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Cell-Cycle-Specific F Plasmid Replication: Regulation by Cell Size Control of Initiation

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F plasmid replication during the *Escherichia coli* division cycle was investigated by using the membrane-elution technique to produce cells labeled at different times during the division cycle and scintillation counting for quantitative analysis of radioactive plasmid DNA. The F plasmid replicated, like the minichromosome, during a restricted portion of the bacterial division cycle; i.e., F plasmid replication is cell-cycle specific. The F plasmid replicated at a different time during the division cycle than a minichromosome present in the same cell. F plasmid replication coincided with doubling in the rate of enzyme synthesis from a plasmid-encoded gene. When the cell cycle age of replication of the F plasmid was determined over a range of growth rates, the cell size at which the F plasmid replicated followed the same rules as did replication of the bacterial chromosome—initiation occurred when a constant mass per origin was achieved—except that the initiation mass per origin for the F plasmid was different from that for the chromosome origin. In contrast, the high-copy mini-R6K plasmid replicated throughout the division cycle.

Plasmids are stably inherited, independently replicating circles of DNA. As they are stably maintained in a culture, their replication must be attuned to the rate of cell growth and division. For low-copy plasmids, the F plasmid being an example, there must be at least one round of replication per division cycle. This requirement, coupled with a specific segregation mechanism, would enable low-copy plasmids to be stably maintained with few plasmid-free segregants. One way of coupling plasmid replication to the rate of cell growth and division would be to have replication of all plasmids occur at one specific time during the division cycle. The minichromosome, as well as the bacterial chromosome, replicates in a cell cycle-specific manner. Even though there are many copies of the minichromosome per cell, the minichromosomes replicate at the same time as the chromosomal origin initiates replication (13).

The most recent results obtained by using the membrane-elution method and quantitative autoradiography to measure F plasmid replication indicate that the F plasmid replicates throughout the division cycle (14); i.e., F plasmid replication is cell-cycle independent. Mini-F plasmids containing only one of several F plasmid replication origins (*oriS*) were also shown to replicate throughout the division cycle. These results are in sharp contrast to the large body of earlier work that had consistently shown a doubling in the ability of a cell to synthesize plasmid-encoded β-galactosidase at a specific time during the division cycle (3, 6, 8, 9, 17, 22). These enzyme experiments indicated that the F plasmid replicates in a cell-cycle-specific manner. How can the random replication of the F plasmid determined by autoradiography be reconciled with cell-cycle-specific replication determined by enzyme induction?

We reinvestigated the problem of F plasmid replication during the division cycle by using the membrane-elution technique with quantitative radiochemical measurements of the plasmid. We found that the F plasmid replicates at a specific time during the division cycle, coinciding with the time of replication determined by induction and assay of the plasmid-encoded β-galactosidase. Determination of F plasmid replication at different growth rates indicated that the ratio of cell size at initiation of plasmid replication to the number of F plasmid origins is constant.

MATERIALS AND METHODS

Bacteria and plasmids. The bacteria used were *Escherichia coli* B/r F26 (*his thy lac*) F′*lac* and B/r F65 (*his thy* Δ*lac:pro*) (provided by A. C. Leonard). The F′*lac* plasmid was transferred from F26 to F65 by mating; the minichromosome pAL49 (7.6 kb, Kan^r; provided by A. C. Leonard) was then transformed into the strain. Thus, one bacterial strain contained both an F plasmid and the minichromosome. Some experiments used a minichromosome containing part of the *lac* operon and a kanamycin resistance gene, pOC89 *lac:kan* (provided by W. Messer). Mini-R6K plasmid pMF26 (Amp^r; provided by D. R. Helinski) and minichromosome pAL49 were transformed into F65 so that a single strain contained both plasmids.

Growth of bacteria. C medium (11) was supplemented with histidine (42 μg/ml), proline (46 μg/ml), and thymine (20 μg/ml) as required or Casamino Acids (0.2%) and one of the following carbon sources: glucose (0.4%), glycerol (0.4%), succinate (0.4%), or acetate (0.4%). The cells were grown for at least six to nine generations in 150 ml of medium to obtain exponential growth prior to the start of a membrane-elution experiment.

Membrane-elution technique. Exponentially growing cells (150 ml, 1.0×10^8 cells per ml) were labeled with [*methyl*-^{3}H]thymidine (60 to 80 Ci/mmol, 5 μCi/ml) for 5% of the doubling time and filtered onto a nitrocellulose membrane at the end of the labeling period. The cells were washed with medium of the same composition containing cold thymidine (100 μg/ml). The membrane apparatus was inverted, and newborn cells were eluted from the membrane with prewarmed C medium pumped at a rate of 2 ml/min in a 37°C

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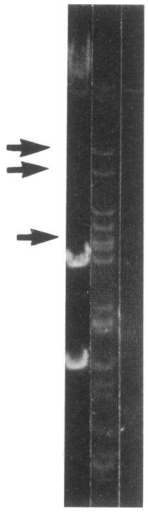

FIG. 1. Agarose gel electrophoresis of the F'*lac* plasmid cut with restriction enzyme *Eco*RI. Left lane: λ *Hin*dIII-digested DNA molecular weight marker. Center lane: F plasmid isolated as described in Materials and Methods, cut with restriction enzyme *Eco*RI, and analyzed by agarose gel electrophoresis. Right lane: DNA isolated from plasmid-free *E. coli* and cut with restriction enzyme *Eco*RI. The F plasmid bands analyzed by cutting the agarose and counting its radioactivity in scintillation fluid are indicated by the arrows.

incubator. Fractions were collected for an interval of time equivalent to the labeling period. Cell numbers were determined by using a particle counter (Coulter Electronics model ZM). The total [*methyl*-^{3}H]thymidine incorporated was determined with a scintillation counter (Beckman Instruments model 3801).

Enzyme assay for plasmid replication. When the time of replication of a plasmid was to be determined by measuring the inducibility of β-galactosidase as a function of the division cycle, the cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) during the labeling period. A 1.0-ml portion of each fraction collected from the membrane elution apparatus was added to 25 μl of toluene and vortexed for 10 s, and the β-galactosidase activity was determined by the endpoint assay described by Miller (16). The optical density at 405 nm was read in a 96-well plate reader (Molecular Devices).

Plasmid isolation and quantitation. Unlabeled cells containing the plasmids of interest were added to each fraction from the membrane elution experiment to visualize the DNA on the agarose gels. The cells were centrifuged at 5,000 × *g* for 15 min, resuspended in 1.5 ml of C medium, and transferred to microcentrifuge tubes. The plasmids were isolated by using an alkaline-detergent procedure (15). When a large plasmid (such as F'*lac*) was being studied, the resulting DNA pellets were dissolved in 25 μl of buffer containing 1 U of *Eco*RI restriction enzyme per μl and digested overnight at 37°C. When plasmids (such as minichromosome pOC89) were small enough to enter the agarose without restriction enzyme cutting, the restriction step was omitted. The fragments were separated by gel electrophoresis on a 0.5% agarose gel run at 2 V/cm for 24 h. The relevant plasmid bands were visualized by ethidium bromide staining and UV fluorescence. Figure 1 is a photograph of an agarose gel with the *Eco*RI-cut F plasmid compared with EcoRI-cut DNA isolated from plasmid-free cells. The multiple bands generated by EcoRI allowed for replicate assays of each fraction collected from the membrane elution apparatus. The F'*lac* plasmid bands at approximately 9 and 13 kb (two bands) were sliced from the gel, dissolved in 10 μl of 70% $HClO_4$ at 70°C for 15 to 30 min, and counted by liquid scintillation. The minichromosome, when present, was also identified by fluorescence, and the counts in the minichromosome were determined as with the F'*lac* plasmid. A slice of gel of equivalent size was removed directly below each DNA band and counted in a scintillation counter. The counts in the background slice were subtracted from the counts in the plasmid band. Control experiments have shown that the radioactivity in the area below each band is an accurate and reproducible measure of the amount of chromosomal contamination present in the plasmid fragment band. This correction is relatively small, and the same conclusions could be made without any background correction. Control experiments also indicated that plasmid isolation was reproducible and consistent.

When autoradiography was performed, the gels were treated with PPO and then dried by the procedure of Laskey (12). The dried gels were exposed to Kodak X-Omat AR film for 2 to 10 weeks at −70°C. The intensities of the plasmid bands were quantitated by using a scanning densitometer.

Identification of F'*lac* bands. It is important to know that the bands being cut out of an agarose gel are actually from the F'*lac* plasmid. To demonstrate this, we purified the F'*lac* plasmid by CsCl-ethidium bromide density gradient centrifugation by concentrating 500 ml of cells and isolating the DNA with an SDS lysis procedure (15). The DNA and 4.4 g of CsCl were dissolved in 4 ml of TE buffer (10 mM Tris, 1 mM EDTA), and 0.2 ml of 10-mg/ml ethidium bromide was added. The samples were centrifuged in a Beckman ultracentrifuge (VTi80 rotor) at 45,000 rpm for 24 h. The band containing the plasmid was isolated by using a 1-ml syringe and a 21-gauge needle. The ethidium bromide was removed from the DNA by washing the DNA four to six times with *n*-butanol, and the DNA was precipitated with 95% ethanol after two- to three-fold dilution with TE buffer. The plasmid was cut with restriction enzyme *Eco*RI and electrophoresed on a 0.5% agarose gel at 2 V/cm. The F'*lac* restriction fragments isolated by CsCl-ethidium bromide density gradients corresponded to those from the membrane elution fractions.

RESULTS

Can plasmid replication be measured by enzyme activity? We evaluated the efficacy of β-galactosidase measurements to determine when a plasmid replicates. We measured the time of replication during the division cycle of *lac*:*kan* minichromosome pOC89 by using radioactivity and enzyme determinations in the same experiment. A minichromosome under control of the chromosomal (*oriC*) region replicates at a discrete time during the division cycle (13). The step in enzyme activity coincided with the peak in radioactivity (Fig. 2), indicating that the enzyme activity is a suitable measure of plasmid replication. Control experiments on enzyme induction from the chromosomal *lac* operon indicated that the doubling in enzyme activity corresponded to the time of gene replication (3, 10). Furthermore, there is no step in activity when a high-copy plasmid, which presumably replicates throughout the division cycle, encodes β-galactosidase (21). This experiment also showed that a minichromosome isolated independently of those of Leonard and Helmstetter (13) replicates in a cell-cycle-specific manner.

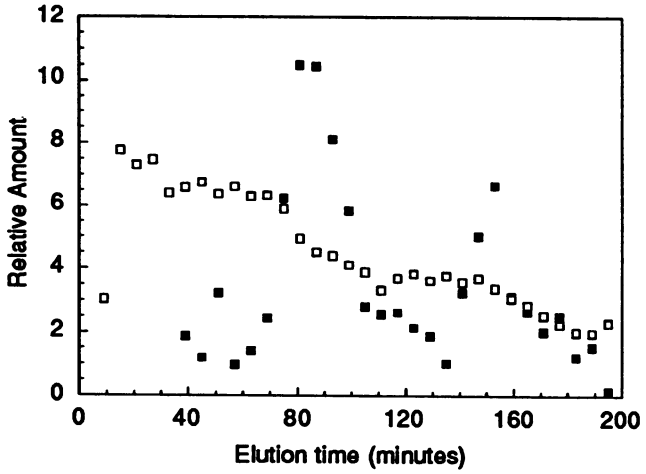

FIG. 2. Cell-cycle-specific replication of minichromosome pOC89. *E. coli* B/r F65 containing *lac:kan* minichromosome pOC89 was grown for several generations in succinate-minimal medium supplemented with histidine, thymine, and proline; labeled with [*methyl*-^{3}H]thymidine; induced with isopropyl-β-D-thiogalactopyranoside for 5 min; and analyzed by membrane elution. The plasmids were isolated by alkaline lysis and purified by agarose gel electrophoresis. The minichromosome bands were cut from the gel, dissolved, and counted by liquid scintillation. The closed squares indicate the relative radioactivity per cell incorporated into the pOC89 plasmid. The open squares indicate the relative amounts of enzyme per cell. The time of plasmid replication during the division cycle, as determined from labeling and counting of the minichromosome, corresponds to a doubling in the capacity to synthesize a plasmid-encoded enzyme.

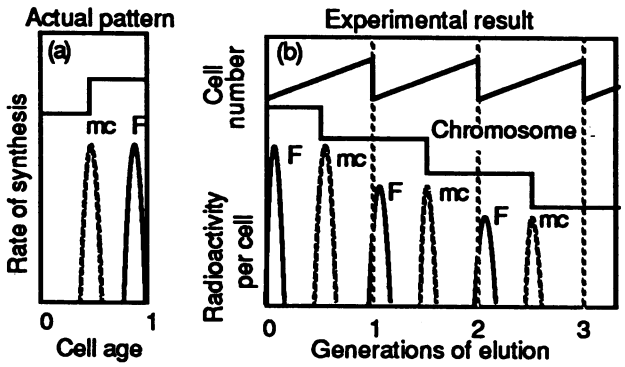

FIG. 3. Idealized membrane-elution experiment for determination of plasmid replication during the division cycle. If the actual pattern were as shown in panel a, then the membrane-elution results would be as shown in panel b. Since, in this example, the F plasmid (F) replicates in cells older than those in which the minichromosome (mc) replicates, the first cells in each generation of elution will have the radioactive F plasmid DNA. This occurs because the first cells eluted are the older cells at the time of labeling, and the younger cells are then eluted, with further growth on the membrane. The step function is the expected rate of chromosomal DNA synthesis. The minichromosome replicates when initiation of chromosomal DNA replication occurs.

Cell-cycle-specific replication of the F plasmid. A remaining possible explanation for the discrepancy between the autoradiographic and enzyme induction results regarding F plasmid replication is that the rate of enzyme synthesis per gene changes during the division cycle when its determinant is carried on an F plasmid and does not change when it is carried on a minichromosome. To check this possibility, we measured the pattern of replication of the F plasmid during the division cycle by liquid scintillation counting of isolated fragments of the F plasmid from cells labeled at different times during the division cycle. An idealized view of the expected pattern of minichromosome and F plasmid replication is illustrated in Fig. 3. For both the minichromosome and the F plasmid, a peaked pattern of radioactivity would indicate a cell-cycle-specific mode of replication.

Replication of the F′*lac* plasmid and minichromosome during the division cycle was monitored in *E. coli* B/r F65(F′*lac* pAL49) by using the membrane-elution technique. To quantitate the amount of radioactivity incorporated into the plasmids during the division cycle, the plasmids were isolated from each fraction of newborn cells eluted from the membrane and cut with restriction enzyme *Eco*RI and the plasmid fragments were separated into individual bands by agarose gel electrophoresis. After trying various plasmid isolation procedures, we determined that the amount of uncut F plasmid entering the gel was significantly less than the total amount of plasmid DNA in the *Eco*RI fragments (data not shown). This led us to believe that a significant portion of the uncut plasmid remained in the well. Restriction cutting of the plasmid allowed most, if not all, of the plasmid to enter the gel, thereby improving radioactive quantitation of the plasmid.

The amount of radioactivity in the plasmid bands was quantitated by using autoradiography and densitometry of the film (Fig. 4). The peaked pattern for the F plasmid and the minichromosome indicates that replication is cell-cycle specific. Desiring more accurate quantitation of the amount of radioactivity incorporated into the plasmids, we cut the bands from the gel and counted them by liquid scintillation. This method allowed us to cut similar slices of agarose below each band of interest to measure the background contamination resulting from the label in the chromosome. The results from one of many consistent experiments are shown in Fig. 5. The patterns for the F plasmid and the minichromosome are more resolved than in Fig. 4, but the results are the same (replication is cell-cycle specific). The peak of radioactivity in the minichromosome band occurred at the same time as initiation of replication of the bacterial chromosome (data not shown), while the peak of radioactivity in the F plasmid occurred at a different time in the division cycle. Since the rises and falls in the F plasmid pattern are in the opposite orientation from the radioactivity in the chromosome, this also argues against the possibility that chromosomal contamination affected our results and is further evidence that the radioactivity in the EcoRI fragments originated in the F′*lac* plasmid. Thus, not only is the F plasmid replication cell-cycle specific, but the F plasmid replicates at a different time during the cycle than the minichromosome.

An additional membrane-elution experiment was performed by using plasmid-free *E. coli* B/r F65 cells to determine whether the pattern of radioactivity incorporated into the F′*lac* plasmid was due to chromosomal contamination. *E. coli* B/r F65 (plasmid free) was pulse-labeled with [*methyl*-^{3}H]thymidine and analyzed by using the membrane-elution technique. Unlabeled cells containing the F′*lac* plasmid and minichromosome were subsequently added to each fraction, and the DNA was isolated, cut with restriction enzyme *Eco*RI, and separated by agarose gel electrophoresis. Bands corresponding to the F′*lac* plasmid and minichromosome were cut from the gel and counted. No clear pattern of

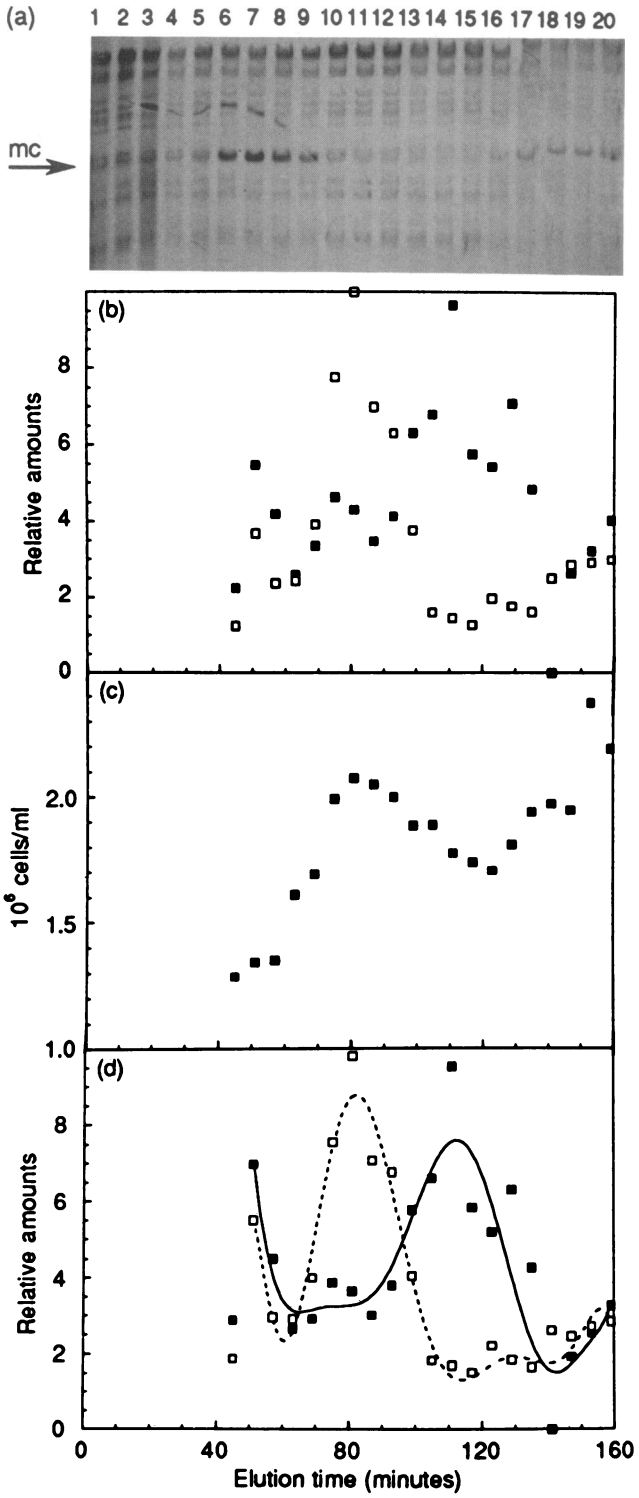

FIG. 4. Cell-cycle-specific replication of the F'*lac* plasmid as determined by autoradiography. *E. coli* B/r F65 containing minichromosome pAL49 and the F'*lac* plasmid was grown in succinate-minimal medium supplemented with histidine, thymine, and proline for several generations, labeled for 6 min with [*methyl*-^{3}H]thymidine, and analyzed by membrane-elution. The plasmids were isolated from each membrane-elution fraction by alkaline lysis and cut with restriction enzyme EcoRI, and the bands were separated by agarose gel electrophoresis. An autoradiogram was prepared by exposing film to the dried gel for 5 weeks and quantitated by using densitometry (quantitated by C. E. Helmstetter). (a) The autoradiogram. The arrow labeled mc indicates the position of minichromosome pAL49 on the gel. All other bands correspond to the F'*lac* plasmid. Lanes 1 to 20 correspond to fractions 7 to 26 collected from the membrane-elution experiment; the DNA was not isolated from fractions 1 to 6. (b) The closed square is the intensity of the F'*lac* band at approximately 20 kb, and the open square is the intensity of the minichromosome (pAL49) band. The plasmids were isolated from the total cells eluted from the membrane during a 6-min period, such that the intensity of the bands represents the total radioactivity incorporated into the plasmids of a membrane-elution fraction. To determine the relative radioactivity per cell incorporated into the plasmids, the intensity must be divided by the cell number in each membrane-elution fraction (c). (d) The relative amounts of radioactivity per cell incorporated into the F'*lac* plasmid 20-kb band (■) and the minichromosome pAL49 band (□) were determined by dividing the intensity in panel b by the cell number in panel c. The first peak in the F'*lac* plasmid occurs prior to membrane-elution fraction 9 (54 min), and the second peak occurs in fraction 20 (120 min). The first peak in the minichromosome occurs in membrane-elution fraction 15 (90 min), and the second peak occurs around fraction 26 (156 min). The data presented here are not as clear as the data in Fig. 4 because of nonlinearities in autoradiography and inaccuracies in densitometry, but they reflect the same trends in F'*lac* and minichromosome replication patterns during the *E. coli* division cycle. The autoradiogram (a) indicates that the differences between our results and the results of Leonard and Helmstetter (14) are due to the problems in agarose gel electrophoresis of large plasmids. Furthermore, it is not possible to correct for any chromosomal contamination in the plasmid bands, even by using the most sophisticated densitometers. These disadvantages led us to use scintillation counting to quantitate the radioactivity incorporated into the plasmids.

radioactivity per cell was observed in the bands corresponding to the F'*lac* plasmid or minichromosome (Fig. 5), indicating that no chromosomal contamination produced a cell-cycle-specific pattern of replication. More important, the normalized amount of radioactivity in the bands isolated from plasmid-free cells was significantly less than the radioactivity in the bands from F'*lac* plasmid-containing cells.

F plasmid replication at different growth rates. The times of minichromosome and F plasmid replication during the division cycle were determined from Fig. 5 and from similar experiments with cells growing at different rates. Although a wide range of growth rates was studied, the best results were obtained in slow-growing cells. Figure 6 graphically summarizes results from our experiments and those of others to indicate when during the division cycle the F plasmid and minichromosome replicate as a function of the growth rate. The results for the F plasmid fall on a line parallel to the line determined by minichromosome and chromosome replication. The chromosome and minichromosome results are consistent with those of earlier studies (5, 9).

Negative control. The replication pattern of high-copy mini-R6K plasmid pMF26 was studied by using the same procedures, under the same growth conditions, and in the same strain used to study F plasmid replication. *E. coli* B/r F65(pMF26 pAL49) was pulse-labeled with [*methyl*-^{3}H]thymidine and analyzed by using the membrane-elution technique. The plasmids were isolated, separated by agarose gel electrophoresis, removed from the gel, and counted by liquid scintillation (Fig. 7). A peaked pattern for radioactivity per cell incorporated into the minichromosome indicates cell-cycle-specific replication. The data for radioactivity per cell incorporated into the mini-R6K plasmid lies along a straight line, indicating that the high-copy plasmid replicates

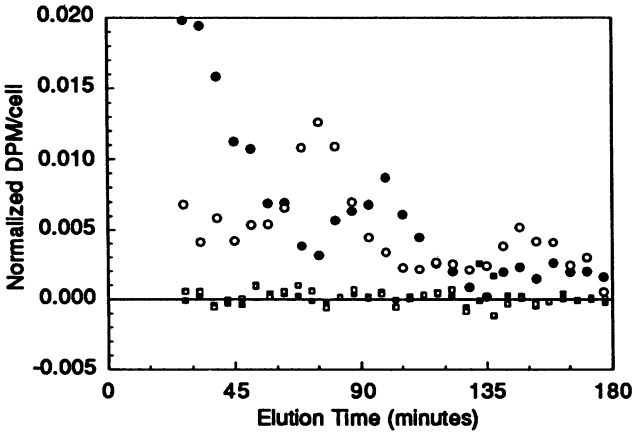

FIG. 5. Cell-cycle-specific replication of the F'*lac* plasmid. *E. coli* B/r F65 containing minichromosome pAL49 and the F'*lac* plasmid was grown for several generations in succinate-minimal medium supplemented with histidine, thymine, and proline; labeled for 6 min with [*methyl*-^{3}H]thymidine; and analyzed by membrane elution. The plasmids were isolated by alkaline lysis and cut with restriction enzyme *Eco*RI, and the bands were separated by agarose gel electrophoresis. The plasmid bands were cut from the gel, dissolved, and counted by liquid scintillation. The closed circles indicate the relative amounts of radioactivity per cell incorporated into the F plasmid. The first peak appears before 45 min of elution, and the second peak occurs at approximately 105 min. The open circles indicate the relative amounts of radioactivity per cell incorporated into the minichromosome. Peaks are seen at 75 and 150 min. The same experiment was performed with *E. coli* B/r F65 (plasmid free), and the results of this analysis are represented by the small squares. The open squares represent the expected amounts of chromosome contaminating the minichromosome band, and the closed squares represent the expected amounts of chromosome DNA contaminating the F plasmid bands. The data for both the plasmid-containing and plasmid-free cells were normalized to the total radioactivity incorporated per cell at a specific point in the second generation so that the data points are shown in their correct absolute relationship. DPM, disintegrations per minute.

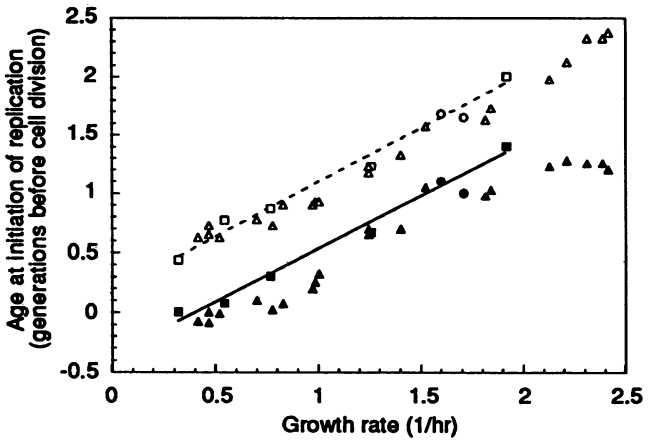

FIG. 6. Variation in cell age at replication of the chromosome and the F plasmid as a function of growth rate. The times of minichromosome and F plasmid replication and chromosome replication initiation during the division cycle were determined from Fig. 4 and other experiments performed at different growth rates. The open symbols indicate when the chromosome initiates replication during the division cycle as a function of growth rate. The squares and circles are the results from this laboratory, while the triangles represent the results of Finkelstein and Helmstetter (9). The closed triangles indicate the replication of an F'*lac* plasmid determined by enzyme induction (9). The closed squares (from scintillation counting) and circles (from enzyme assay) indicate the replication of the F'*lac* plasmid determined in this laboratory. Linear regression of the circles and squares (data from this laboratory) indicated that the two lines are approximately parallel.

throughout the division cycle. The results of this experiment are evidence that the membrane-elution technique does not produce artificial periodicities in plasmid replication.

DISCUSSION

For over 20 years, investigations of F plasmid replication during the *E. coli* division cycle have indicated that replication is cell-cycle specific (2, 3, 8, 9, 17, 18, 22). Most of this work used the step in the rate of synthesis of a plasmid-encoded enzyme as a measure of the time of plasmid replication. Recently, a number of workers have reported that F plasmid replication occurs throughout the division cycle; i.e., replication is not cell-cycle specific (1, 14, 19). One of these experiments (14) used autoradiography as a direct measure of plasmid replication. A control plasmid, the minichromosome, was included within the same cell as a measure of cell-cycle-specific replication. The autoradiographic analysis indicated that although the minichromosome replicates in a cell-cycle-specific manner, the F plasmid (and a single-origin mini-F plasmid derivative) does not replicate in a cell-cycle-specific manner (14).

There are several problems in the quantification of large plasmids in agarose gels by using autoradiography that could account for the discordant results: nonlinearities in autora-

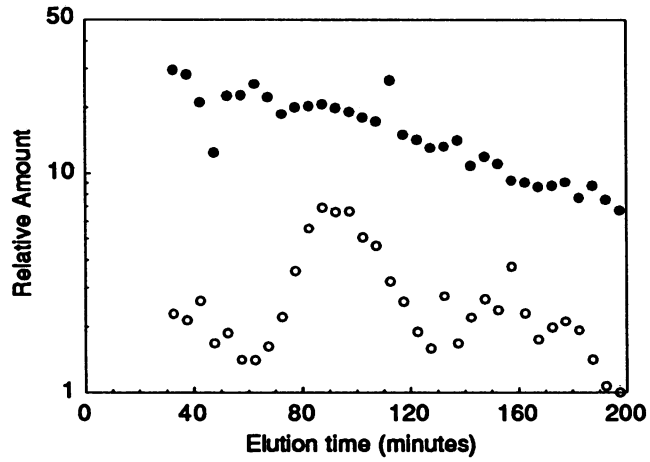

FIG. 7. Cell-cycle replication pattern of mini-R6K plasmid pMF26. *E. coli* B/r F65 containing minichromosome pAL49 and plasmid pMF26 was grown for several generations in succinate-minimal medium supplemented with histidine, thymine, and proline; labeled for 6 min with [*methyl*-^{3}H]thymidine; and analyzed by membrane elution. The plasmids were isolated by alkaline lysis, and the bands were separated by agarose gel electrophoresis. The plasmid bands were cut from the gel, dissolved, and counted by liquid scintillation. The closed circles represent the relative amounts of radioactivity per cell incorporated into the mini-R6K plasmid. The data lie along a straight line, indicating that the mini-R6K plasmid replicates in a cell-cycle-independent manner. The open circles represent the relative amounts of radioactivity per cell incorporated into the minichromosome. Peaks are seen at 90 and 160 min, confirming that the minichromosome replicates in a cell-cycle-specific manner.

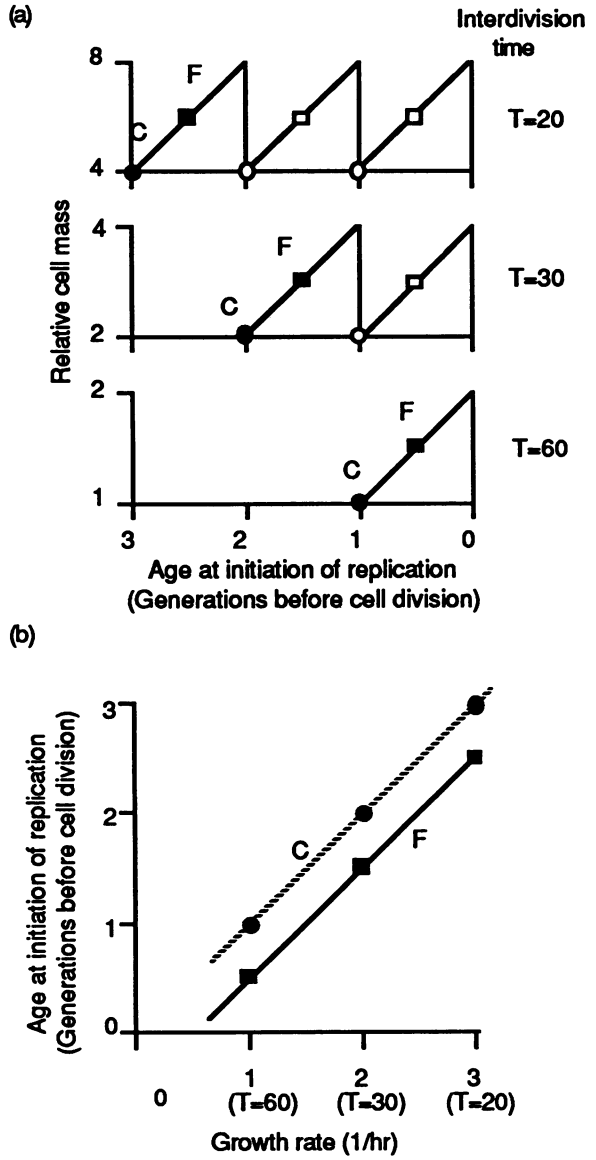

FIG. 8. Interpretation of patterns of F plasmid replication at different growth rates. (a) The timing of initiation of chromosome replication and F plasmid replication during the division cycle is shown for three idealized interdivision times. The filled circles indicate times of initiation of chromosome replication with respect to a cell division at the right. For each growth rate, there is 60 min between initiation of chromosome replication and a division (5); this is equivalent to one interdivision period in 60-min cells, two interdivision periods in 30-min cells, and three interdivision periods in 20-min cells. Each closed square is the time of F plasmid replication if the F plasmid replicates precisely in the middle of the division cycle. For the three cases drawn here, the F plasmid replicates 0.5, 1.5, and 2.5 interdivision periods prior to a division. If for the three growth rates illustrated the mass at initiation of the chromosome varies as 1:2:4 and the number of origins varies in a similar manner, a constant mass per origin for initiation of the chromosome is obtained (7). The cell masses are related by the factors 1:2:4 in the middle of the division cycle as they are related at the beginning of the division cycle. If the number of F plasmid origins varies as 1:2:4 for doubling times of 60, 30, and 20 min, respectively, then the mass per plasmid origin at replication is also constant. (b) When the cell age during the division cycle for F plasmid replication or chromosome replication initiation was plotted as a function of growth rate (bottom graph), two parallel lines were obtained. To generalize this result, if any plasmid replicates at a constant mass per origin, a parallel line is obtained in the lower graph; the actual displacement of the parallel line, however, depends on the amount of cell mass per plasmid origin at the time of initiation.

diography (20), difficulties in correcting for any chromosomal contamination, and problems with inconsistent isolation and separation of large plasmids in agarose gels. The problems with autoradiography can be eliminated by slicing the plasmid bands from the gels and directly counting the label incorporated into plasmid by liquid scintillation counting. Unlabeled carrier cells can be added to each sample to allow visualization of the plasmid bands. Because of the large size of the F'*lac* plasmids (~100 kb), a low percentage of the plasmid DNA may enter the gel during standard gel electrophoresis. We have experimental evidence that this is actually the case, since the total fluorescence from the F'*lac* plasmid entering the gel is much less than the total fluorescence from the smaller bands arising from restriction cutting; this means that much of the uncut F'*lac* plasmid does not enter the gel, possibly leading to a lack of quantitative recovery of the plasmid. To overcome this problem, the plasmid DNA can be cut with specific restriction enzymes to produce fragments that easily and quantitatively enter the agarose gel. Since our autoradiography results are consistent with the direct counting results, the problems with purifying large plasmids by standard agarose gel electrophoresis may account for discrepencies between our results and those of Leonard and Helmstetter (14). Finally, the problem of chromosome contamination can be solved by cutting out interband gel slices to determine how much chromosomal contamination is present within each plasmid band.

Our results demonstrate that the F'*lac* plasmid replicates during a specific period in the division cycle. These findings agree with previous work indicating that doubling of the ability to synthesize a plasmid-encoded enzyme occurred during a specific interval of the cell division cycle. Why do our results differ from those of Leonard and Helmstetter (14)? In addition to the suggestions that (i) direct counting is better than autoradiography, (ii) restriction cutting leads to a more quantitative recovery of plasmid material, and (iii) it is important to have a direct measure of contaminating chromosomal material so that it can be subtracted from the plasmid band, we feel that (iv) performing the experiment with slow-growing cells led to more definitive determination of the pattern of plasmid replication. The data of Cooper (3) support this finding.

Overriding all of these arguments is the experimental evidence (Fig. 2) that plasmid replication (or, more precisely, gene duplication) can be measured by using an enzyme assay. Thus, the finding of steps in plasmid-encoded enzyme synthesis when there is pulse induction is consistent with the quantitative radiochemical measurement of plasmids presented here and inconsistent with the results of Leonard and Helmstetter (14). The presence of steps in β-galactosidase activity per cell in earlier studies argues against the suggestion that the minichromosome imposes an artificial cell-cycle dependence on F plasmid replication, since the minichromosome was not present in F'*lac*-containing cells in these early studies. The finding that a doubling in the capacity to produce β-galactosidase occurs at different times during the division cycle, depending on the location of the gene for the enzyme (3, 6, 9, 10, 21, 22; this work), argues against the possibility that the steps in enzyme

activity occur because the method of cell-cycle analysis affects cell physiology.

When the cell ages at initiation of chromosome replication and F plasmid replication were plotted as a function of growth rate, two parallel lines resulted (Fig. 6). What is the meaning of these parallel lines? In Fig. 8 we present a schematic illustration of the relationship of chromosome replication and plasmid replication as a function of growth rate. It has been shown previously (5), and confirmed here, that the chromosome initiates replication at a constant time prior to cell division (C + D or 60 min), so that there is a variation in the number of interdivision periods between replication initiation and cell division. It has also been shown that the cell mass at initiation varies with growth rate so that there is a constant mass per origin at initiation (7). These two relationships are summarized by the closed circles in Fig. 8. If a plasmid replicates at a constant mass per plasmid origin, then we would expect a similar situation, as illustrated by the closed squares in Fig. 8a. For a doubling time of 60 min, one chromosome origin initiates replication 1.0 generation prior to division, when the cell mass per chromosome origin becomes 1/1 or 1.0, and one plasmid replicates 0.5 generation prior to division, when the cell mass per plasmid origin becomes 1.41/1 or 1.41. This calculation assumes that the mass of the cell increases exponentially during the division cycle (4). For a doubling time of 30 min, two chromosome origins initiate replication 2.0 generations prior to division, when the cell mass per chromosome origin becomes 2/2 or 1.0, and two plasmids replicate 1.5 generations prior to division, when the cell mass per plasmid origin becomes 2.82/2 or 1.41. For a doubling time of 20 min, four chromosome origins initiate replication 3.0 generations prior to division, when the cell mass per chromosome origin becomes 4/4 or 1.0, and four plasmids replicate 2.5 generations prior to division, when the cell mass per plasmid origin becomes 5.64/4 or 1.41. Thus, the chromosome initiates replication when the cell mass per origin equals 1.0 and the plasmid replicates when the cell mass per plasmid origin equals 1.41. When the times of plasmid replication and chromosome replication initiation from the three upper graphs were plotted in Fig. 8b, we obtained a result similar to the experimental findings. The two lines are parallel. We interpret this to mean that the F plasmid replicates when there is a constant mass per plasmid origin.

At plasmid replication, the number of plasmids doubles from one to two per cell in the 60-min culture, from two to four in the 30-min culture, and from four to eight in the 20-min culture. However, the line corresponding to the F plasmid in Fig. 8 may be drawn at different heights relative to the chromosomal replication pattern. If, rather than one, two, and four plasmids in the newborn cells in the upper graphs, there were two, four, and eight plasmids in the newborn cells, then the plasmid line in Fig. 8 would be displaced upward by an integer value such that replication would occur more generations prior to a cell division. The case illustrated in Fig. 8 is the minimal case, in which there is one plasmid going to two plasmids at the slowest growth rates.

The cell-cycle-independent replication pattern found for the high-copy mini-R6K plasmid (Fig. 7) indicates that the experimental approach used here does not artificially produce periodicities in plasmid replication. Although Leonard and Helmstetter (14) included high-copy plasmid pBR322 in their experiments, the large number of bands produced by restriction cutting the F'*lac* plasmid and the minichromosome made it difficult to include any more plasmids in our experiments. A final possible explanation for discrepancies between our results and those of Leonard and Helmstetter is that the pBR322 plasmid induced artificial cell-cycle-independent replication of the F plasmid in their experiments.

To summarize, the F plasmid replicates in a cell-cycle-specific manner. The timing of the replication is similar to that of the chromosome in that initiation occurs when there is a constant amount of mass per plasmid origin present. We conjecture that all naturally occurring low-copy plasmids not only have cell-cycle-specific replication, but their replication is also regulated by a size control mechanism formally analogous to the mechanisms proposed here for the F plasmid and by others for the chromosome.

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