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Coordinate Initiation of Chromosome and Minichromosome Replication in *Escherichia coli*

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***Escherichia coli* minichromosomes harboring as little as 327 base pairs of DNA from the chromosomal origin of replication (*oriC*) were found to replicate in a discrete burst during the division cycle of cells growing with generation times between 25 and 60 min at 37°C. The mean cell age at minichromosome replication coincided with the mean age at initiation of chromosome replication at all growth rates, and furthermore, the age distributions of the two events were indistinguishable. It is concluded that initiation of replication from *oriC* is controlled in the same manner on minichromosomes and chromosomes over the entire range of growth rates and that the timing mechanism acts within the minimal *oriC* nucleotide sequence required for replication.**

The origin of replication on the *Escherichia coli* chromosome, *oriC*, has the property, apparently unique among bacterial replicons, that initiation of replication is rigidly coupled to the growth of the cell (reviewed in reference 25). Furthermore, chromosome replication and cell division are coordinated so that the frequency of initiation from *oriC* determines the frequency of cell division during steady-state growth (8). Thus, critical aspects of bacterial proliferation are controlled by the molecular mechanism that governs the timing of initiation of chromosome replication.

We have recently shown that minichromosomes, i.e., plasmids that initiate synthesis from a resident copy of *oriC*, replicate during a restricted interval in the division cycle of *E. coli* (12). In cells growing with a mean generation time of 28 min at 37°C, minichromosomes harboring approximately 2 kilobases (kb) of *oriC*-containing chromosomal DNA replicated at a mean cell age which was similar to or slightly younger than the cell age at initiation of chromosome replication. These findings, along with observations that minichromosomes replicate once per generation (11, 26) and require the same gene products as the chromosome for their synthesis (3–5, 9, 13, 26–28), suggest that the initiations of minichromosome and chromosome replication are timed coordinately. Unambiguous determination of a timing relationship requires comparison of the mean cell ages and the distribution of cell ages at initiation from *oriC* replicons in cells growing at different rates. We now report that chromosomes and minichromosomes initiate replication simultaneously in *E. coli* cells growing with a variety of generation times. Furthermore, this coordinate initiation was achieved with minichromosomes which contained *oriC* within as few as 327 nucleotides of chromosomal DNA. It thus appears that the molecular basis for the control of initiation of chromosome replication may be identified through analysis of the replication properties of these small, dispensable minichromosomes.

MATERIALS AND METHODS

Bacteria and growth conditions. All experiments were performed with *E. coli* B/r F (*thyA his*). The minichromosomes were pAL49 (12) and pAL55. pAL55 (3.9 kb) comprises DNA restriction fragments from three sources: (i) an *Acc*I restriction fragment derived from pUC-4K (24) which harbors the kanamycin resistance transposon Tn*903*, (ii) an *Sma*I-*Acc*I restriction fragment from pK01 (16) carrying the *galK* gene lacking the promoter region, and (iii) a chromosomal *Hae*III-*Acc*I restriction fragment on which is found the *oriC* nucleotide sequences from coordinates −41 to 286, based on the map of Oka et al. (18). Cultures were grown in minimal salts medium (6) containing either glucose (0.1%) or glycerol (0.2%). In some experiments the minimal medium was supplemented with mixtures of amino acids, which included methionine, histidine, arginine, proline, leucine, and threonine, each at a final concentration of 50 μg/ml, or 0.2% Casamino Acids (Difco Laboratories). For each experiment, 100 ml of medium containing the appropriate carbon sources and 100 μg of kanamycin per ml was inoculated with bacteria and incubated at 37°C in a shaking water bath for approximately 18 h. The initial inoculum was such that the cells were in the late log phase of growth by 18 h. The cultures were then diluted 1:1,000 in 100 ml of the same medium lacking kanamycin and grown at 37°C for approximately 4 h until they reached 5×10^7 to 10×10^7 cells per ml for use with the membrane-elution procedure.

Cell cycle analyses. The exponentially growing cultures were exposed to 10 μCi of [*methyl*-^{3}H]thymidine (70 to 80 Ci/mmol; New England Nuclear Corp.) per ml for 4 min. Unlabeled thymidine (100 μg/ml final concentration) was added at the end of the labeling period, and the timing of chromosome and minichromosome replication in the cell cycle was determined at 37°C with the membrane-elution technique (7). Briefly, the cells were filtered onto the surface of a type GS nitrocellulose membrane filter (142-mm diameter; Millipore Corp.) and washed once with an additional 100 ml of medium containing 100 μg of thymidine per ml. The filter was inverted and elution was begun, with the identical medium lacking kanamycin, at a rate of 15 ml/min for the first minute and then at 2.0 ml/min thereafter. After a delay of 4 min to allow release of weakly attached cells, 24 to 30 consecutive samples of newborn cells were collected from the effluent. A 0.5-ml portion was removed from each sample for measurement of cell concentration with a model ZB Coulter electronic particle counter, and 0.5 ml was removed and placed in ice-cold 5% trichloroacetic acid for measurement of total [^{3}H]thymidine incorporation per cell. The remaining portion of each sample was lysed by the procedure of Projan et al. (19), except that lysostaphin was

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replaced by lysozyme. Agarose electrophoresis of the samples and fluorography were performed as described previously (12). The dried agarose gels were exposed to Kodak X-OMAT X-ray film at −70°C for 5 to 30 days. The densities of the bands on the films were determined with an LKB laser densitometer.

RESULTS

DNA replication during the division cycle of cells growing at various rates. The relationship between minichromosome replication and initiation of chromosome replication in *E. coli* B/r growing at various rates was examined with the membrane-elution technique (12). Cultures growing exponentially at 37°C with doubling times between 25 and 60 min were pulse-labeled with [^{3}H]thymidine, bound to the surface of a nitrocellulose membrane filter, and then flushed continuously with culture medium. The timing of chromosome and minichromosome replication during the division cycle was determined by measuring the radioactivity present in newborn cells as they were continuously released from the surface of the membrane due to the growth and division of the bound cells. Radioactivity in the newborn cells released during each generation of elution reflected the amount of [^{3}H]thymidine incorporated into the oldest through the youngest cells of the original exponentially growing population. The amount of radioactivity in minichromosome DNA was assayed by agarose gel electrophoresis of whole-cell lysates of the newborn cells (19). Figure 1 shows fluorographs of agarose gels after electrophoresis of newborn cell samples collected at 4-min intervals from the effluents of membrane-bound cultures of *E. coli* B/r F(pAL49) growing with mean generation times of about 25, 35, and 43 min. The radioactive bands corresponding to closed circular minichromosome DNA and the concentrations of cells in the effluents are shown for each of the growth rates. Under all growth conditions, the radioactivity in pAL49 minichromosome DNA fluctuated periodically in consecutive samples of the newborn cells.

The timing of minichromosome replication with respect to the cell division cycle can be seen in Fig. 1 by comparing the periodicities in radioactivity to the periodicities in the curves showing the concentration of cells in the effluents. The shape of the cellular concentration curve in each generation of elution reflects the age distribution of cells initially bound to the membrane (6). The mean generation times of the membrane-bound cells can be determined from the periodicities in the curves, as indicated by the vertical interrupted lines in each frame. The amount of [^{3}H]thymidine incorporated into minichromosome DNA as a function of the cell division cycle, i.e., cell age in the cycle, is read from right to left in each generation of elution. It can be seen that the peaks of minichromosome DNA radioactivity in newborn cells appeared at different times during the division cycle in cells growing at different rates. This is, however, an approximation of the timing of minichromosome replication in the cell cycle because it assumes that radioactive labeling took place at the instant the cells were bound to the membrane. Since the total time between the addition of [^{3}H]thymidine and the start of elution was approximately 4 min, minichromosome replication was actually earlier in the cell cycle by a time which was not longer than the duration of this pulse-labeling interval. Nevertheless, the data show that the minichromosomes replicated in a burst in the second half (Fig. 1a), first half (Fig. 1b), and toward the middle (Fig. 1c) of the division cycle in cells growing with generation times of 25, 35, and 43 min, respectively. The data also show that the periodicities in radioactive minichromosome DNA in newborn cells were unrelated to the periodicities in newborn cell concentrations, since the positions of the maxima for minichromosome radioactivity varied with respect to the maxima for the cellular concentrations.

The relationship between minichromosome replication and initiation of chromosome replication in cells growing at different rates is shown in Fig. 2. The upper curve in each frame shows the total amount of cold trichloroacetic acid-precipitable radioactivity in the newborn cells, which corresponds to the amount of [^{3}H]thymidine incorporated into chromosomal DNA during the pulse-labeling. The stepwise increases in incorporation, again read from right to left, reflect initiation of rounds of chromosome replication. The lower curve in each frame shows the relative amount of radioactivity in minichromosome DNA as determined by densitometric analysis of fluorographs from experiments of the type shown in Fig. 1. Based on visual inspection of the curves, it is evident that the pAL49 minichromosomes replicated at the same time as the chromosomes initiated replication at each growth rate.

Cell cycle replication of the small minichromosome pAL55. The minichromosome pAL49 carries the *oriC* region of the chromosome and the adjacent *mioC* gene, which serves as a positive effector of minichromosome copy number (5, 14, 22, 23, 25). It was of interest to delineate the regions of the minichromosome which were involved in the coordinate

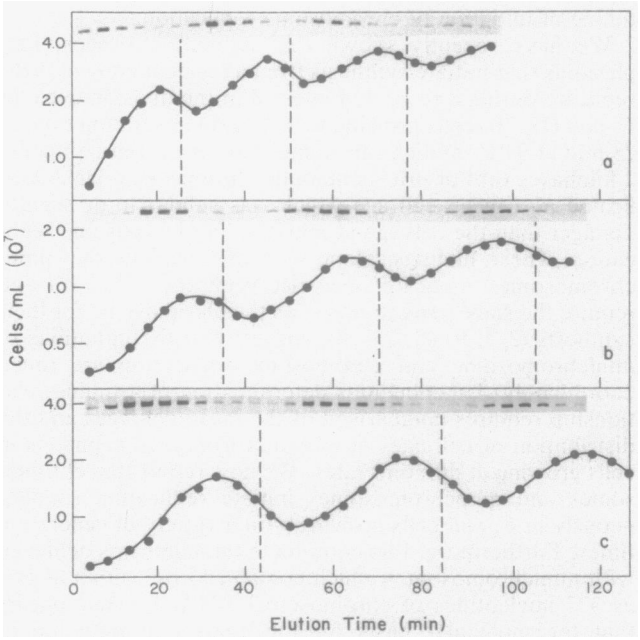

FIG. 1. Minichromosome replication during the division cycle of *E. coli* B/r F(pAL49) growing at different rates. Cells growing exponentially in minimal medium containing glucose plus Casamino Acids (a), glucose plus six amino acids (b), or glucose alone (c) were pulse-labeled with [^{3}H]thymidine for 4 min, bound to a membrane filter, and eluted with minimal medium of the same composition. Whole-cell lysates of the newborn cells released into the effluent were subjected to agarose gel electrophoresis and fluorography. The radioactive bands corresponding to closed circular pAL49 minichromosome DNA are shown for consecutive 4-min samples of the effluent at each growth rate. The concentrations of cells in the effluent at each growth rate are also shown, and the vertical interrupted lines indicate the end of each generation of growth on the membrane.

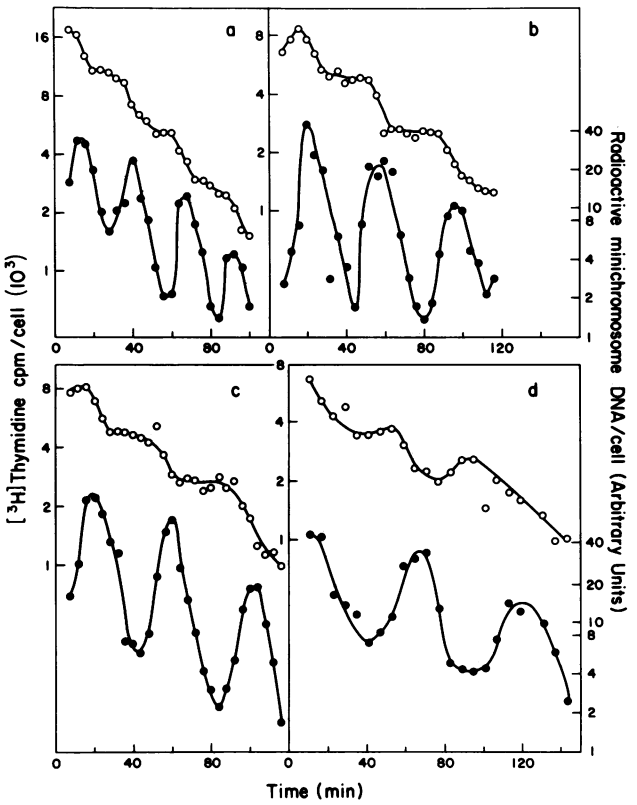

FIG. 2. Comparison between the timing of chromosome and pAL49 minichromosome replication during the division cycle. Exponential-phase cultures of *E. coli* B/r F26(pAL49) growing in glucose plus Casamino Acids (a), glucose plus six amino acids (b), glucose (c), or glycerol (d) were pulse-labeled and treated as described in the legend to Fig. 1. The radioactivity per cell in minichromosome DNA (●) and total radioactivity per cell (○) in newborn cells collected from the effluents of membrane-bound cultures are plotted at the midpoints of the 4-min collection intervals.

replication response. We have previously shown that this *mioC* gene is not a determinant of cell cycle-specific replication of pAL49 (12). The replication properties of pAL55, a much smaller minichromosome containing only 327 base pairs (bp) of *oriC* DNA, are shown in Fig. 3. As with pAL49, the burst of pAL55 replication paralleled initiation of chromosome replication at this and all growth rates examined. In addition, the distribution of the bursts of replication for both plasmids was indistinguishable (compare Fig. 2 and 3), indicating that 327 bp or less were sufficient to establish coordination between minichromosome replication and initiation of chromosome replication.

Cell age at initiation of chromosome and minichromosome replication. To quantitate the relationship between initiation of chromosome and minichromosome replication at all growth rates, a number of membrane-elution experiments of the type shown in Fig. 2 and 3 were performed. In each experiment, the mean cell ages and age distributions of the two initiation events in the division cycle were measured and compared. Figure 4 shows an example of the means by which the data from these experiments were analyzed. Since the time required for replication of the minichromosomes at 37°C would only be a few seconds, assuming the rate of DNA polymerization is the same as for the chromosome, measurement of [^{3}H]thymidine incorporation into minichromosome DNA is effectively a measure of the timing of initiation of replication. Thus, the mean cell age at initiation of minichromosome replication in the division cycle was considered the midpoint of the peak in minichromosome replication, as shown in Fig. 4. The mean cell age at initiation of chromosome replication was considered the midpoint of the stepwise increase in the rate of total [^{3}H]thymidine incorporation. The age distributions of the two initiation events in each generation are best compared by relating the minichromosome replication curves to the differential of the [^{3}H]thymidine incorporation curve, which gives the rate of initiation (Fig. 4). As indicated by these examples, the distributions were so similar as to be essentially indistinguishable.

Quantitative comparisons of the timing of initiation of chromosome and minichromosome replication in the division cycle of cells growing at various rates were accomplished by calculating the mean cell ages at initiation (a_I) by the equation $a_I = [(n + 1)\tau - (C + D)]/\tau$ (8), where n is the smallest integer to yield a positive value. The membrane-bound generation time (τ) substituted into the equation for each experiment was the average of the durations of the first three generations of elution, determined as described in Fig. 1. $C + D$ is the time interval between initiation of a round of chromosome replication and the division following completion of that round, and it is known to equal approximately 60 min in B/r growing with generation times between 25 and 60 min at 37°C (8). For calculation of cell age at initiation of chromosome replication, the actual value of $C + D$ in each experiment was measured as shown in Fig. 4. Since newborn cells released immediately after the culture was attached to the membrane (time zero) were the progeny of ancestral cells that initiated a round of replication $C + D$ min earlier, the duration of $C + D$ in this example is given by the interval

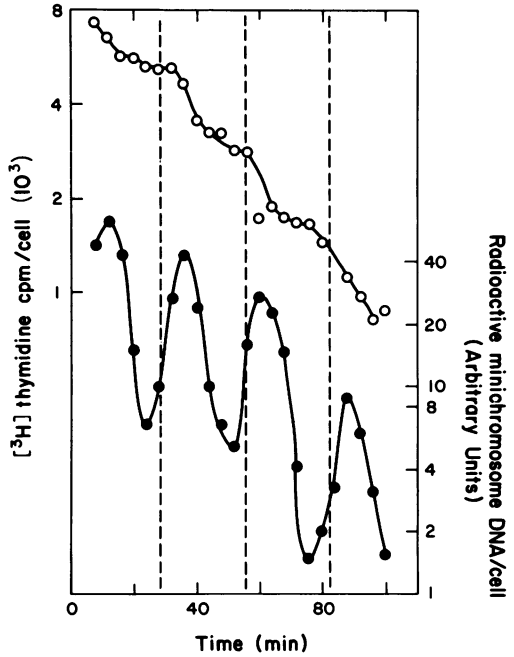

FIG. 3. Comparison between the timing of chromosome and pAL55 minichromosome replication during the division cycle. Exponential-phase cultures of *E. coli* B/r F26(pAL55) growing in glucose plus Casamino Acids were pulse-labeled and treated as described in the legend to Fig. 1. Symbols are as described in the legend to Fig. 2.

between time zero and the initiation identified in the third generation of elution. Cell age at minichromosome replication was calculated in identical fashion by measuring the time between the start of elution and the minichromosome replication burst at $C + D$ min.

Figure 5 shows cell age at initiation of minichromosome replication versus cell age at initiation of chromosome replication at all growth rates examined. Since $C + D$ varies little between generation times of 25 and 60 min at 37°C, cell age at initiation of chromosome replication changes continuously with growth rate, and as a consequence, a range of cell ages at initiation are represented. If chromosomes and minichromosomes initiated replication at the same time in the division cycle at a given growth rate, all the points would lie on the 45° line shown in the figure. The measured ages fell along the 45° diagonal with no obvious tendency to be consistently above or below the line, indicating that the initiations of replication were coincident at all growth rates.

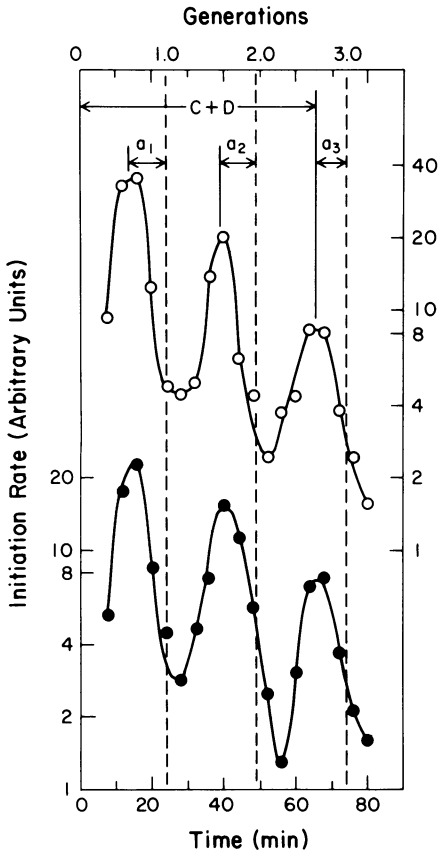

FIG. 4. Cell age at initiation of chromosome and minichromosome replication. Exponential-phase cultures of *E. coli* B/r F26(pAL49) growing in glucose plus Casamino Acids were pulse-labeled and treated as described in the legend to Fig. 1. The rates of initiation of minichromosome replication (●) and chromosome replication (○) during the division cycle are seen by reading from right to left in each generation of elution (interrupted vertical lines). The rate of initiation of chromosome replication was determined by drawing a smooth curve through the data points representing total radioactivity per cell in the effluent, as in Fig. 2, and calculating the difference in radioactivity at consecutive 4-min intervals. Measurement of the time between initiation of a round of chromosome replication and the division upon completion of that round ($C + D$) is shown, as well as measurements of the cell age (a_I) at initiation during the division cycle in three consecutive generations of elution.

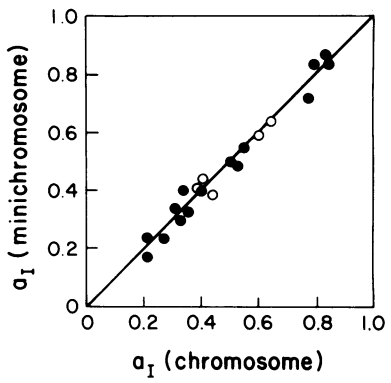

FIG. 5. Coordination between initiation of chromosome and minichromosome replication in cells growing at various rates. Cell ages at initiation of chromosome replication (in fractions of a generation) are shown as a function of cell ages at initiation of minichromosome replication for cells containing pAL49 (●) or pAL55 (○) growing with mean generation times between 25 and 60 min at 37°C. The cell ages (a_I) were determined as described in the text.

DISCUSSION

Initiation of chromosome replication in *E. coli* is coupled to cell growth so that, over the range of growth rates examined in this paper, there is little variation in cell mass per chromosomal copy of *oriC* at the time of initiation (2, 8, 25). Our results suggest that minichromosome replication is subject to the same stringent control. Within the limits of the sensitivity of the method employed, minichromosomes replicated coincident with initiation of chromosome replication in the cell division cycle at all growth rates examined. This was found to be true for both pAL49, which contains the entire *oriC* region in addition to the adjacent *mioC* gene, and pAL55, which contains only 327 bp of *oriC* DNA. Not only were the mean cell ages at initiation of replication identical for pAL49 and pAL55, but the age distributions of the minichromosome replication bursts were indistinguishable from each other and from the distribution of initiation of chromosome replication. It is concluded from the results reported here and elsewhere (10–12, 26) that the cell growth-coupled, coincident initiation of chromosome and minichromosome replication is specified by an *oriC* sequence which is not more than 327 bp in length.

It was also found that the presence of minichromosomes had no discernible effect on cellular growth properties. Minichromosome maintenance did not affect the growth rates of the cultures, mean cell age at initiation of chromosome replication, or the duration of $C + D$. Furthermore, the degree of selection of newborn cells from the membrane-bound cultures was not noticeably altered by the presence of the minichromosomes, indicating normal coupling between chromosome replication and cell division. The membrane-elution technique is very sensitive to alterations in chromosome replication-cell division coupling. Any randomization of either chromosome replication or cell fission would be seen as a change in the pattern of release and size distribution of the newborn cells. Since no changes were detected, it is concluded that the minichromosomes did not affect the normal process of cell duplication.

We have previously shown that the periodicity of minichromosome radioactivity in newborn cells released from membrane-bound cultures could not have been introduced

by any aspect of the DNA isolation or electrophoresis procedures (12). The present cell cycle analyses of cultures growing at various rates also dispel concern that the periodicity could be accounted for by fluctuations in the newborn cell concentration. Such variations should theoretically be removed by expressing all of the data in terms of radioactivity per cell. However, it was still considered important in these experiments, as well as in those performed earlier to measure chromosome replication in the division cycle (6, 7), to demonstrate that fluctuations in cell concentration did not introduce artificial periodicities in radioactivity per cell. The studies presented clearly showed that the fluctuations in cell concentration were unrelated to the observed periodicity in the rates of minichromosome replication. The positions of the peaks of radioactivity in minichromosome DNA varied with respect to the maxima or minima for cell concentrations in the effluents of cultures growing at different rates.

Our results suggest that information on the control of the timing of initiation of chromosome replication from *oriC* may be obtained through analysis of the replication properties of dispensable minichromosomes, in a manner similar to studies which have identified controlling components of other bacterial plasmids (reviewed in reference 20). The nature of this control process must be such that it allows cells to initiate replication simultaneously at all *oriC* origins, chromosomal and plasmid, without appreciably affecting the normal growth and division processes of the cell. Two fundamentally different control mechanisms can be considered. The control system might communicate with the *oriC* replication origins as either autonomous units or members of an interconnected assembly. In the former situation, each origin would respond independently to the initiation signals, with the targets for these signals residing within 327 bp or less of *oriC* DNA. An obvious example of such a timing mechanism would be the creation of a prepriming complex (1) on each individual *oriC*, with initiation of the priming stage and subsequent DNA polymerization taking place when formation of the limiting component of the complex was completed. In this case the origins would respond independently to a multisubunit control signal. In an interconnected assembly, all origins would have the capacity to read the same rate-limiting controller. This situation would obtain if all the prepriming complexes were turned on by a single event, such as the activation of a membrane territory suitable for replication of all membrane-bound *oriC*-containing replicons (15, 17). As an alternative, one or a few master origins could, upon activation, direct the passive initiation of the remaining replicons. It is not possible to distinguish between these types of control mechanisms at present. However, the observations that large increases in the number of *oriC* replicons in the cell had no measurable effect on growth and division, that chromosomes and multicopy minichromosomes initiated replication coordinately, and that individual chromosomal origins in the cell initiated replication with remarkable synchrony (21) lend support to the concept that the origins respond to the control system as an assembly.

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