules treated with the *Eco*RI restriction endonuclease. Electron microscopy was performed as described previously (2). Frames (a) 1–8 represent molecules of fragment B arranged in order of increasing extent of replication. Frames (b) 1–4 represent molecules of fragment A arranged in order of increasing extent of replication. The total length of eye molecules of fragment B was calculated by use of the equation $T = U_1 + U_2 + (R_1 + R_2)/2$, where T is total length, U_1 and U_2 are the unreplicated segments, and R_1 and R_2 are the two segments of replicated DNA which comprise the bubble. The total length of Y structures was determined by use of the equation $T = U_1 + (R_1 + R_2)/2$, where T is total length, and U_1 is the single unreplicated segment, and R_1 and R_2 are the two replicated segments. The extent of replication of both eye and Y structures was $\frac{1}{2}(R_1 + R_2)/T$.

from an *Eco*RI end, before extensive synthesis proceeds away from the origin in the other direction.

Y structures of fragment B

The structures of 37 of 51 of the Y structures of fragment B represented in Fig. 3b are consistent with the conclusion drawn from analysis of the looped structures of fragment B, namely, that replication advances first to a unique termination point located 7.5% from an *Eco*RI end before beginning replication from the origin in the other direction. These 37 molecules have a short unreplicated segment and two long replicated segments. The structures of molecules numbered 38–42 in Fig. 3b are consistent with replication having progressed from the origin in the direction away from the terminus. Molecules numbered 43–47 could either represent cases where replication has proceeded from the origin towards, and past the terminus, or, like molecules numbered 48–51, could represent cases where replication progressed

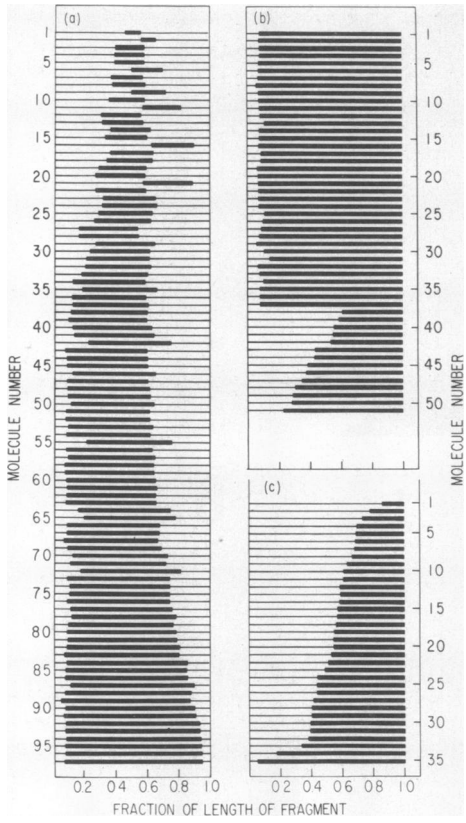

FIG. 3. Representation of the two *Eco*RI endonuclease generated fragments of replicating R6K DNA. Molecules are numbered in order of increasing extent of replication. The replicated portion of each molecule is indicated by the heavy bar. (a) Eye structures of fragment B. (b) Y structures of fragment B. (c) Y structures of fragment A.

from the origin initially in both directions. Molecules 48–51 could also have resulted from cases where replication went past the terminus and at a later time began replication from the origin away from the terminus. The possibility that molecules numbered 43–51 represent cases of replication from another origin, however, cannot be excluded.

Replication of fragment A

Only Y structures with one unreplicated segment and two replicated segments have been observed (Fig. 3c). The fact that only Y structures with one unreplicated branch are found indicates that the replication of fragment A in each case has progressed in one direction. The finding of Y structures exclusively for fragment A is consistent with the idea that both the origin and terminus of replication of R6K reside in fragment B.

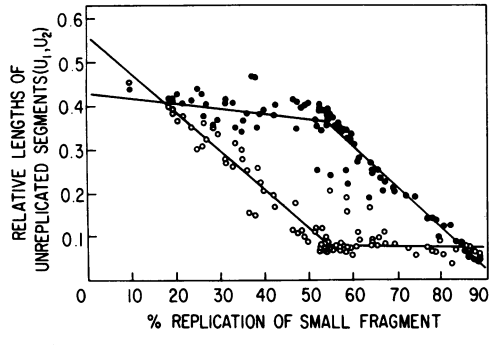

FIG. 4. Analysis of the length of the two unreplicated segments of the eye structures of fragment B as a function of the extent of replication. Eye structures of fragment B, identified as described in the *text*, were used in this analysis. U_1 and U_2 are the unreplicated segments joining an internal bubble comprised of two segments of replicated DNA, R_1 and R_2. The relative lengths of U_1 (●) and U_2 (○) for each molecule and the percent replicated were determined as described in Fig. 2.

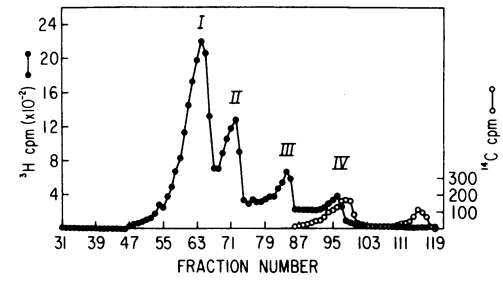

FIG. 5. Site of the strand-specific break in the relaxed R6K DNA–protein complex. The open circular DNA form of the induced R6K relaxation complex, labeled with [^{3}H]thymine, was prepared from *E. coli* P678-54 Thy$^-$ (R6K) as described elsewhere (5). After treatment with *Eco*RI endonuclease, the relaxed R6K DNA was denatured with alkali and analyzed by electrophoresis in a 0.7% agarose gel for 160 min at 100 V. Agarose gel electrophoresis or denatured DNA was performed by the method of Hayward and Smith (13) as described previously (1). ^{14}C-Labeled internal markers with molecular weights of 2.25×10^6 and 1.1×10^6, respectively, were obtained from the two *Eco*RI fragments of a plasmid, pML21, constructed from the 4.5×10^6 dalton kanamycin-resistance fragment of pSC105 and a 2.2×10^6 dalton mini-ColE1 plasmid (14) (M. Lovett and D. Helinski, manuscript in preparation). Fragments I and II represent the denaturation products of the unbroken strands of the larger (A) and smaller (B) of the *Eco*RI digestion products of R6K DNA, respectively. Fragments III and IV are derived from the strand of fragment B possessing the uniquely located break. Under the conditions employed there is a linear relationship between mobility and the logarithm of molecular weight for fragments ranging in size from 1.75×10^5 daltons up to at least 2.34×10^6 daltons as determined by use of restriction endonuclease fragments of bacteriophage λ and the ColE1 and ColE2 plasmids.

Site of the single-strand break in the open circular DNA form of the relaxation complex of R6K DNA and protein

It has been shown that the site of the nick in the open circular DNA of the relaxed R6K complex is located at a unique

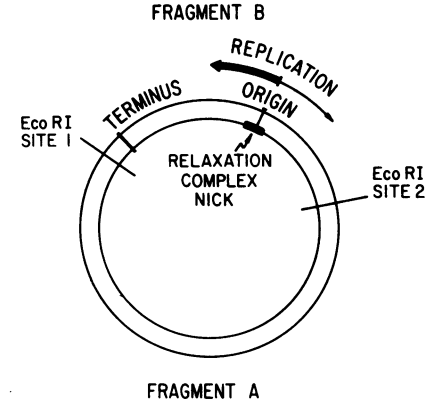

FIG. 6. Model of the R6K genome. The positions of the two *Eco*RI cleavage sites are indicated. The size of fragment A is 15.0 $\times 10^6$ daltons. The size of fragment B is 9.3×10^6 daltons. The relative positions of the origin, the terminus, and the site of the relaxation complex nick are indicated for fragment B, as described in the *text*. Fragments III and IV shown in Fig. 5 are derived from fragment B of the *Eco*RI cleaved DNA of the relaxed R6K complex.

position in fragment B of R6K DNA (5). To map the nicked site precisely in relation to the position of the origin of R6K replication the sizes of the fragments derived from the presence of the uniquely located nick in one strand of fragment B derived from relaxed R6K DNA after treatment with *Eco*RI have been estimated by electrophoresis of the denatured fragments in an agarose gel. As shown in Fig. 5, in addition to the denatured strands I and II derived from fragments A and B, respectively, there are two smaller strands, III and IV, derived from fragment B (5), whose relative sizes determine the position of the relaxation complex nick in fragment B. The molecular weights determined for strands III and IV are 3.5×10^6 and 2.3×10^6, respectively. The length of strand IV, therefore, could range from 40% to 48% of the length of fragment B depending on whether the gel estimate or the electron microscopic estimation of the size of fragment B were accepted. Nevertheless, the location of the relaxation event is very similar to the location of the origin of R6K replication in fragment B, shown to be approximately 42% of the size of fragment B from an *Eco*RI end.

DISCUSSION

Bidirectional replication has been described for several circular DNA replicons including bacteriophage λ (15), the chromosomes of *E. coli* (16), *Salmonella typhimurium* (17), and *Bacillus subtilis* (18), and the genomes of the viruses simian virus 40 (10) and polyoma (11). The bidirectional replication in each case appears symmetrical in the sense that the terminus is diammetrically opposed to the origin. It has been shown for the chromosomes of *B. subtilis* (18) and *E. coli* (19) that the two replication forks reach the termination point at approximately the same time.

The structure of plasmid R6K DNA has several unusual features, summarized in Fig. 6. Replication of this molecule is asymmetric, proceeding from an origin in fragment B in two directions to a unique termination point or region of fragment B. The shorter distance between origin and terminus is about 20% of the R6K genome. The replication in the two directions appears to be sequential in the case of most of the molecules, proceeding first from the origin to the terminus in one direction, and then from the origin in the other direction with the consequence that fragment A is replicated unidirectionally. The significance of this asymmetric, sequential mode of replication is unknown. It is of interest that in a similar study carried out with a derivative of R6K that has been deleted of 8.5×10^6 daltons of DNA, asymmetric and bidirectional replication also was observed, but in this case two origins of replication were found (J. H. Crosa, L. K. Luttropp, F. Heffron, and S. Falkow, manuscript submitted for publication).

Finally, both the origin of R6K DNA replication and the site of the nick in the open circular DNA form of the R6K relaxation complex are approximately the same distance from one end of fragment B. The procedures used made no distinction between the ends of fragment B, but it is most likely as shown in Fig. 6 that the origin and relaxation complex sites are the same distance from the same end of the molecule, as has been suggested for the relation between the origin-terminus of ColE1 DNA replication and the site of the nick in the ColE1 relaxation complex (1, 2). The correspondence between the origin of replication of the plasmids R6K and ColE1 and the position of the nick in the relaxed complexes of these plasmids strongly suggests a role for the relaxation complex in the early events of plasmid replication. A role of relaxation complex in plasmid ColE1 replication also is indicated by the recent demonstration of an altered ColE1 relaxation complex in a temperature-sensitive replication mutant of this plasmid (J. Collins and D. Helinski, manuscript in preparation).

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