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Cell cycle-specific replication of *Escherichia coli* minichromosomes

(*oriC*/plasmids/cell division)

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ABSTRACT **The timing of *Escherichia coli* minichromosome replication in the cell division cycle was examined using an improved procedure for studying plasmid replication frequency. Cultures growing exponentially in glucose/Casamino acids minimal medium were pulse-labeled with [^{3}H]thymidine, and the radioactivity incorporated into plasmid DNA in cells of different ages was analyzed. At the end of the labeling period the bacteria were bound to the surface of a nitrocellulose membrane filter, and the radioactivity in new daughter cells, which eluted continuously from the membrane, was quantitated following agarose gel electrophoresis. The minichromosomes replicated during a discrete interval in the cell division cycle that appeared to coincide with initiation of chromosome replication. In contrast, plasmid pBR322 replicated throughout the division cycle at a rate that increased gradually as a function of cell age. The difference in minichromosome and pBR322 replication was clearly discernible in cells harboring both plasmids. It was also found that the 16kD gene adjacent to *oriC* was not a determinant of the timing of minichromosome replication during the division cycle. The results are consistent with the conclusion that minichromosome replication frequency is governed by the same mechanism that controls chromosome replication.**

Minichromosomes of *Escherichia coli* are plasmids whose autonomous replication ensues solely from a resident copy of the chromosomal origin of replication, *oriC* (1–5). Research on minichromosomes is predicated on the idea that characterization of the replication properties of these small molecules should yield new information on the mechanisms and control of *E. coli* chromosome replication. This expectation has been supported by numerous findings that minichromosomes require a number of the same gene products for initiation of replication as the chromosome, including the DnaA protein (6–10). On the other hand, it has also been found that minichromosomes are present in higher copy numbers than the chromosome and are not stably maintained (1, 2, 4–7).

If minichromosomes are to serve as authentic models for the control of initiation of chromosome replication *in vivo*, they would be expected to initiate replication in coordination with the cell growth cycle, as the chromosome does. In a previous study on this issue, we measured the incorporation of radioactivity into closed circular minichromosomes isolated by cesium chloride/propidium iodide gradient centrifugation from synchronously dividing cultures (8). The data were interpreted to indicate that minichromosome replication took place throughout the division cycle in the synchronous cells. Subsequently it was reported that minichromosome interreplication times were nonrandom in exponential-phase cultures, as determined by density shift experiments (11). Although these latter findings indicate a level of minichromosome replication control that could be similar to the chromosome, they do not address the relationship between the timing of minichromosome replication and either initiation of chromosome replication or the cell division cycle.

We have reexamined the replication of minichromosomes during the division cycle using a procedure that is significantly more sensitive than the synchronous growth study reported previously. It is based on the technique of Projan *et al.* (12) for measurement of plasmid copy numbers by electrophoretic separation of DNA in whole cell lysates. The timing of minichromosome replication during the division cycle was determined by coupling this electrophoretic analysis with the method originally used to establish the relationship between chromosome replication and the division cycle (13, 14). An undisturbed, exponentially growing culture was pulse-labeled with [^{3}H]thymidine and attached to a membrane filter. The radioactivity in minichromosomes was then measured in new daughter cells withdrawn at intervals from the membrane-bound culture. Any uncertainties as to the physiological state of minichromosome-containing cells in synchronous culture were thus eliminated, and clear identification of the rate of radioactive thymidine incorporation into minichromosome DNA was made possible. The results show unequivocal evidence that minichromosomes replicated during a specific interval in the division cycle that appeared to correspond to the time of initiation of chromosome replication.

MATERIALS AND METHODS

Bacteria, Plasmids, and Growth Conditions. The strains employed were *E. coli* B/r F26 (*thyA his*) or *E. coli* B/r F44 (*his thr pro rpsL recA1*). These strains contained the minichromosomes pAL4, pAL43, or pAL49 (Fig. 1), and plasmid pBR322 either alone or in combination with the minichromosomes. Cultures were grown in minimal salts medium (13) supplemented with 0.1% glucose and 0.2% Casamino acids, and when necessary, 10 μg of thymine per ml. For each experiment, 100 ml of minimal medium containing kanamycin (100 μg/ml) was inoculated with bacteria and incubated in a shaking water bath at 37°C for at least 18 hr until the culture was in the early stationary phase of growth. Bacterial concentrations were determined with a model ZB Coulter electronic particle counter.

Cell Cycle Analyses. The cultures were diluted 1:1000 in 100 ml of minimal medium lacking kanamycin and grown at 37°C for approximately 4 hr until they reached 5–10 × 10^7 cells per ml. The cultures were then exposed to 10 μCi of [^{3}H]thymidine per ml (New England Nuclear, 70–80 Ci/mmol; 1 Ci = 37 GBq) for 4 min. Unlabeled thymidine (100 μg/ml, final concentration) was added at the end of the labeling period, the cells were filtered onto the surface of a type GS, 142-mm diameter nitrocellulose membrane filter (Millipore) and washed twice with an additional 100 ml of minimal medium containing 100 μg of thymidine per ml (13). The filter was inverted, and elution was begun at a rate of 2.0 ml/min at 37°C with minimal medium lacking kanamycin. After a delay of 12–16 min to allow release of weakly attached cells, 18–20

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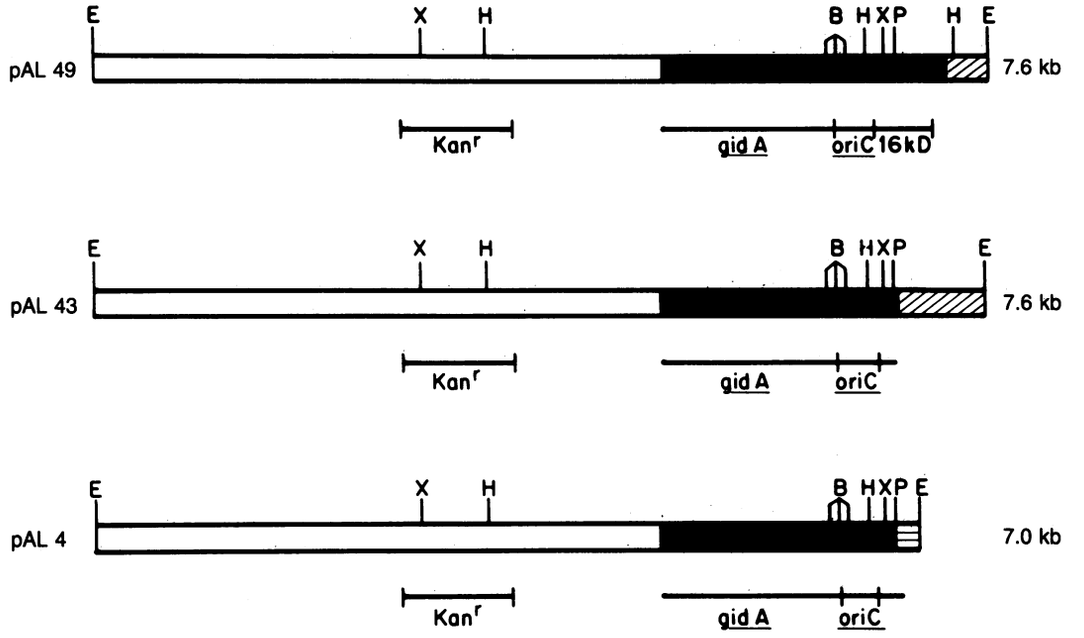

FIG. 1. Genetic and physical map of the *E. coli* minichromosomes. Restriction enzyme-generated fragment sizes were determined by agarose gel electrophoresis. The circular maps for each plasmid were opened at their unique *Eco*RI sites. The open boxes represent DNA derived from pML21 that harbors Tn*903* and confers resistance to kanamycin. Diagonal hatches indicate DNA that was derived from pK01 (15). Filled boxes represent contiguous *E. coli* chromosomal DNA, and horizontal hatches designate DNA derived from the *E. coli* chromosome but which is not contiguous. *Eco*RI (E), *Hin*dIII (H), *Pst* I (P), *Xho* I (X), and *Bam*HI (B). The DnaA protein binding site and promoter of the 16kD gene found on pAL49 have been removed from minichromosomes pAL43 and pAL4. The *Eco*RI–*Pst* I fragment of pAL43, derived from pK01, harbors the promoter region of the β-lactamase gene from which transcription is initiated toward *oriC*.

consecutive 4-min samples were collected from the effluent. A 0.5-ml portion was removed from each sample for measurement of cell concentration, and 0.5 ml was removed and placed in ice-cold 5% (vol/vol) trichloroacetic acid for measurement of total radioactive incorporation per cell (13). The remaining portion of each sample, ≈7.0 ml, was placed in a 15-ml centrifuge tube in an ice bath. Each tube contained 100 μg of thymidine per ml and 0.1 M sodium azide. For analysis of radioactivity incorporated into plasmid DNA in these samples, whole cell lysates were prepared as described by Projan *et al.* (12) except that the final sample volume was approximately 120 μl. A 40- to 70-μl volume of lysate was loaded into each well of a horizontal, 1% agarose slab gel (International Biotechnologies, Inc., New Haven, CT) and electrophoresed at 40 volts for 18–20 hr in Tris/borate/EDTA buffer (16). Gels were prepared for fluorography as described by Laskey (17). The dried gels were exposed to Kodak X-Omat AR x-ray film at −70°C for 1–3 hr (for chromosomal bands) and 6–15 days (for plasmid bands). Autoradiographs were scanned with a Helena recording densitometer. To correct for variations in cell concentrations in each sample, as well as for any variations in the amount of cell lysate added to each well, the radioactivity in minichromosome DNA per cell was calculated as: the ratio of the photographic densities of the plasmid to chromosomal bands in each lane multiplied by the total trichloroacetic acid-precipitable radioactivity per cell determined for each sample.

RESULTS

The timing of minichromosome replication during the division cycle of *E. coli* was determined by pulse-labeling an exponentially growing culture with [^{3}H]thymidine, binding the labeled culture to a nitrocellulose membrane filter, and measuring the radioactivity present in the minichromosome DNA of new daughter cells eluted continuously from the membrane. The first cells to be eluted were progeny of cells at the end of the division cycle at the time of membrane attachment, and those eluted at one generation were progeny of cells at the start of the cycle. Therefore, radioactivity in the new daughter cells during each generation of elution reflected the amount incorporated in the oldest through the youngest cells of the original exponentially growing population. Radioactivity in minichromosome DNA was assayed by subjecting whole cell lysates of the new daughter cells to agarose gel electrophoresis. Fig. 2 shows fluorographs of a gel after electrophoresis of samples collected at consecutive 4-min intervals from the effluent of a membrane-bound population of *E. coli* B/r F26 containing the minichromosome pAL49 (Fig. 1). The radioactivity in bands corresponding to chromosomal DNA and closed circular minichromosome DNA in the new daughter cell samples are shown. The radioactivity in chromosomal DNA decreased progressively in new daughter cells as a function of elution time, whereas the radioactivity in pAL49 minichromosome DNA fluctuated periodically.

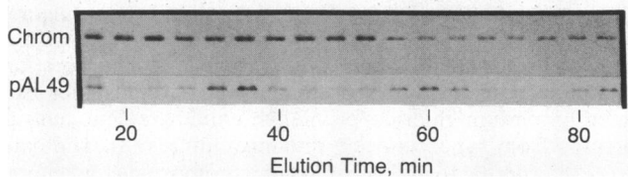

FIG. 2. Electrophoretic separation of labeled chromosome and pAL49 minichromosome DNA from new daughter cells in the effluent of a membrane-bound culture of *E. coli* B/r F26 (pAL49). Cells growing exponentially were labeled with [^{3}H]thymidine for 4 min, bound to a membrane filter, and eluted with glucose/Casamino acids minimal medium. Whole-cell lysates of new daughter cells in the effluent were subjected to agarose gel electrophoresis and fluorography. The radioactive bands corresponding to chromosomal and pAL49 DNA are shown for consecutive 4-min samples of the effluent. Exposure times to the x-ray films were 3 hr for the chromosomal bands and 10 days for the minichromosome bands.

Quantitative analysis of the relationship between minichromosome replication and the division cycle of *E. coli* is shown in Fig. 3. The lower curve in the figure gives the concentration of new daughter cells in the effluent from a membrane-bound population of B/r F26 (pAL49). The shape of the elution curve in each generation reflects the age distribution of the cells initially bound to the membrane (13). The mean generation time of the cells growing on the membrane was determined from the periodicity in the curves—i.e., by the midpoint of the decrease in cell concentration (18)—as indicated by the vertical interrupted lines. The middle curve shows the relative amount of radioactivity in minichromosome DNA as determined by densitometric tracing of the closed circular plasmid bands in a fluorograph of an agarose gel. The rate of [^{3}H]thymidine incorporation into minichromosome DNA as a function of the division cycle is read from right to left in each generation of elution. Thus, the pAL49 minichromosomes replicated in a burst toward the end of the division cycle in cells growing with a doubling time of approximately 28 min at 37°C.

The upper curve in Fig. 3 shows the total amount of cold trichloroacetic acid-precipitable radioactivity in the new daughter cells, which essentially corresponds to the amount of [^{3}H]thymidine incorporated into chromosomal DNA during the pulse-labeling. The rate of chromosome replication during the division cycle is again read from right to left in each generation of elution. The step-wise increases correspond to initiation of a round of chromosome replication (13, 14, 18, 19). It is evident that the burst of minichromosome replication coincided with initiation of a round of chromosome replication.

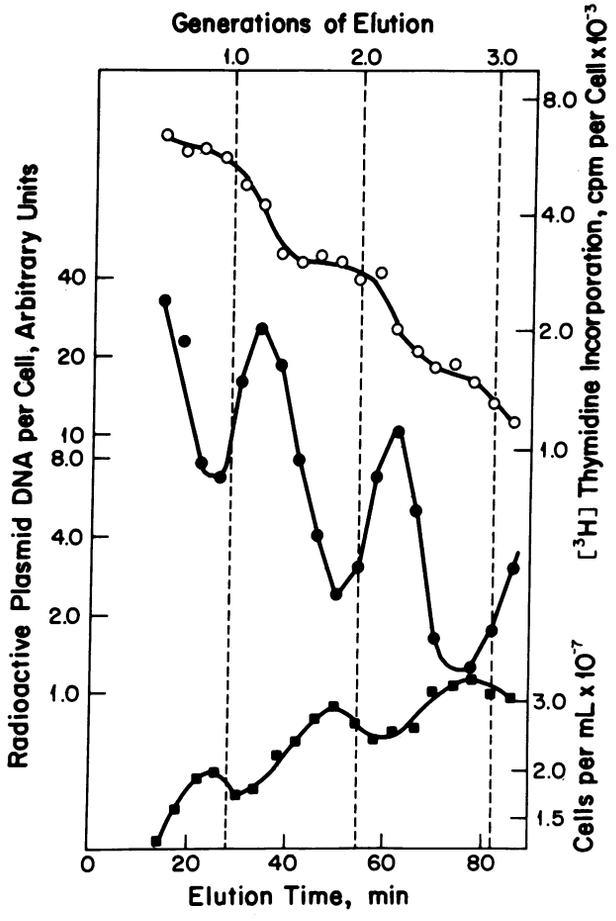

FIG. 3. Radioactivity in minichromosome and total DNA in new daughter cells in the effluent from a membrane-bound culture of *E. coli* B/r F26 (pAL49). Exponential-phase cells were pulse-labeled and treated as described in the legend to Fig. 2. Experimental points are plotted at the midpoints of the 4-min collection intervals from the effluent. The vertical interrupted lines indicate generations of elution. ■, Concentration of cells in consecutive 4-min samples of the effluent; ●, radioactivity in minichromosome DNA per cell; ○, total radioactivity per cell.

A gene located adjacent to *oriC*, designated 16kD and present on pAL49, has been shown to be a determinant of minichromosome incompatibility and to modulate copy number levels (20–24). Since transcription from the promoter of 16kD can pass into *oriC* and is under negative control by DnaA protein (20, 23, 24), this gene could conceivably play a role in regulation of initiation from *oriC* and perhaps be a determinant of the cell cycle-specificity of minichromosome replication. Accordingly, experiments identical to those described in Fig. 3 were performed with a minichromosome that lacked a functional 16kD gene, pAL4 (Fig. 4). The results were basically indistinguishable from those shown in Fig. 3. Again, minichromosome replication took place in a burst that was coincident with the time of initiation of chromosome replication in the culture. The burst of minichromosome replication appeared to be slightly later in the division cycle of these cells, but initiation of chromosome replication was also later due to the slightly lower growth rate of the *recA* mutant employed in this experiment.

The current technique for measuring plasmid replication in the division cycle can be validated further by demonstrating the absence of cell cycle-specific replication for plasmids that would not be anticipated to show such specificity (25). Consequently, we examined the replication of plasmid

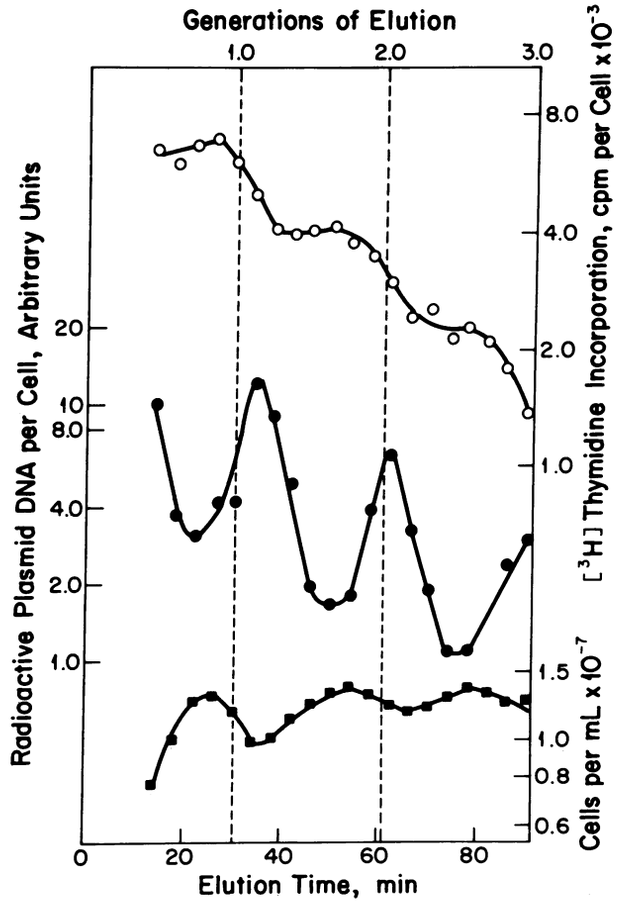

FIG. 4. Radioactivity in minichromosome and total DNA in new daughter cells in the effluent from a membrane-bound culture of *E. coli* B/r F44 (pAL4). Experimental details and symbols are as described in the legend to Fig. 3.

pBR322. Of particular importance are studies on cells that contained both a minichromosome and pBR322 since the replication pattern of both plasmids could be compared directly in the same experiment. The results of such an experiment on cells containing both pBR322 and the minichromosome pAL43 are shown in Fig. 5. pAL43 lacks a functional 16kD gene, but this gene was replaced with the promoter of the β-lactamase gene derived from pK01 (15). Transcription initiates from this promoter toward *oriC*. As shown in the figure, replication of this minichromosome was also cell cycle specific. On the other hand, pBR322 replication took place throughout the division cycle, at a rate that increased gradually during the cycle, with no evidence of cell cycle-specific replication.

DISCUSSION

E. coli minichromosomes were found to replicate in a burst during the cell division cycle. The timing of the minichromosome replication burst, and its distribution in the cell cycle, were qualitatively similar to the timing and distribution of initiation of chromosome replication. This correspondence between minichromosome replication and initiation of a round of chromosome replication was evident in all experiments in B/r F26 *rec*$^+$ and B/r F44 *recA*, with both replication events being slightly later in the cycle of the *recA* mutant cells due to their slower growth rate. In some experiments, the midpoint of the minichromosome replication interval may have been slightly earlier in the division cycle than the midpoint of initiation of chromosome replication, but additional studies on cells growing at different rates will be required to evaluate this possibility. The replication burst in each cell cycle consisted of a 5- to 10-fold variation between the maximum and minimum of [^{3}H]thymidine incorporation into minichromosome DNA. Considering that at least 10% of the cells in the effluent from a membrane-bound culture are not new daughter cells, and some initiation of chromosome replication between the steps in total incorporation is always evident (13, 18, 19), it appears justified to conclude from our findings that minichromosome replication is restricted to a discrete interval in the cell growth cycle and that it is probably timed by the same mechanism as initiation of chromosome replication.

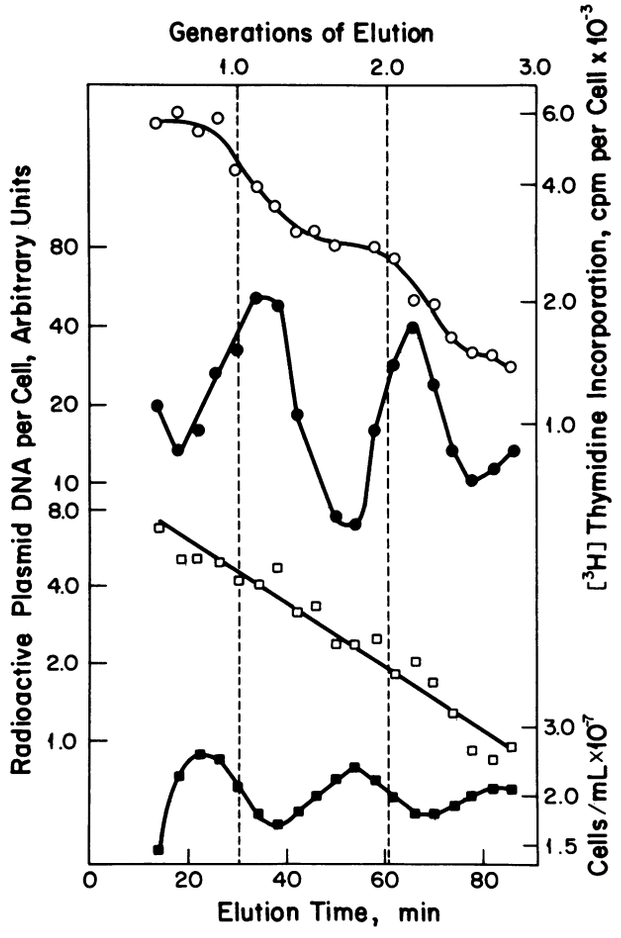

FIG. 5. Radioactivity in plasmid and total DNA in new daughter cells in the effluent from a membrane-bound culture of *E. coli* B/r F44 containing pAL43 and pBR322. Experimental details and symbols are as described in the legend to Fig. 3 except open squares, radioactivity in pBR322 DNA per cell.

Our inability to detect cell cycle-specific minichromosome replication in earlier studies was very likely a consequence of the inadequacies of the methods that had been used to study plasmid replication during the division cycle. The earlier work was performed on synchronously dividing cultures. Studies on such cultures are influenced by possible growth perturbations caused by the synchronization procedure, even when minimal manipulations are involved to obtain synchronously dividing cells. In fact, the original establishment of the pattern of chromosome replication during the division cycle required use of the labeling protocol employed here, i.e., pulse-labeling of an exponentially growing culture prior to any treatment and subsequent measurement of radioactivity in new daughter cells by membrane elution (13, 14). This procedure avoids use of synchronously dividing cells entirely, and only requires that the membrane-bound cells divide in the same sequence as they would in suspension culture. Plasmid replication studies utilizing this protocol were made possible by electrophoretic analysis of whole cell lysates of the new daughter cells (12), thereby enabling quantitation of lower levels of radioactivity in plasmid DNA than could be accomplished with cesium chloride/propidium iodide gradient centrifugation (8). A further advantage of the current technique is that separation of cellular DNA by agarose gel electrophoresis allowed identification of the amount of radioactivity incorporated into different plasmid molecular species in the same cell. The clear evidence for periodic synthesis of one plasmid (the minichromosome) and the absence of periodic synthesis for a second plasmid (pBR322) when present in the same cell provides additional strong evidence for cell cycle-specific *E. coli* minichromosome replication. Thus, the periodicities in minichromosome radioactivity in the identical new daughter cell samples could not have been introduced by the isolation or electrophoresis procedures, or fluctuations in cellular precursor pools, because corresponding periodicities were not observed in pBR322 replication.

The cell cycle-specific replication of minichromosomes takes place in spite of their instability and the absence of a plasmid-encoded partition system for segregation of minichromosomes into daughter cells at division (4–7, 26). It was also shown that the 16kD gene adjacent to *oriC*, which is not required for, but positively affects, replication (11, 20, 22–24), did not determine the timing of the initiation of minichromosome replication during the division cycle of cells grown in glucose/Casamino acids. The loss of the function of this gene or the replacement of the 16kD promoter with that of the β-lactamase gene promoter had no effect on minichromosome replication control. Further studies with the procedure described in this paper will be required to identify any nonessential minichromosome nucleotide sequences that are required for cell cycle replication specificity.

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