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Replication of the Nonconjugative Plasmid RSF1010 in *Escherichia coli* K-12

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Replicating DNA molecules of the nonconjugative R plasmid RSF1010 (Sm^r Su^r) were cleaved with the *Eco*RI restriction endonuclease and examined with the electron microscope. Results of this analysis indicated that replication is initiated from an origin located at about 19% of total genome size from one of the *Eco*RI ends. Replication proceeded either unidirectionally or bidirectionally with equal frequency. Results of the analysis of replicative intermediates of RSF1010 containing the Ap^r-transposable sequence (Tn) are also presented.

Small nonconjugative R plasmids present as multicopy pools and conferring resistance to sulfonamides (Su) and streptomycin (Sm) are relatively common in clinical isolates of enteric bacteria. The prototype of this plasmid group is NTP2, initially described by E. S. Anderson and his colleagues (1-3). Smith et al. (28) showed that 19 of 26 Su Sm resistance plasmids from various salmonellae belonged to the same compatibility type. Barth and Grinter (4) examined a number of Su Sm plasmids which were representative of a broad host and geographical range. They found that 10 of 12 of these plasmids possessed an identical molecular mass and shared a high proportion of their nucleotide sequences in common. On this basis it was suggested that a single plasmid evolved once and had spread efficiently with relatively few modifications around the world.

We have examined the molecular nature of one Su Sm plasmid, RSF1010, which is identical to the original NTP2 isolate of Anderson and Lewis (1-3). RSF1010 has been used in molecular cloning experiments (26) and as a recipient of the 3.2×10^6-dalton DNA transposition sequence, Tn*A* (16), which carries the structural gene for the TEM-1 β-lactamase (17). Since the insertion of Tn*A* causes mutation when it occurs within structural genes and is polar when it occurs within an operon (25), it has been possible to map the structural genes for Su and Sm resistance and a locus which has a profound effect upon plasmid copy number (25). Moreover, Tn*A* insertion can occur at a minimum of 19 distinct sites on the RSF1010 genome (16).

Replicating plasmids (8), animal virus (12, 23, 27), and mitochondrial DNA (6) have been shown to share certain properties in common. These common features are consistent with a model that replication proceeds by the progressive unwinding of superhelical turns of a covalently closed circular molecule accompanied by replication in which two open circular branches of DNA containing the nascent DNA are generated. This continuous reduction in superhelical content proceeds until two equivalent open circular daughter molecules containing a "nick" or "gap" are formed (5, 8, 18). These molecules are then covalently closed, and superhelical turns are once more introduced until two covalently closed circular molecules are formed (9, 30). Despite these common features, each DNA species has several unique properties. For example, it has been shown that the plasmids ColE1 (19) and pSC101 (7) replicate unidirectionally from a unique origin, whereas plasmid cointegrates may initiate replication at either of the two (or more) origins of the cointegrated replicons (24). RSF1040 and R6K, conjugative, multicopy plasmids, possess two initiation sites which are occasionally utilized simultaneously (10), although, in general, replication proceeds exclusively from one or the other of two initiation sites. Perhaps the most unique feature of R6K and RSF1040 replication is that overall replication is asymmetric and bidirectional in the sense that replication first proceeds to a unique terminus in one direction and then proceeds from the same origin in the other direction to the terminus to complete the replicative process (8, 20).

Because RSF1010 is representative of a common naturally occurring plasmid type and has been widely used in laboratory studies, we

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thought it worthwhile to examine its replicative properties. The results reported in this communication indicate that in the vast majority of cases RSF1010 initiates replication from a unique origin and that replication may proceed unidirectionally or bidirectionally with about equal probability.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* K-12 W1485-1, F^- *thy nal* was used throughout this study. RSF1010 (Su Sm), 5.5×10^6 daltons, has been described in detail previously (13). RSF1103 (RSF1010::Tn*1*) and RSF1210 (RSF1010::Tn*3*) are 8.7×10^6-dalton derivatives of RSF1010 into which Tn*A* had been inserted (15). M9 salts medium supplemented with 0.5% glucose, 0.2% Casamino Acids (Difco), 2 μg of thiamine per ml, and 1.7 to 3 μg of thymine per ml was the medium used to grow cells (10).

Growth and labeling conditions, the isolation of plasmid DNA replicative intermediates, and the conditions of centrifugation were identical to those described previously in detail (8, 10). Essentially, cells in the logarithmic phase of growth were harvested and resuspended in a medium lacking thymine and incubated for 30 min (limiting thymine was used to reduce the DNA chain elongation rate and result in an increased number of initiating molecules). The culture was shifted to 25°C and pulsed for 30 s with [^{3}H]-thymidine, and incorporation was stopped by the addition of sodium azide and immediate freezing. Plasmid DNA was isolated by dye-buoyant density centrifugation, and material banding at a location intermediate between mature covalently closed circular and open circular DNA was pooled, dialyzed, and used for the analysis of replicative intermediates.

Analysis of *Eco*RI-cleaved replicative intermediates. The *Eco*RI restriction endonuclease introduced a single double-stranded break at the same unique site in RSF1010, RSF1103, and RSF1210 DNAs (15). Purified covalently closed circular plasmid DNA or pools containing plasmid replicative intermediates were cleaved with *Eco*RI as previously described (10), and the DNA was prepared for electron microscopy by the aqueous method of Davis et al. (11). The DNA was picked up on Parlodion-coated grids, rotary shadowed with platinum-paladium (80:20) in a JEOL vacuum evaporator, and examined with a JEOL 100B electron microscope. Molecules were measured by projecting the images from electron micrographs onto tracing paper and measuring their length with a map measurer. The measurements were calibrated with a carbon-grating replica (21,575 lines/cm) or by reference to uncleaved RSF1010 open circular DNA spread on the same grid. Cleaved replicative intermediates were seen as linear molecules composed of a symmetrical loop or "eye" of replicated DNA bounded by two unreplicated branches.

RESULTS

Mode of replication of RSF1010. We have used measurements of the length of the unreplicated linear branches and the replicated part of *Eco*RI-cleaved molecules to examine the mode of replication of RSF1010. A total of 172 replicating molecules were examined, for which we measured a length of 2.67 ± 0.18 μm, which was the same as the average contour length of uncleaved open circular RSF1010 DNA or nonreplicating *Eco*RI-cleaved linear molecules. Figure 1 depicts the short unreplicated branch L_1 and the longer unreplicated branch L_2 plotted as a percentage of the total length of individual molecules versus the percentage of replication. Even a superficial examination of these data suggested that the mode of replication of RSF1010 was not homogeneous. The most likely interpretation of these results (given the fact that the heterogeneity could not be attributed to variations in the measurement of individual molecules) was that there were two subpopulations of replicating molecules. In one case (denoted by ○ in Fig. 1), the longer unreplicated branch remained essentially invariant in length, whereas the shorter unreplicated branch decreased in size with in-

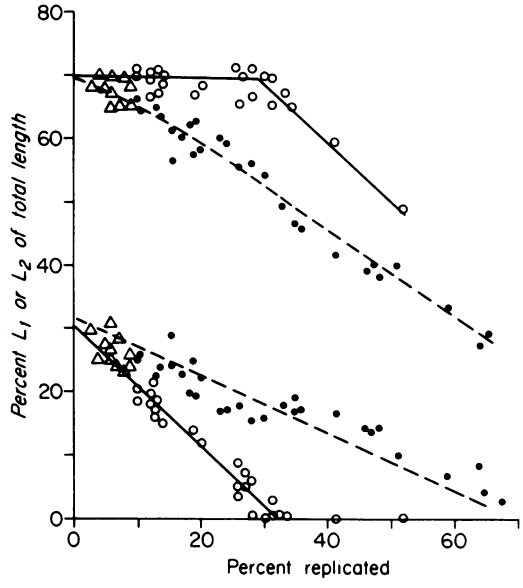

FIG. 1. *Analysis of the mode of replication of RSF1010. EcoRI-cleaved, replicating RSF1010 DNA molecules were photographed and measured.* L_1 *and* L_2 *are the unreplicated segments joining an internal loop comprised of two segments of replicated DNA,* R_1 *and* R_2*. The total length of molecules with an internal loop is then:* $T = L_1 + L_2 + [(R_1 + R_2)/2]$*. The extent of replication was* $(R_1 + R_2)/2$ *T. Relative length of* L_1 *or* L_2 *for each molecule is plotted as a function of the percentage of replication. Two subgroups are shown: unidirectional subgroup (●); bidirectional subgroup (○). The same symbols are used for both* L_1 *and* L_2 *in each subgroup. Molecules that could belong in either of the two subgroups are represented by the symbol △.*

creasing replication. This mode of replication, illustrated in Fig. 2A to F, was compatible with a model which assumes a single, fixed origin from which replication proceeded unidirectionally. Linear regression analysis of this subpopulation indicated that the origin of replication of this subpopulation was located 30.7% from the arbitrarily designated left-hand end of the RSF1010 molecule and that termination of replication should occur at a site closely adjacent to the origin of replication. The other subpopulation of replicating RSF1010 molecules (denoted by ● in Fig. 1) showed reduction in the length of both linear branches as replication proceeded, although the rate at which the replication proceeded along each arm (as evidenced by the slope of the line) was not identical. These data, also illustrated in Fig. 3A to F, were consistent with the interpretation that replication was proceeding from a fixed origin bidirectionally, and linear regression analysis indicated that the origin of replication for this mode was 31% from the arbitrarily designated left-hand end of the RSF1010 molecule and that replication should

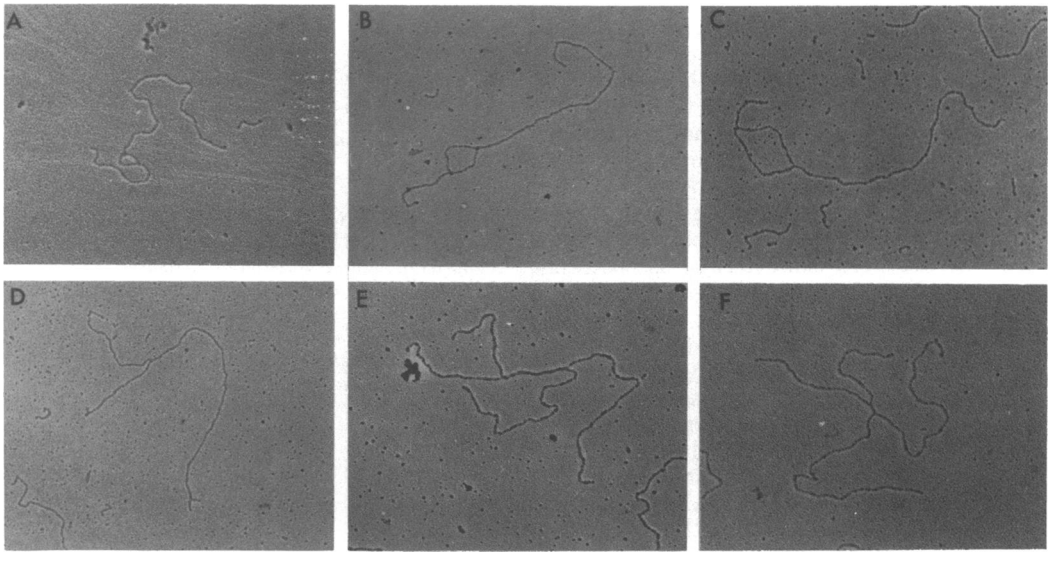

FIG. 2. *Replicating RSF1010 molecules treated with the EcoRI restriction endonuclease. Unidirectional subgroup; electron microscopy was performed as described in the text. (A) through (F) represent molecules arranged in order of increasing extent of replication.*

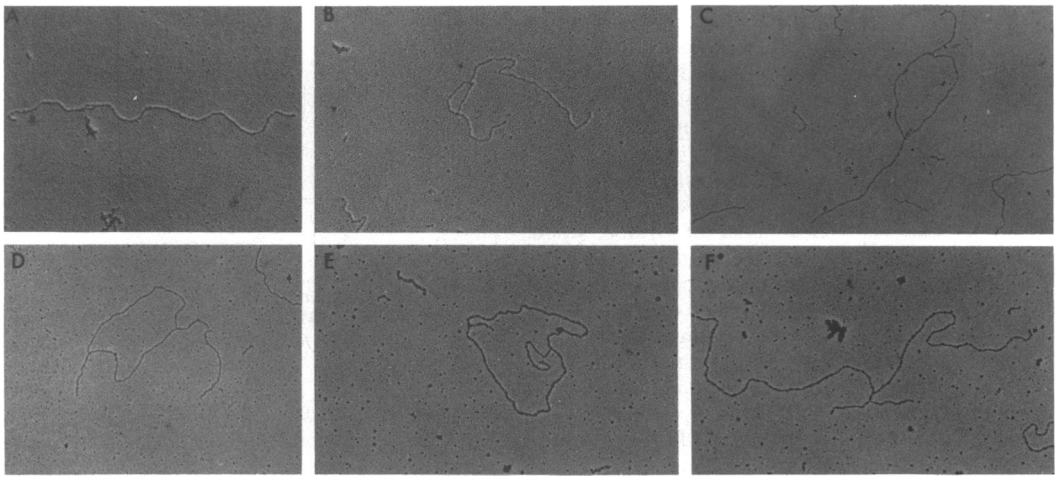

FIG. 3. *Replicating RSF1010 molecules treated with the EcoRI restriction endonuclease. Bidirectional subgroup; (A) through (F) represent molecules arranged in order of increasing extent of replication.*

terminate some 90% from the left-hand end. Molecules which have replicated to only a limited extent (between 1 and 12%) could not be unequivocally assigned to either subpopulation and were ignored for the purposes of these calculations (these points are designated as △ in Fig. 1).

The examination of the mode of replication of RSF1010 was consistent with the model that replication was initiated from a fixed origin and proceeded unidirectionally or bidirectionally with about equal probability.

Replication of RSF1010::Tn derivatives. As noted earlier, we have used RSF1010 as a recipient of the 3.2×10^6-dalton transposition sequences Tn*1* and Tn*3* which carry the structural gene for the TEM-1 β-lactamase (15). RSF1010::Tn derivatives retain their single site susceptible to *Eco*RI cleavage, and the relationships of the site of Tn insertion in the cleaved molecule can be unequivocally determined with relation to the site of *Eco*RI cleavage and to the Su Sm structural genes (23). Thus, the plasmid RSF1210 has been characterized as possessing Tn*3* inserted 3.36% from a designated left-hand *Eco*RI-generated end within the structural gene for Sm resistance. The plasmid RSF1103 has been characterized as possessing Tn*1* inserted 95% from the designated left-hand *Eco*RI-generated end within the structural gene for Su resistance. Hence, the two RSF1010 derivatives containing Tn*A* have 3.2×10^6 daltons of DNA inserted at opposite ends of the RSF1010 genome, so that analysis of *Eco*RI-cleaved replicative intermediates of these derivatives permits the unequivocal assignment of the site of the origin of replication relative to the Su^r and Sm^r genes and also permits confirmation of the nature and mode of RSF1010 replication.

Figure 4 shows the short unreplicated branch L_1 and the longer unreplicated branch L_2 plotted as a percentage of the total length of individual molecules versus the percentage of replication for RSF1103. Figure 5 depicts similar results obtained for RSF1210.

The apparent location of the replication origin in these RSF1010::Tn*A* derivatives as measured from L_1 and L_2 will depend, of course, upon whether the Tn has inserted at a site between the short branch L_1 and the replication origin or in the longer branch L_2 and the replication origin. The data show that the origin of replication for RSF1210 was located 42.9% from the shortest *Eco*RI unreplicated branch, whereas the origin of replication for RSF1103 was located 19.4% from the arbitrary left-hand end of the molecule. Assuming that RSF1010 has a mass of 5.5×10^6 daltons and that Tn*1* and Tn*3* possess a mass of 3.2×10^6 daltons, the locations of the origin in

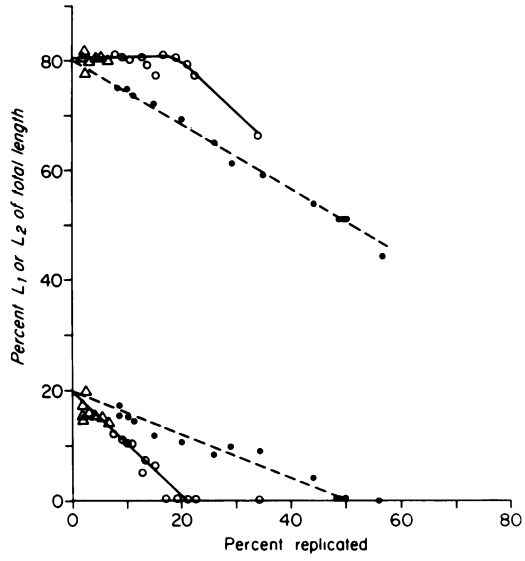

FIG. 4. *Analysis of the mode of replication of RSF1103. EcoRI-treated, replicating RSF1103 molecules were photographed and measured. The average length of the molecules was 4.0 ± 0.18 μm. Plotting of the measurements was carried out as described in Fig. 1. Symbols:* (○) *unidirectional subgroup;* (●) *bidirectional subgroup;* (△) *molecules that could belong in either subgroup.*

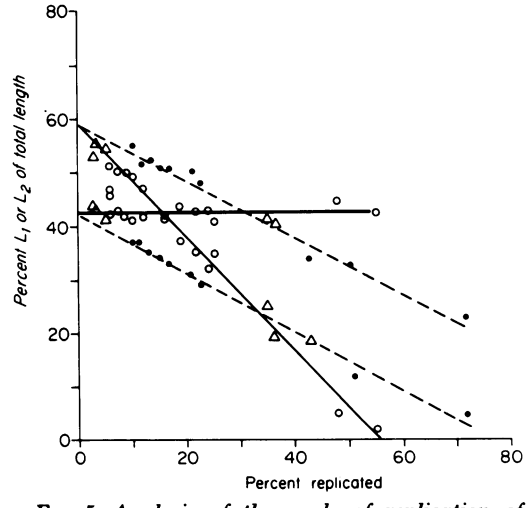

FIG. 5. *Analysis of the mode of replication of RSF1210. See legend to Fig. 4. Symbols:* (○) *unidirectional subgroup;* (●) *bidirectional subgroup;* (△) *molecules that could belong in either of the two subgroups.*

both RSF1210 and RSF1103 are precisely as would be predicted (see Fig. 4 and 5). Moreover, regardless of the site of Tn insertion and the change in the apparent location of the origin of replication, two subpopulations (one unidirec-

tional, one bidirectional) of replicating molecules were observed. It is of interest that the additional DNA to be replicated (the inserted Tn) did not change the mode of bidirectional replication. That is, there was no unique replication terminus in RSF1010 (or presumably in Tn) so that replication proceeded bidirectionally from the origin until the two replication forks reached each other at a site determined by the rate of replication for each replicating arm. Thus, if one has determined the origin and has calculated the slope of L_1 and L_2 (for Fig. 1, 4, and 5), the theoretical location of the replication terminus was quite close to that found experimentally (Table 1).

DISCUSSION

The results of this study show that RSF1010 initiates its replication from a unique origin and that replication proceeds either unidirectionally or bidirectionally with about equal frequency. Unidirectional replication is a prominent feature of several small, multicopy plasmids such as ColE1 (19), whereas bidirectional replication has been described as the predominant mode for the chromosome of several bacterial species (15, 21, 22), animal viruses (18), and the bacterial plasmids R6K, and its derivative RSF1040 (8, 19), and F (12), as well as bacteriophage λ (26). It is perhaps noteworthy that whereas phage λ and the plasmid F replicate in a predominantly bidirectional replication mode, in both instances a significant proportion of molecules (about 20%) also replicated unidirectionally. Hence, the finding that RSF1010 displays both modes of replication need not be considered unique. The near-equal distribution between the unidirectional and bidirectional mode of replication does seem rather unique, however, and is intriguing in terms of the possible biochemical factors at play in selecting the alternative replicative mode at the time that replication is initiated.

Information concerning the replicative properties of RSF1010 has been accumulating slowly. It has been established that RSF1010 has a dependence for DNA polymerase I for its maintenance, though not necessarily for its replication (14). This partial dependence upon DNA polymerase I is in contrast to other small multicopy plasmids such as ColE1, which appears to have an absolute dependence upon polymerase I for both replication and maintenance. The results of this study establish the site of the origin of replication and the mode(s) of replication of RSF1010, as well as imply that replication termination does not occur at a unique genetic site. Whereas it might be thought that any deletion or interference with the stretch of DNA located about 30% from the left-hand *Eco*RI end of RSF1010 would be a lethal event, recent evidence (unpublished observation) suggests that an alternative replication origin is present some 40% from the left-hand *Eco*RI-cleaved end of RSF1010. It is, perhaps, of interest that insertions of Tn*A* at a site closely adjacent to this alternative origin of replication have been previously shown by us (25) to have a profound effect on the copy number of RSF1010 within host cells. Thus, we can identify a stretch of DNA, minimally 1,000 nucleotides in length, that is intimately associated with the replicative functions of RSF1010. The nature of these replicative functions and their control are still highly speculative. It is our hope that subsequent studies on the structural features and biochemical nature of the RSF1010 replication will provide an interesting study in similarity (or contrast) to other plasmid systems. Moreover, the further study of the replicative properties of RSF1010 may permit us to better appreciate how plasmids like RSF1010 have become so widely distributed in nature among a wide variety of bacterial species.

TABLE 1. *Summary of the location of origin, termination, and mode of replication for RSF1010 and RSF1010::Tn derivatives*[a]

Plasmid	Replication	Origin (%)	Termination (%)	
			Calculated	Found
RSF1010	Unidirectional	30.7	32	34
RSF1010	Bidirectional	30.7	90	86
RSF1103	Unidirectional	19.4	20	19
RSF1103	Bidirectional	19.4	21	23
RSF1210	Unidirectional	42.1	45	42
RSF1210	Bidirectional	42.1	15	15

[a] The calculations for the theoretical location of the termination site were done by using the origin and slope of L_1 and L_2 from Fig. 1, 4, and 6, respectively.

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