Replication of Prophage P1 Is Cell-Cycle Specific

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P1 prophage replication during the Escherichia coli division cycle has been analyzed by using the membrane-elution technique to produce cells labelled at different times during the division cycle and scintillation counting for quantitative analysis of radioactive prophage DNA. P1 prophage replicates during a restricted portion of the bacterial division cycle, like the minichromosome, but at a time during the division cycle different than the time at which the minichromosome replicates in the same cell. A high-copy mini-R6K plasmid present in the same cell replicates throughout the division cycle. Over a wide range of growth rates, the P1 prophage replicates approximately one-half generation after the minichromosome replicates. Thus, the mechanisms underlying P1 replication are similar to those for the F plasmid and the chromosome. Replication occurs when some property related to cell size or cell mass reaches a constant value per origin.

The P1 prophage exists as a low-copy plasmid at approximately one copy per chromosome equivalent (8, 9). The stable maintenance of a low-copy plasmid requires that its replication be attuned to the rate of cell growth and division. One possible mechanism of coupling plasmid replication to cell division is to have the plasmid replicate at a specific time during the division cycle, as is found for initiation of chromosome replication and minichromosome replication (4, 7, 14). Recently, we have shown that the low-copy F plasmid replicates in a cell-cycle-specific manner and that replication occurs when a constant cell mass per plasmid origin is achieved (10). These results have been supported by density-shift experiments demonstrating that F plasmid replicates nonrandomly (13). The F plasmid, however, does not replicate at the same time during the division cycle that the chromosome initiates replication or that the minichromosome replicates (10). A mini-F plasmid also replicates in a cell-cycle-specific manner, similar to that of the parental F plasmid, when it contains two origins of replication, but appears to lose cell-cycle-specific replication when the oriV origin of replication is deleted (12). We now turn to another low-copy plasmid, the P1 prophage, to determine its pattern of replication during the division cycle.

The timing of P1 prophage replication during the bacterial division cycle has been investigated. Abe (1), using a dnaA(Ts) mutant to synchronize cell growth and DNA-DNA hybridization to monitor P1 replication, reported that P1 prophage replicates throughout the division cycle. These results are equivocal, since synchronization can lead to incorrect results (3), particularly when studying DNA replication initiation and using the temperature-sensitive initiator of DNA replication to synchronize cells. Prentki et al. (19) studied P1 replication relative to two chromosomal markers in unperturbed cells and concluded that replication of prophage P1 was not simultaneous with initiation or termination of chromosome replication. Prentki et al. (19) proposed that the timing of P1 replication varies, relative to chromosome replication initiation, as a function of growth rate.

We have reinvestigated P1 prophage replication during the division cycle by using the membrane-elution technique with a direct quantitative measurement of label incorporated into prophage DNA. We find that P1 prophage replicates at a specific time during the division cycle, approximately one-half generation after initiation of chromosome replication. This replication pattern is observed over a wide range of growth rates. This result implies that the P1 prophage replicates when a constant mass per plasmid origin is achieved and that the initiation mass per origin is different for the P1 prophage than it is for the chromosome origin.

**MATERIALS AND METHODS**

**Bacteria and plasmids.** The host bacterium used was *Escherichia coli* B/r NC3 (provided by F. C. Neidhardt). The P1 phage (P1Cm0 cl.100 Cm"*" [referred to as P1]; provided by D. I. Friedman) was plated onto a bacterial lawn of NC3 cells, and lysogens were selected (17). The lysogenic strain is temperature sensitive, and thus all experiments with this plasmid strain were carried out at temperatures at or below 30°C. The minichromosome pAL49 (7.6 kb, Kan"*"; provided by A. C. Leonard) and the mini-R6K plasmid pMF26 (Amp"*"; provided by D. R. Helinski) were then transformed into the strain by the procedure of Davis et al. (5). Thus, one bacterial strain contained the P1 prophage, the minichromosome, and the mini-R6K plasmid.

**Growth of bacteria.** C medium (7) was supplemented with one of the following carbon sources: glucose (0.4%) plus Casamino Acids (0.2%), glucose (0.4%), succinate (0.4%), or acetate (0.4%). The cells were grown for at least six to nine generations in 100 ml of medium at 20 or 30°C in order to obtain exponential growth prior to starting a membrane-elution experiment and to avoid induction of the P1 lytic cycle.

**Membrane-elution technique.** Exponentially growing cells (100 ml, 1.0 × 10⁸ cells per ml, incubated at 20 or 30°C) were labelled with [³H]methyl thymidine (60 to 80 Ci/mmol, 10 μCi/ml) for 5% of the doubling time and filtered onto a nitrocellulose membrane at the end of the labelling period. The cells were washed with medium of the same composition and temperature containing unlabeled thymidine (100 μg/ml). The membrane apparatus was inverted, and new-

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born cells were eluted from the membrane with prewarmed C medium pumped at a rate of 2 ml/min in an incubator set at the same temperature as the exponential culture. Fractions were collected for an interval of time equivalent to the labelling period. Cell numbers were determined by using a particle counter (Coulter Electronics, model ZM). The total [3H]methyl thymidine incorporated was determined by liquid scintillation counting (Beckman Instruments, model 3801).

Plasmid isolation and quantitation. Unlabelled cells containing the plasmids of interest were added to each fraction from the membrane-elution experiment in order to visualize the plasmid DNA fragments on agarose gels. A constant volume of cells containing the plasmids of interest which had been labelled overnight with [14C]thymine (54 mCi/mmol, 0.25 μCi/ml) and deoxyadenosine (100 μg/ml) (18) was added to each fraction from the membrane-elution experiment in order to correct for any DNA losses or variations in plasmid yield during the isolation procedure. The combined cells ([H]-labelled cells eluted from the membrane-elution apparatus, unlabelled cells, and [14C]-labelled 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 (16). The resulting DNA pellets were dissolved in 20 μl of buffer containing 1 U of EcoRI restriction enzyme per μl and digested overnight at 37°C. The fragments were separated by gel electrophoresis on a 0.75% agarose gel run at 2 V/cm in buffer (54 g of Tris base per liter, 0.001 M EDTA, 27.5 g of boric acid per liter) for 24 h. The relevant plasmid bands were visualized by ethidium bromide staining and UV fluorescence. The sizes of the bands generated by the EcoRI restriction digestion agree with the published EcoRI restriction map of the P1 plasmid (20). The bands corresponding to the mini-R6K plasmid, the minichromosome, and the P1 prophage were sliced from the gel, digested in 10 μl of 70% HClO4, at 70°C for 20 min, and counted by liquid scintillation. Plasmid replication during the division cycle was determined by dividing the relative radioactivity incorporated into plasmid DNA isolated from each membrane-elution fraction by the cell number in each fraction [i.e., (H disintegrations per minute [dpm]/[14C] dpm) per cell]. A slice of gel of equivalent size was removed below or above the DNA band of interest and counted by liquid scintillation. The counts in these background slices were subtracted from the counts in the plasmid bands in order to correct for chromosomal contamination in the plasmid bands. Control experiments have shown that the radioactivity in this 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 our conclusions could be made without any background correction. Control experiments also indicated that plasmid isolation was reproducible and consistent.

RESULTS

Cell-cycle-specific P1 prophage replication. Replication of the P1 prophage, the minichromosome, and the mini-R6K plasmid during the division cycle was monitored in E. coli B/r NC3 (P1 pAL49 pMF26) cells by using the membrane-elution technique to label cells of various ages and by direct quantitative measurement of label incorporated into prophage DNA. The results of one such experiment, in which the cells were grown in glucose-minimal medium, are shown in Fig. 1a. The peaked patterns for radioactivity per cell incorporated into the P1 prophage and the minichromosome indicate that replication is cell-cycle specific (10). The data for radioactivity per cell incorporated into the mini-R6K plasmid lies along a straight line, indicating that this high-copy plasmid replicates throughout the division cycle (exponential, cell-cycle-independent replication). The ratios of radioactivity per cell incorporated into the P1 prophage and into the minichromosome relative to that incorporated into

![FIG. 1. Replication pattern of the P1 prophage during the E. coli cell cycle. E. coli B/r NC3 (P1 pAL49 pMF26) was grown for several generations in glucose-minimal medium at 30°C and analyzed by using the membrane-elusion technique and quantitation of label incorporated into plasmid DNA as outlined in Materials and Methods. (a) ⌃, cells per milliliter; ⌡, total 3H dpm per cell; ◊, (3H dpm/14C dpm) per cell in minichromosome pAL49; ■, (3H dpm/14C dpm) per cell in prophage P1; △, (3H dpm/14C dpm) per cell in plasmid pMF26. The peaked patterns in the minichromosome pAL49 and the prophage P1 data indicate cell-cycle-specific replication. The time of minichromosome replication is when the chromosome initiates replication (indicated by a step in the total radioactivity incorporated per cell). The data for the pMF26 plasmid fall on an exponential line, indicating that this plasmid replicates throughout the division cycle. (b) The data for radioactivity per cell incorporated into the minichromosome and the P1 prophage were divided by the data for radioactivity per cell incorporated into the pMF26 plasmid. ⌡, P1 divided by pMF26; ◊, pAL49 divided by pMF26.](http://jb.asm.org/)
the mini-R6K plasmid are represented in Fig. 1b. Presenting the data for the cell-cycle-specific plasmid relative to that for a cell-cycle-independent plasmid allows for more accurate determination of replication timing in late generations eluted from the membrane, because the ratio yields peaks of equal height; this makes the measurement of timing more accurate. Again, it is clear that replication of both the P1 prophage and the minichromosome is cell-cycle specific, but the replication times during the division cycle are different. P1 replication occurs midway between the replication of the minichromosomes in the same cell.

**P1 prophage replication at different growth rates.** Minichromosome and P1 prophage replication were monitored at various growth rates in cells growing on different carbon sources (Fig. 2 and 3), using the same methods used for the experiment presented in Fig. 1. The data for radioactivity per cell incorporated into the P1 prophage and into the minichromosome relative to that incorporated into the mini-R6K plasmid for cells grown in succinate are shown in Fig. 2, and similar data for cells grown in glycerol are shown in Fig. 3. The times of minichromosome and P1 prophage replication during the division cycle have been determined from the data presented in Fig. 1, 2, and 3 and from other experiments (data not shown) and are summarized in Fig. 4a. The results for the P1 prophage fall on a line parallel to the line determined by minichromosome replication and initiation of chromosome replication. An alternative way of considering replication is to plot the time of P1 replication as a fraction of the time between subsequent rounds of minichromosome replication, as in Fig. 4b. This graph indicates that at all growth rates the pattern of P1 plasmid replication is the same relative to the timing of minichromosome replication. Independent of the precise timing reported here, the significant observation which the data support is that replication of the P1 prophage occurs between the replication of the minichromosomes. This qualitative observation is supported by the horizontal line in Fig. 4b.

**DISCUSSION**

Plasmid replication during the bacterial division cycle has been repeatedly investigated in the past 25 years, with conflicting results. The replication pattern of the high-copy plasmids has been consistently found to be cell-cycle independent (10, 11, 15, 21), whereas the replication pattern of the low-copy plasmids (F, P1, and R1) has been controversial. Recently, the contradictory reports of F plasmid replication were resolved by using the membrane-elution technique and quantitative measurement of label incorporated into plasmid DNA in cells grown at different rates (10). The minichromosome was used as an internal (i.e., in the same cell with the F plasmid) control for cell-cycle-specific replication, and the mini-R6K plasmid was used in conjunction with the minichromosome as an external (i.e., not in the same cell with the F plasmid) control for cell-cycle-independent replication. The analysis demonstrated that the F plasmid replicates in a cell-cycle-specific manner in *E. coli* and that F plasmid replication occurs when a constant cell mass per plasmid is achieved. Support for this conclusion has come from density-shift experiments indicating that the F plasmid replicates in a nonrandom manner (13).

**Cell-cycle-specific replication of P1.** Investigations of P1 prophage have reported that its replication pattern is cell-cycle independent (1) or cell-cycle specific with a growth rate-dependent replication time relative to initiation of chromosome replication (19). We used the membrane-elution technique, quantitative radiolabelling, and the minichromosome and the mini-R6K plasmid as internal controls (three plasmids in the same cell) to investigate P1 prophage replication during the *E. coli* division cycle. The minichromosome was chosen as a control for cell-cycle-specific replication because this oriC plasmid replicates in a cell-cycle-specific manner at the time of chromosome replication initiation (14). Thus, it is a good indicator for the time when chromosome replication is initiated. The mini-R6K plasmid was chosen as a control for cell-cycle-independent replication because this high-copy plasmid replicates in a cell-cycle-independent manner (10, 11). The data in Fig. 1, 2, and 3 indicate that the P1 prophage replicates in a cell-cycle-specific manner.

FIG. 2. Replication pattern of the P1 prophage during the *E. coli* cell cycle. *E. coli* B/r NC3 (P1 pAL49 pMF26) was grown for several generations in succinate-minimal medium at 30°C and analyzed by using the membrane-elution technique and quantitation of label incorporated into plasmid DNA as outlined in Materials and Methods. (a) (H dpm/4C dpm) per cell in minichromosome pAL49; (H dpm/4C dpm) per cell in prophage P1; (H dpm/4C dpm) per cell in plasmid pMF26. (b) The data for radioactivity per cell incorporated into the minichromosome and the P1 prophage were divided by the data for radioactivity per cell incorporated into the pMF26 plasmid. pAL49 divided by pMF26; P1 divided by pMF26.
exposure

With autoradiography methods for cell-cycle-specific generations cycle.

Correct for recovery of minichromosome and the P1 prophage were divided by the data for radioactivity per cell incorporated into the plasmid DNA as outlined in Materials and Methods. The data for radioactivity per cell incorporated into the minichromosome and the P1 prophage were divided by the data for radioactivity per cell incorporated into the pMF26 plasmid. □, pAL49 divided by pMF26; ■, P1 divided by pMF26.

That replication of the P1 prophage is altered by the presence of the minichromosome and the mini-R6K plasmid is unlikely. The timing of chromosome replication initiation (or the cell size at initiation of chromosome replication) is unaffected by the presence of the minichromosome (14), the F plasmid (10), and/or the R6K plasmid (11). Furthermore, the F plasmid and the R6K plasmid have the same replication patterns when they are alone in a cell as do when they are present with the minichromosome in a cell (10, 11).

**Experimental requirements for successful demonstration of cell-cycle-specific replication.** Several criteria have been established which must be satisfied in order to accept the results for the replication pattern of a plasmid. (i) The cell concentration eluted from the membrane must have a pattern reflective of the expected age distribution in a culture. (ii) The pattern of chromosome replication rate per cell must agree with previous determinations of the pattern of chromosome replication (4, 7). (iii) The radioactivity per cell incorporated into the minichromosome must have a peaked pattern, and the peaks must correspond with initiation of chromosome replication (14). (iv) The data for radioactivity per cell incorporated into the mini-R6K plasmid must fall on an exponential line. Once these criteria have been met, the results for the plasmid under investigation (P1 in this case) can be accepted. The experiments presented here have met these criteria.

We have not used autoradiography to study plasmid replication during the division cycle because we feel that autoradiography is not as good a method as direct counting. With autoradiography one must adhere to very rigid criteria of exposure in the linear region of the film, use adequate methods for densitometry, and solve other problems which are not present with direct counting using double labels to correct for recovery of plasmid DNA. More important, with autoradiography it is not possible to use double labels to correct for minor differences in plasmid recovery in different fractions. Furthermore, the use of autoradiography has led to contradictory findings, as the cell-cycle-independent replication of the F plasmid determined by autoradiography (15) is not consistent with the pattern of plasmid replication determined by using enzyme induction, whereas the direct counting method has been directly shown to be consistent.
with the enzyme induction experiments. We feel that while autoradiography can give a correct result, it is not as reliable as direct scintillation counting of identified plasmid DNA bands.

**Size control of initiation of P1 plasmid.** The mechanism or rule underlying P1 prophage replication was investigated by determining the cell-cycle timing of prophage and minichromosome replication at different growth rates. The time of P1 prophage and minichromosome replication during the division cycle was taken to be the peak in radioactivity per cell incorporated into prophage and minichromosome DNA relative to cell division. The times of minichromosome and P1 replication in terms of generations prior to cell division are plotted as a function of growth rate in Fig. 4a. A multiple of one was added to the times of replication, depending on the growth rate. The slope of the best line through the points representing the times of initiation of chromosome replication suggests that the C and/or D periods have lengthened disproportionately relative to the doubling time with the decrease in temperature. Linear regression of the P1 and minichromosome data results in nearly parallel lines. These parallel lines have been previously interpreted to mean that the plasmid initiates replication, as does the chromosome, when a critical mass per origin is reached. Thus, P1 plasmid replicates when a critical mass per origin is achieved. This is a formal statement of the observations, and the underlying mechanism for initiation is not yet known.

The relationship of P1 replication to minichromosome replication can be seen most clearly if the time of P1 prophage replication as a fraction of the time between subsequent rounds of initiation of chromosome replication (and minichromosome replication) is determined from Fig. 1, 2, and 3 (and other data) as plotted in Fig. 4b. Since initiation of chromosome replication is the key event regulating the division cycle, it is logical to determine plasmid replication timing relative to the beginning of the division cycle (initiation of chromosome replication) rather than relative to the end of the division cycle (cytokinesis), when other variables (C and D) become important. Plotting the data in this form allows us to eliminate temperature-dependent variables that may be affected disproportionately relative to temperature effects on mass synthesis, on the C period (because of temperature-dependent changes in the DNA elongation rate), or on the D period (because of temperature-dependent changes in cell division). The data in Fig. 4b fall on a straight line of zero slope, indicating that the P1 prophage replicates approximately one-half generation after initiation of chromosome replication at all growth rates and temperatures. Since the chromosome initiates replication at a constant cell mass per origin, since the cell mass grows at an exponential rate during the division cycle, and since the number of P1 prophages per chromosome equivalent in an exponential culture is approximately one at all growth rates, the straight line is slope zero in Fig. 4b implies that the P1 prophage must also replicate at a constant mass per plasmid origin. Any other proposal would be inconsistent with some of the aggregate data.

An analysis of the replication pattern is schematically illustrated in Fig. 5. (The replication patterns are represented for a cell growing at 37°C, whereas the experiments were performed at 30°C. Since a decrease in temperature only increases the doubling time but does not affect the relative timing of events during the cell cycle [3], the experimental and theoretical results are easily comparable.) Three possible mechanisms are proposed to explain the experimental observation that replication of the P1 plasmid occurs between rounds of minichromosome replication at all growth rates. The proposal that initiation occurs at a constant time prior to division agrees with the experimental findings only at 20-, 30-, and 60-min interdivision times, and the proposal...
that initiation occurs at a constant fraction of a division cycle prior to division agrees with the experimental findings only at 60-, 40-, 30-, and 20-min interdivision times. At other growth rates these proposals predict that replication will sometimes occur simultaneously with minichromosome replication. Only the constant mass per origin proposal agrees with the experimental findings at all growth rates. When the data are replotted as in the rightmost diagram in Fig. 5, the replication times fall on parallel lines, as does the experimental data in Fig. 4a. The constant-mass proposal results in a plasmid replication pattern in which the time of plasmid replication is a constant fractional interval between the times of minichromosome replications. In this respect, replication of the P1 prophage is under control of a mechanism that is formally similar to, and perhaps mechanistically similar to, those mechanisms controlling F plasmid replication and the initiation of chromosome and minichromosome replication.

Note that the timing of P1 prophage replication in Fig. 4a is drawn as occurring at a higher mass per origin than chromosome replication initiation; replication of the P1 plasmid occurs after the initiation of the chromosome or minichromosome replication. Placement of the line for P1 prophage replication is arbitrary and does not affect the conclusions. If the line for P1 prophage were placed to the left (below) the line for chromosome replication (Fig. 5), it would be equivalent to having initiation of P1 prophage occur before chromosome replication or at a lower mass per plasmid origin. In either case, replication of prophage P1 occurs at a constant mass per origin. As drawn in Fig. 5, one plasmid per cell replicates to make two plasmids at the slowest growth rates (0.0 to 1.5 doublings per h), two plasmids replicate to make four plasmids between 1.5 and 2.5 doublings per h, four plasmids replicate to make eight plasmids between 2.5 and 3.5 doublings per h, etc. Any integral multiple of this plasmid value per cell agrees with the observations reported here.

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