Nonrandom F-Plasmid Replication in *Escherichia coli* K-12

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Both the autonomous and chromosomally integrated F plasmids were found to replicate in a nonrandom fashion after a density transfer from heavy medium (\(^{13}\text{C}\)glucose, \(^{15}\text{N}\)H\(_4\)) to light medium (\(^{12}\text{C}\)glucose, \(^{14}\text{N}\)H\(_4\)). The data are consistent with the hypothesis that both the chromosome and the F plasmid are replicated in a cell cycle-specific manner. Thus, these data support the proposal (J. D. Keasling, B. O. Palsson, and S. Cooper, *J. Bacteriol.* 173:2673–2680, 1991) that plasmids replicate in a cell cycle-specific manner.

The fertility factor F of *Escherichia coli* is a 94.5-kb plasmid which belongs to the F1 incompatibility group. The F plasmid replicates independently of the chromosome such that there is less than one copy per chromosomal origin (2, 6, 12, 14). Early experiments with F'lac indicated that replication was confined to the final stage of the division cycle, coincident with termination of chromosome replication (3, 4, 17). However, more recently, a variety of methods have been used to suggest that replication of the same plasmid would be evenly spread throughout the cell division cycle (1, 5, 10, 15). Recently, Keasling et al. (6) used the membrane elution method and direct scintillation counting to determine the cell cycle replication pattern of the F plasmid. They found that the F plasmid replicated in a cell cycle-specific manner at a point midway between initiations of chromosomes or minichromosomes in the same cell. This led to the conclusion that initiation of F-plasmid replication occurs when the mass-origin of the F plasmid reaches a constant size, similar to the timing of chromosomal replication. Previous experiments by Leonard and Helmstetter (10), also using the membrane elution method, led to a different conclusion. One difference is that the latter experiments (10) used autoradiography to measure plasmid radioactivity in agarose gels. The autoradiographic results are inconsistent with the previous series of results demonstrating steps in IPTG (isopropyl-\(\beta\)-D-thiogalactopyranoside) induction which had been taken as a measure of cell cycle-specific replication of the F plasmid (3, 4, 17). Keasling et al. (6) used a model system with a lac-containing minichromosome to demonstrate that the IPTG method was a good method for determining the timing of plasmid replication during the division cycle.

To determine whether the F plasmid replicated randomly or nonrandomly during the division cycle I decided to reinvestigate F-plasmid replication after a density shift using a different approach: transfer of a culture growing in minimal medium with \(^{13}\text{C}\)glucose and \(^{15}\text{N}\)H\(_4\) to the same medium with \(^{12}\text{C}\)glucose and \(^{14}\text{N}\)H\(_4\). The results are not compatible with random replication of the fertility factor. The recent report by Keasling et al. (6) is in accordance with this conclusion.

(A preliminary draft of this report was presented by K. Nordström at the EMBO Workshop on the Molecular Basis of Bacterial Growth and Division in Segovia, Spain, 1987 [abstr. p. 61].)

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MATERIALS AND METHODS

Strain CM796 = ER(\(\Delta\)asnB) was used in an earlier study of minichromosome replication (8). The presence of F was verified by sensitivity to the male-specific bacteriophages M13 and R17. The autonomous state of the F factor was detected by soft agarose gel electrophoresis of total cellular DNA (such as that prepared for Southern blotting hybridization). If a significant fraction of F was integrated in the chromosome, then the autonomous plasmid would have been rapidly lost by incompatibility; in addition, a possible Hfr genotype would be unstable in strains in which oriC was not inactivated (cf. LK476).

Strain LK217 = CM1588 del-1071 (\(\text{gidAB oriC asnA}\)) has also been used before (7). Strain JC(pED810) was obtained from N. Willetts; its plasmid pED810 contains about 10 kb from the transfer region of F. Strain LK476 was constructed from LK217 and plasmid pLK6 del-2, replacing the 5-kb deletion of the oriC region on the chromosome with the 16-bp deletion in oriC on the plasmid. Plasmid pLK6 del-2 in turn was constructed from pLK6 (7) by limited digestion with restriction endonuclease *PstI*; it lacks the minimal R1 replicon of pLK6 but retains its kanamycin resistance gene. The identity of LK476 was verified in several ways: (i) Southern blotting analysis; (ii) incompatibility with F::\(\Delta\)Tn10; (iii) sensitivity of growth to a low concentration of acridine orange.

The minimal medium was MOPS (morpholinepropanesulfonic acid) (13) with the addition of 10 mM NaHCO\(_3\). \(^{13}\text{C}\)glucose was obtained from MSD Isotopes, Montreal, Quebec, Canada. The method of density shift is outlined in Fig. 1 (7). The hybridization technique has been described before (8).

RESULTS

In a preliminary experiment, strain CM796 harboring the F plasmid was pulse labeled with \(^{3}\text{H}\)thymidine and then shifted from heavy medium (\(^{13}\text{C}\)glucose, \(^{15}\text{N}\)H\(_4\)) to light medium (\(^{12}\text{C}\)glucose, \(^{14}\text{N}\)H\(_4\)). CsCl gradient centrifugation was used to separate the unreplicated DNA of heavy density from the replicated DNA of hybrid density. The sudden appearance of label at hybrid density in the gradient at about one doubling time after the shift represents the cumulative distribution of interreplication time of the total DNA (Fig. 2).

To determine the kinetics of replication of F-specific radioactivity, the fractions with heavy DNA were pooled, and those with hybrid-density DNA were pooled. Next, these pools were hybridized to an excess of pED810, which contains a 10-kb insert derived from the *tra* operon of plasmid F. The results show that the timing of F replication...
reinitiation is similar to that of the chromosome, i.e., non-random, although not as precise (Fig. 2). Plasmid R1 retains its random replication characteristic when driving replication of the chromosome (7), while oriC minichromosomes replicate synchronously with the chromosome (8, 9). It was therefore possible to address the question of the timing of replication of plasmid F by analyzing replication of the chromosome under control of plasmid F. Strain LK476 lacks 16 bp from the essential origin, oriC, which are replaced by the kanamycin resistance gene of plasmid R1. Consequently, chromosome replication starts at F'ilv integrated between the rncC and idv genes in the vicinity of oriC. The result of the density shift experiment is shown in Fig. 3. Again, the interreplication time of the chromosome shows a high degree of precision, whereas that of plasmid R1 shows a random spread after a delay of about 0.2 generation time. The replication pattern of the chromosome under control of plasmid F'ilv appears to be in between that of the wild-type chromosome and that of plasmid R1. The same result was obtained with strain LK217, in which a 5-kb FIG. 1. Outline of a density shift experiment to study timing of replication. (A) To the left is pictured a replication fork in heavy HH DNA at the end of the pulse-labeling with [3H]thymidine but immediately before the chase and the shift to light medium (time zero). The pulse-labeled heavy strands will keep their HH density until they are replicated again during the chase in light medium. From then on, they are associated with light strands and keep a hybrid HL density. (B) DNA in samples taken during the chase in light medium is sheared into small pieces and separated by equilibrium centrifugation into unreplicated components of HH density and replicated ones of HL density. (C) The fraction of the total radioactivity in the HL position of the density gradients, HL/HH (HL + HH), is plotted as a function of time after pulse-labeling and density shift. The slope of the line is a measure of the precision of timing of replication (the dispersion of interreplication time). The x-axis intercept is a measure of the minimal time interval between successive replication forks. Reprinted from Cell (7) with permission of the publisher.

FIG. 2. Timing of F-plasmid replication after a density shift. The upper panels (A and B) show the replication kinetics for strain ER harboring both the minichromosome λoriC212 and plasmid F. The curves were calculated on the assumption that 93% of the pulse-labeled DNA rereplicated with a coefficient of variation of 29%. The lower panels (C and D) show the replication kinetics for strain CM796(λasn53) harboring only the F plasmid. The curves were calculated assuming 100% rereplication with a coefficient of variation of 19%. (A and C) Total input DNA, i.e., mainly chromosomal; (B and D) F-plasmid-specific DNA, i.e., after hybridization to pED810. The doubling time was 81 min at 30°C.

FIG. 3. Summary of density shift experiments with strains in which chromosomal replication is under normal control or under control of integrated plasmid R1 or F. ○, replication kinetics of chromosomal (total) DNA for the wild-type strain ER (doubling time, 78 min at 30°C); ●, replication kinetics for strain LK476 (doubling time, 60 min at 34°C) in which F drives the chromosome; the kinetics of strain LK217 (doubling time, 105 min at 30°C) was indistinguishable; △, □, replication kinetics for the control strains LK211 (ΔoriC::R1, clockwise) and LK348 (ΔoriC::R1, counterclockwise), respectively. Their doubling time was 77 min at 37°C.
deletion abolished not only oriC but also two genes at each side (data not shown).

DISCUSSION

The nonrandom replication of F replicons described here is consistent with previous work on F'lac which used pulse induction of β-galactosidase by IPTG (3, 4, 17). This approach has been explicitly validated by control experiments using a minichromosome containing the lac gene (6). In recent years, some experiments have proposed, in contrast to the IPTG results, that F plasmids replicate randomly during the division cycle (1, 5, 10, 15). The experiment of Leonard and Helmstetter (10) is particularly crucial as it used the membrane elution method to analyze the division cycle and autoradiography to determine the radioactivity in plasmids; in these experiments, a random pattern of F-plasmid replication was observed. Keasling et al. (6) also used the membrane elution method but cut each band and counted the radioactivity directly. They found cell cycle-specific replication which followed the rule that initiation of plasmid replication occurred when the cell mass or size per plasmid origin reached a particular value. The autoradiographic method has problems with the linearity of the photographic emulsions, and large plasmids may not enter the agarose gel; thus, this method may lead to problematic results. These problems were overcome by Keasling et al. (6), who cut the large F plasmid with restriction enzymes to allow the smaller pieces to enter the gel in a quantitative manner and also cut out the bands for scintillation counting. In addition, the presence of a constant amount of counterlabeled material corrected for all plasmid isolations. This was not possible in the autoradiographic approach. Thus, it seems that the most consistent view of the previous data is that the F plasmid replicates in a cell cycle-specific manner as described by Keasling et al. (6).

The data in this report support the findings of Keasling et al. (6) by demonstrating, in a direct manner, that the F plasmid replicates nonrandomly. The density shift used here was very nonperturbing, and the cesium chloride experiments and hybridization methods are straightforward. Such density shift experiments do not specify cell cycle timing without additional assumptions: the intermediate degree of nonrandomness of recombination shown in Fig. 3 could lead to randomization of the cell cycle timing of replication in the population. However, such asynchrony is unlikely because Keasling et al. (6) showed that, at a number of growth rates, F initiation is related to the same size-origin formalism that has, for many years, described chromosome replication. In other words, fluctuations of replication timing during the first cell cycle must be compensated by opposite fluctuations during the second cell cycle (incremental size timing [unpublished data]).

The similar timing of replication of plasmid F and the chromosome is in accordance with the similar genetic organization at the origin regions: both replicons contain a series of direct repeats that bind the essential initiator protein, RepE and DnaA, respectively (16). It has been proposed that the repeats are in fact a timing device titrating the initiator during steady growth and directing it toward the origin. Thus, they allow cooperativity in the assembly of the replisome and precision of timing with respect to cell division (11). This in turn is important to ensure equal partition of plasmid copies between siblings at cell division (16). In this view, the partition (sop), killing (ccd), and site-specific recombination (pif) system described for F would be of secondary importance to secure inheritance and could be regarded as compensating for the reduced size of the replicon compared with that of the chromosome.

In conclusion, the data presented here are consistent with the hypothesis that F plasmid replicates in a cell cycle-specific manner. This has been proposed earlier on the basis of cell cycle experiments by Keasling et al. (6).

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