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Control of Cyclic Chromosome Replication in *Escherichia coli*

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INTRODUCTION

The biochemistry of initiation of chromosome replication in bacteria has been frequently reviewed, including the function of the origin of replication of the bacterial chromosome, *oriC*, and the role of the *dnaA* gene product in this process (11, 31, 57, 59, 82, 92). In those reviews, the generation of cyclic initiation activity was not specifically addressed, but it was generally assumed that DnaA protein plays a crucial role in this control.

Earlier investigators assumed that identical control mechanisms should apply to chromosome and plasmid replication (41, 66). Now it has become apparent that most plasmids initiate replication randomly in time, although not F (46), whereas initiation of chromosome replication, as well as of plasmids which use *oriC* to initiate replication, occurs at discrete times separated by nearly constant intervals (49). Therefore, chromosome replication, but generally not plasmid replication, requires a mechanism which generates cyclic initiation activity. An essential problem of the control of chromosome replication is to find the biochemical basis of this oscillator.

Pritchard et al. (66) discussed two models of an oscillator, one based on negative control and the other based on positive control. Those authors argued in favor of a negative control factor. All reported models of initiation control, including those of Pritchard et al., assume that the *oriC* region is involved in the oscillator, either as the location for the gene of the initiation control factor or as a target for that factor or both. Theoretically the origin need not be directly involved in the cycle generator; in fact, Jacob et al. (41) assumed that the replication "clock" is generated on the cell membrane.

Recently the idea that the protein product of the *dnaA* gene is controlling the time of initiation has found widespread acceptance (54). This model assumes that DnaA monomers gradually accumulate at the chromosomal origin of replication (*oriC*). Once a certain number of DnaA molecules per *oriC* has accumulated, initiation occurs. The ensuing replication of the origin then reduces the DnaA/origin ratio by 50%, so that a new cycle of accumulation begins. Since DnaA is required for initiation, this implies a positive control mechanism.

The following observations are difficult to reconcile with this model. (i) If DnaA were the positive factor driving the oscillator, continued overexpression of DnaA should lead to continued overinitiation, i.e., runaway replication. Excess DnaA does indeed stimulate initiation, but only temporarily and to a very limited extent, leading to a 20% reduction in the initiation mass (which is the cell mass which must accumulate for initiation of replication to occur at a single origin) (54). (ii) The initiation mass changes with changes in growth rate and temperature (23, 29). According to the DnaA model, these changes should correlate with changes in the amount of DnaA protein. This is not observed (20, 29). (iii) According to the DnaA model, the presence of *oriC* plasmids in the cell should cause initiation to occur at an increased cell mass, since it would require more protein to saturate all origins with DnaA molecules and then trigger initiation. However, this is not observed (83), which implies that *oriC* itself is not part of the mechanism involved in generating cyclic initiation activity. (iv) During continuous overexpression of DnaA, when the initiation mass is reduced, some other factor must become limiting. Since the synchrony of initiation remains perfect under these conditions (54), the other factor apparently provides a backup oscillator as perfect as that assumed to be governed by DnaA. This seems unlikely, and it also poses the new problem of identifying this second oscillator.

The emphasis in this review is on the theoretical and biochemical aspects of potential oscillator mechanisms and the possible role of DnaA in this oscillator. The inconsistencies with the DnaA model make it desirable to reexamine the evidence for the role of DnaA in the control of replication, to evaluate possible explanations for these inconsistencies, and to consider alternatives. Because of the complexity of this problem, we first provide a critical discussion of the pertinent observational and theoretical background. This is not intended to be a comprehensive review of the literature, but focuses on aspects which are directly relevant to arguments about initiation control. We show that positive control models, in contrast to negative control, explain the observed cyclic initiation, and we compare amount- and concentration-driven systems. Finally we discuss other alternatives to the DnaA model to explain the cyclic initiation of chromosome replication.

OBSERVATIONS

Chromosome Replication

Cyclic replication. Meselson and Stahl (58) were the first to observe the strict regularity of bacterial DNA replication: one generation after shifting a culture from heavy to light medium, all DNA was found to have hybrid density. This means that any segment of DNA which replicates at a given moment replicates again after exactly one generation time. It can be shown that such results of density shift experiments are independent of the replication velocity (*C*-period [25]) as long as the replication velocity does not change during the experiment. Furthermore, the results are also independent of the number of replication origins or their distribution on the chromosome and of the degree of synchrony of initiation at multiple origins. The data therefore suggest that time intervals between successive initiations of replication at a single origin do not vary from cell to cell.

The Meselson-Stahl experiment was repeated by Koppes and Nordström (49), who improved the sensitivity of the technique by incorporating radioactive pulse-labeling. They found that the density of the labeled DNA shifted stepwise to the hybrid value after one generation. This unambiguously showed that initiation of chromosome replication occurs in intervals of nearly exactly one mass doubling time. In addition, they replaced the chromosomal *oriC* by the replication origin of plasmid R1 and applied the same pulse-label plus density shift protocol. Instead of assuming hybrid density in a sharp step, the pulse-label now approached hybrid density in a gradual, exponential fashion, such that 37% (1/*e*) was still unreplicated after one generation. This is the expectation for random replication (1/*e* is the zero term

of the Poisson distribution for $m = 1$), meaning that replication is initiated at each plasmid origin with a constant probability per unit time.

Synchrony of replication from *oriC*. As a consequence of overlapping rounds of replication, *Escherichia coli* bacteria growing with doubling times shorter than 60 min have at least two, and up to eight, chromosomal replication origins at the time of initiation of rounds of replication (25). The density shift experiment showed that the time from initiation at one origin to the next initiation at the same origin is invariant (49). Since it would be difficult to envision separate clocks with the same cycle period but different phase shifts for every origin in a given cell, the density shift result makes synchronous initiation at *oriC* likely but does not prove it.

Synchrony of initiation at multiple origins in the same cell was first suggested by observations on age-fractionated cultures obtained by the membrane elution technique (36). However, since replication events are only partly synchronized in such cultures, the observed variability of the initiation age is also affected by the variabilities of division and replication fork movement, making it difficult to assess the degree of synchrony in such experiments.

The most direct measurement of the synchrony of initiations was obtained by inhibiting initiation of replication with the antibiotic rifampin, followed by observation of the amount of DNA in individual cells with the aid of a flow cytometer after all rounds of replication had gone to completion (75). Most cells had two, four, or eight fully replicated chromosomes, whereas cells with three, five, six, or seven chromosomes were rare. Such data indicate that the rifampin-sensitive step in the initiations at multiple origins in one cell occurs within a few minutes, corresponding to a small percentage of the cell cycle.

Protein requirement for initiation of chromosome replication. After inhibition of protein synthesis in a bacterial culture by amino acid starvation or treatment with antibiotics (e.g., chloramphenicol or rifampin), DNA continues to accumulate until the ongoing round of replication is completed (67, 75). A quantitative evaluation of the observed "run-out" kinetics of DNA accumulation is consistent with the assumption that initiations of replication cease essentially immediately after the onset of inhibition, whereas replication forks and termination events continue unimpeded (Fig. 1) (14). Thus, initiation requires the continued accumulation of a stable or an unstable protein or the dilution by growth of a replication inhibitor. Rifampin might, in addition, inhibit synthesis of an RNA primer for replication, or it might inhibit transcription in the vicinity of the origin, which facilitates local unwinding of DNA (transcriptional activation) (3).

After relief from a short period of thymine starvation or from heat inactivation of thermolabile DnaA or DnaC protein, a burst of initiations occurs (rate stimulations) (15, 33, 91), as if the initiations which had been skipped during the inhibitory condition were made up at the time of release from the inhibition. If, as we argue in this review, control of initiation is positive, the rate stimulation suggests that initiation of replication requires the accumulation of a stable protein.

Since initiation ceases as soon as protein synthesis ceases, the required protein must act stoichiometrically, rather than catalytically. The protein could be DnaA or alternatively a protein that is active before the DnaA step, i.e., a control factor of the oscillator, or a protein required after the DnaA step. Despite the importance of this question, it has not been possible, so far, to design an experiment to distinguish among these alternatives.

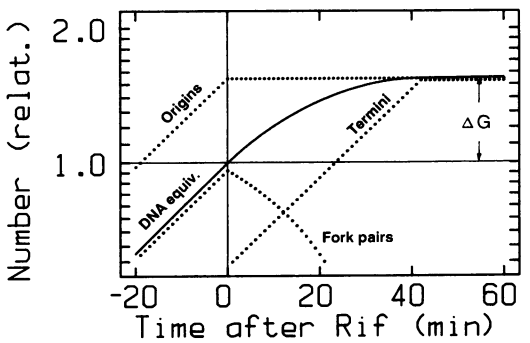

FIG. 1. Chromosome replication in a culture growing exponentially with a 30-min mass doubling time. At zero time, initiation of new rounds of replication is inhibited by rifampin (Rif). Dotted lines: theoretical curves show relative numbers of replication origins and termini per unit of volume of culture, drawn under the assumption that initiation stops instantly and that DNA elongation and termination are not affected. The curve labeled Fork pairs represents the number of replication fork pairs, calculated as the difference origins minus termini, since one fork pair is created at each initiation and disappears at every termination event. The fork curve also represents the rate of DNA synthesis (each fork pair functions at the rate of one genome equivalent per C min; $C = 41$ min [25]). The curve labeled DNA equiv. represents the accumulation of DNA, obtained as the integral over the rate (fork) curve. The ordinate for all curves was normalized so that the amount of DNA at $t = 0$ is one genome equivalent of DNA per unit of volume of culture. The amount of DNA is seen to increase 1.55-fold after the inhibition of initiation. This increase factor (ΔG; see text) is a function of only C/τ and is equal to the ratio origins/genome during exponential growth (origins at zero time since genomes = 1.0 at $t = 0$).

Growth rate dependence of initiation mass. By combining estimates of the average initiation age for *E. coli* B/r (36) with measurements of the DNA/mass ratio from *Salmonella typhimurium* (72), Donachie (26) calculated the average cell mass per *oriC* site at initiation (initiation mass). These estimates suggested that the initiation mass was constant and did not vary with the growth rate.

Direct measurements of the initiation mass were obtained by measuring the relative increase in DNA, ΔG (ratio of DNA after inhibition to DNA before inhibition), following the inhibition of initiation by rifampin (23). The amount of DNA in the culture, measured in genome equivalents, then becomes equal to the number of replication origins present at the time of inhibition, so that ΔG equals the number of origins per genome present during exponential growth (Fig. 1). The initiation mass, or mass per origin, can be found from ΔG:

$$\frac{\text{mass}}{\text{origin}} = \frac{\text{mass/genome}}{\text{origin/genome}} = \frac{\text{OD/DNA}}{\Delta G}$$

OD/DNA is the reciprocal of the amount of DNA, expressed in genome equivalents, per optical density (OD) unit of culture mass, or per amount of total protein, observed during exponential growth. In this manner, the initiation mass was found to increase with increasing growth rate in slowly growing cultures and to reach a plateau at growth rates above one doubling per hour, corresponding to 4×10^8 amino acid residues in cellular protein per *oriC* site (23).

Cell-to-cell variation of initiation mass. The cell-to-cell variation of initiation mass has been measured by pulse-

labeling DNA in exponential cultures with [^{3}H]thymidine, followed by performing autoradiography to determine the labeling rate in individual cells. After electron-microscopic determination of the cell size, the DNA synthesis rate per cell was plotted as a function of cell size (50). The result showed a 16% variability of the initiation mass within the cells of a culture growing with a doubling time of 102 min. Because of the overlapping rounds of replication in fast-growing bacteria, these measurements can be done only with slow-growing bacteria.

Dam methylation requirement for synchronous initiation. The minimal *oriC* region has 11 GATC deoxyadenosine methylation (Dam) sites. The time for hemimethylated DNA, produced by passage of a replication fork, to become fully methylated has been measured directly at a number of different chromosomal sites (19). For both *oriC* and the promoter region of the *dnaA* gene, this time is unusually long: approximately one-third of the doubling time in cells growing faster than one doubling per hour. This is in contrast to other regions, which become fully methylated after 2 to 3 min. If Dam$^-$ strains are transformed with fully methylated *oriC* plasmids, hemimethylated DNA accumulates (69). Hemimethylated, but not unmethylated or fully methylated, *oriC* DNA binds to cell membrane fractions in vitro (62). Most probably, initiation is prevented when the origin is bound to the membrane, so that the temporary hemimethylation produces a refractory period after the initiation of a round of replication during which no new initiations can occur.

Dam$^-$ (null) and Dam-overproducing strains display a loss of synchrony of initiation, as shown by flow cytometry following rifampin treatment (9) and pulse-labeling plus density shift experiments (4). The loss of cyclic initiation might be a consequence of the loss of the refractory period as a result of the absence of hemimethylated DNA. Alternatively, the oscillator mechanism itself might depend on Dam methylation.

Replication after DnaA overproduction. When DnaA protein was expressed from the inducible *lacUV5* promoter on a multicopy plasmid, it was found to restore replication in a thermosensitive *dnaA46* mutant at the nonpermissive temperature, but it did not visibly stimulate chromosome replication in a *dnaA*$^+$ host (24). This suggested that *dnaA* is not normally a limiting factor for initiation of replication and that the timing of initiation is determined in a step preceding DnaA action. However, when extra DnaA was induced from the p_L promoter of phage λ, one extra initiation at *oriC* was observed in a *dnaA*$^+$ strain, again without leading to an increased DNA concentration. The oversupply of DnaA caused a two- to threefold increase in the origin/mass ratio within about 60 min, which then remained approximately constant (2). This suggested that DnaA is a limiting factor for initiation but that the induced extra initiations are aborted close to the origin.

For the experiments involving induction of DnaA from the *lacUV5* promoter, rich (LB) medium was used without a temperature shift, whereas DnaA induction from the λ p_L promoter was brought about by heat inactivation of the temperature-sensitive λ repressor, and glycerol minimal medium was used. It seems that the use of a poor growth medium and an elevated temperature facilitates the induction of extra initiations by excess DnaA (88). A doubling of the whole chromosome number per cell was observed when minimal medium was used and when the induction of excess DnaA synthesis from the *lac* promoter was accompanied by a temperature upshift from 30 to 42°C. When cells growing in glucose minimal medium are treated with low concentrations of rifampin, which only partially inhibit transcription, and are shifted to 42°C, there is a transient stimulation of initiation but no increase in whole chromosome number per cell (32). Under these conditions, extra initiations are observed without induction of excess DnaA protein; they require functional heat shock genes (32).

Pierucci et al. (63, 64) measured the effect of induction of DnaA synthesis from either the λ p_L promoter, using a temperature shift, or the strong *tac* promoter, using isopropyl-β-D-thiogalactopyranoside (IPTG), on chromosome replication. In these experiments, rifampin was added to stop further initiation and the amount of DNA synthesized during the completion of rounds of replication was measured. Their results indicate that in the presence of excess DnaA, in the absence of a temperature shift, a single extra round of replication can initiate in a considerable fraction of the population during prolonged incubation in 100 μg of rifampin per ml. This extra initiation can occur several hours after the addition of rifampin. After a temperature shift, the majority of the cells immediately initiate one extra round of replication, independent of the stage during the cell cycle in which the cells happened to be. Similar results have been reported by Skarstad et al. (76).

During prolonged growth at 42°C, in the presence of different levels of DnaA protein expression, the initiation mass was reduced, but only by 20% (54). Further increases in DnaA protein expression did not lead to further reductions in the initiation mass, implying that some other factor had become limiting for initiation under these conditions. Surprisingly, the reduction in initiation mass was accompanied by a decrease in the replication velocity (increase in the *C*-period) so that the total amount of DNA per cell mass remained constant (54). This suggested that under conditions of continuous overexpression of DnaA protein, prematurely induced initiation events can lead to the synthesis of whole chromosomes but that the replication forks pause somewhere on the chromosome rather than being aborted.

Taken together, these observations indicate that with an unphysiological excess of DnaA a single extra round of chromosome replication can be induced at any (or nearly any) time during the cell cycle. This induction is followed by a period of reduced initiation so that, during continuous overexpression of DnaA, the *oriC*/mass ratio is increased only 20% over the normal value.

Under conditions of continuous overexpression of DnaA, when the initiation mass is about 20% decreased, the synchrony of initiation remains perfect (54). Under such conditions, initiations can no longer be limited by DnaA; i.e., the timing of initiation must be controlled by another factor. Thus, if the oscillator mechanism normally involves DnaA, then, under conditions of continuous oversupply of DnaA, the timing of initiation of replication must be controlled by a backup oscillator that is as precise as the DnaA oscillator. Alternatively, there is only one oscillator in the cell which does not involve DnaA.

In a cold-sensitive *dnaA*(Cs) strain, which carries an intragenic suppressor of a *dnaA46* mutation (13, 47), several rounds of initiation occur upon a temperature downshift from 42 to 30°C (47). Unlike the situation when DnaA protein is overexpressed, these initiations observed with the *dnaA*(Cs) mutant are lethal. Apparently, the mutant protein is capable of causing the initiation of several extra rounds of replication.

Involvement of RNA polymerase and *mioC* in replication initiation. As mentioned, initiation of chromosome replica-

tion stops instantly after inhibition of RNA polymerase by rifampin (14, 75). This suggests that RNA polymerase might be involved in the synthesis of a primer during the initiation of a round of replication. Numerous transcription pause sites which coincide with RNA-DNA junctions have been found within the *oriC* region; most of these transcripts originate at the *mioC* promoter, at a distance of about 0.5 kb from *oriC* in the clockwise direction (45, 48). The direction of transcription from *mioC* in the counterclockwise direction agrees with the predominant direction of the first replication fork formed during replication initiation at *oriC* (90), consistent with the idea that these transcripts are, indeed, primers for replication.

The *mioC* promoter is under stringent control (20, 68) and is repressed by DnaA (79), control features which *mioC* shares with the *dnaA* gene (see below). This might suggest that DnaA also controls synthesis of the replication primer. However, the significance of these observations is obscure since deletion of the *mioC* promoter on the chromosome has no effect on the timing and synchrony of replication initiation (8), and transcription from *mioC* is affected by DnaA only when the gene is on a plasmid (20). Furthermore, when *mioC* is expressed from an inducible promoter on *oriC* plasmids, its transcription frequency shows no consistent relationship to the *oriC* plasmid copy number (see below).

DnaA-dependent in vitro replication of *oriC* plasmids can be primed by either DnaG primase or RNA polymerase or both (61). Therefore, it is not clear whether chromosome replication is primed by RNA polymerase. It has also been observed that the partial unwinding of the *oriC* DNA during initiation is facilitated by transcription in the vicinity of *oriC*, a phenomenon which has been described as transcriptional activation (3). This suggests another possibility for the involvement of RNA polymerase in the initiation process. It is conceivable that RNA polymerase plays a double role in the initiation of replication, i.e., that initiation requires the coincident occurrence of transcriptional activation near *oriC*, the accumulation of sufficient DnaA molecules at *oriC*, and the pausing of RNA polymerase in the *oriC* region from a transcript originated either at *mioC* or further away, at the *asnC* promoter.

Replication of *oriC* Plasmids

Structure and function of *oriC*. The origin of replication of the *E. coli* chromosome was localized originally by measurements of gene frequency (7, 56) and later identified by determining the smallest restriction fragment which, in combination with an antibiotic resistance marker, allows replication as an *oriC* plasmid or minichromosome (60, 89). In this manner, a 245-bp segment of the bacterial chromosome was defined as a minimal *oriC* region. This segment includes four 9-bp DnaA protein-binding sites, one of three 13-bp A+T-rich sites at which the local unwinding of DNA begins at initiation, 11 GATC Dam sites, two adjacent promoters pointing in opposite directions, and two membrane protein-binding sites. There are no primosome assembly sites in the *oriC* or adjacent regions.

In vitro replication of *oriC* plasmids. In vitro replication studies of *oriC* plasmids suggested the following steps in the initiation process (11). First, DnaA-ATP complexes bind to the four DnaA-binding sites. Then further DnaA-ATP complexes bind to the origin DNA, presumably involving both protein-protein and protein-DNA interactions, until 20 to 40 such DnaA-ATP complexes have bound. At the same time the *oriC* DNA wraps around the cluster of DnaA proteins. This causes the A+T-rich 13-mers, one after the other, to unwind and thereby allows two DnaB helicases and two DnaC molecules to enter the helix (84, 85). These events also require the proteins HU, SSB, and DNA gyrase. Priming by either DnaG primase or RNA polymerase can then occur (61). The replication fork moving counterclockwise on the chromosome (direction defined on the conventional genetic map) forms first (90).

In vivo replication of *oriC* plasmids. (i) Synchrony. *oriC* plasmids replicate in synchrony with the chromosomal *oriC* (52), suggesting that *oriC* plasmids are models for studying the control of chromosome replication. An important question to answer would be whether the synchrony of *oriC* plasmid replication persists in the absence of cyclic chromosome replication, i.e., when the chromosome replicates under the control of a plasmid origin. If the oscillator is reset following initiation of replication of the chromosome, then *oriC* plasmids should not be able to replicate in a cyclic manner on their own. However, if the idea is correct that the accumulation of DnaA at *oriC* (including *oriC* plasmids) triggers initiation, or if the oscillator is not located on the DNA, the replication of *oriC* plasmids should be cyclic and independent of chromosome replication.

(ii) Copy number. The copy number of *oriC* plasmids is 8 to 10 per chromosomal *oriC* (53). Since fast-growing bacteria have up to eight chromosomal origins, there may be up to 80 *oriC* plasmids per cell. This number represents an average for an average cell between two divisions. Owing to the cell-to-cell variation in the copy number, the maximum number of *oriC* plasmids per cell is estimated to be around 200. If the *oriC* sequence is not involved in generating the cyclic activity, a *trans*-acting cyclic signal from the oscillator must not be limiting since it can serve such a large number of *oriC* plasmids in addition to the chromosomal *oriC* sites.

(iii) Compatibility. The presence of *oriC* plasmids in a cell has no effect on the cell mass per chromosomal *oriC* (82, 83). Models of replication control that involve the origin as part of the oscillator predict that the presence of *oriC* plasmids should affect the cell mass at initiation, no matter whether the model is based on positive or negative control factors (see below). Thus, a 10-fold increase in the number of *oriC* sites as a result of *oriC* plasmids should lead to a 10-fold increase in the cell mass. Since this is not observed, it seems that only the chromosomal *oriC* concentration is controlled. This suggests that *oriC* plasmids do not carry the oscillator.

(iv) Copy number control and partitioning. A few generations after transformation with *oriC* plasmid DNA, most transformed bacteria contain one plasmid, about 10% of the cells carry two, and 1% carry three. During further growth under selective conditions, the average copy number per cell gradually increases over hundreds of generations, so that at any time all copy numbers (per cell) occur with equal frequency up to a maximum number. This maximum number increases with increasing number of generations, presumably as a result of random partitioning of plasmids during cell division and loss of plasmid-free cells in selective medium. These results indicate that neither the copy number nor the segregation at division of *oriC* plasmids is controlled (44).

(v) Effect of DnaA overexpression. Overexpression of DnaA by pulse induction from the *tac* promoter on a multicopy plasmid has been shown to produce an immediate increase in the rate of *oriC* plasmid replication at any time during the cell cycle, resulting in the induction of one extra round of replication in all cells (64). After the induction, there was a period of severalfold-reduced replication rate. During continuous induction, the rate of *oriC* plasmid repli-

cation increased again at about the time during the cell cycle when replication would also have occurred in the uninduced control culture. The results are consistent with the idea that all *oriC* plasmids have already undergone some preparation for the following round of replication which allows them to prematurely initiate that round in the presence of an excess of DnaA.

(vi) Effect of *mioC* transcription. Immediately adjacent in the clockwise direction from the minimal *oriC* region lies an open reading frame for a 16-kDa protein. Transcription of this gene (*mioC*) can be reduced to 6% of its normal value with no effect on the copy number, but further reduction reduces the copy number (53). Also, disruption of the coding sequence has no effect, but substitution of a stronger or weaker promoter of this gene on an *oriC* plasmid or insertion of a transcription terminator between the promoter and *oriC* reduces its copy number by 50 to 70% (10, 79, 80). The gene has therefore been named *mioC* (modulation of initiation from *oriC*). The significance of these observations is obscure since there seems to be no consistent correlation between *mioC* transcription and *oriC* plasmid copy number.

Control of DnaA Synthesis

Effector-binding sites. The 52-kDa protein product of the *dnaA* gene has two effector-binding sites, one for ATP or ADP and the other for cyclic AMP (38, 73). The DnaA-ATP complex is a double-stranded DNA-binding protein that binds to specific 9-bp target sequences in the *oriC* region (30), near the *mioC* (79) and *dnaA* (12) promoters, and near the replication origins of many plasmids. The DnaA-ATP complex is the form that is active in initiation, although the ADP-bound protein can also bind DNA (73). ADP is released from DnaA by either cyclic AMP (38) or cardiolipin (74), which thereby allows reactivation of DnaA. The percentages of total DnaA protein in the cell complexed with these nucleotides and of free and bound DnaA, respectively, are not known.

Autoregulation. The *dnaA* gene has two promoters, p_1 and p_2, with a DnaA-binding site located between them (35). In vivo and in vitro, over 90% of *dnaA* transcription originates from p_2 (21, 51, 65). Excess DnaA blocks transcription from both promoters in vivo and in vitro, indicating that the *dnaA* gene is autoregulated (1, 12, 51, 86).

Studies of whether autoregulation of DnaA plays a role at physiological DnaA concentrations have given seemingly contradictory results. (i) β-Galactosidase (β-Gal) synthesis expressed from the *dnaA* promoter increased fivefold after heat inactivation of mutant thermolabile DnaA46 protein (1, 12). This suggested that, under normal physiological conditions, the *dnaA* gene is about 80% repressed by its own protein product. (ii) Removal of the DnaA-binding site between the two *dnaA* promoters abolishes repressibility by excess DnaA of either tetracycline resistance or β-Gal expressed from *dnaA* p_2, as expected. However, in the absence of excess DnaA, tetracycline resistance or β-Gal expressed from *dnaA* p_2 did not increase (1, 12). If the *dnaA* gene were 80% repressed by DnaA at physiological concentrations, removal of the DnaA-binding site should produce a fivefold stimulation of expression from the *dnaA* promoter, which was not observed.

The following observation was equally puzzling (1). Although heat inactivation of DnaA caused an immediate cessation of initiation of replication, the increase in β-Gal synthesis expressed from the *dnaA* promoter after a shift to the nonpermissive temperature occurred only gradually over a period of 4 to 5 h. This slow increase in β-Gal specific activity suggested a slowly decreasing autorepression, in contrast to the fast inactivation of initiation activity.

β-Gal activity is measured per total protein synthesis, and therefore it is determined by the amount of β-Gal mRNA per total (bulk) mRNA. Thus, the relative rate of β-Gal synthesis depends as much on bulk mRNA synthesis as on the synthesis of specific β-Gal mRNA. After a shift to the nonpermissive temperature of a *dnaA*(Ts) strain, the rate of culture growth (mass increase) gradually declines over a 4- to 5-h period, as the decreasing DNA concentration becomes limiting for RNA synthesis. Owing to the control of ribosome synthesis, one might expect that the relative rate of stable RNA synthesis would gradually increase after the temperature shift relative to the rate of mRNA synthesis because the culture grows under conditions of an internal nutritional shift-up (70, 71). Therefore, a gradual increase in the rate of p_{dnaA}-directed β-Gal synthesis after a temperature shift-up of a thermosensitive *dnaA* strain would be expected as a result of gradual changes in the global cell physiology under these conditions. This would explain the slow response of *dnaA* gene expression after heat inactivation of *dnaA* and, in addition, the apparent contradiction between the temperature shift result and the absence of an effect upon removal of the DnaA-binding site. In agreement with this interpretation, there is no increase in *dnaA* gene expression when a *dnaA46* temperature-sensitive strain is shifted to 42°C if the chromosome is replicated by an integrated R1 plasmid or P2 prophage (65). Under these conditions, DNA replication continues at the high temperature, and so RNA synthesis does not become limited by a decreasing DNA concentration.

We suggest that the physiological concentration of DnaA protein in the cell is too low to produce a significant repression or autoregulation of the *dnaA* gene. The transcription of the *dnaA* gene is repressed only at an artificially increased concentration of DnaA as a result of overexpression from an inducible promoter or from the normal promoter on a multicopy plasmid. This conclusion is supported by a recent analysis of the effects of point mutations within the DnaA-binding site between the two *dnaA* promoters (65), which shows that although the minor p_1 promoter can be repressed by DnaA protein, the major p_2 promoter is not. Mutations in the DnaA-binding site actually reduce expression from P_2. When both promoters are present and active, mutations in the DnaA-binding site have no effect on the levels of transcription and repression of *dnaA* transcription is observed only when excess DnaA protein is present.

Growth rate and stringent control. The percentage of total protein that is DnaA protein has been determined by immunoblotting; it increased almost in direct proportion to the growth rate, from 0.009% or 100 DnaA molecules per *oriC* at a growth rate of 0.5 doubling per h to 0.035% or 400 DnaA molecules per *oriC* at 2.5 doublings per h (20). Thus, there is no indication of a constant level brought about by autoregulation of the *dnaA* gene.

At the transcriptional level, the amount of mRNA from both *dnaA* promoters per total RNA increases in direct proportion to the growth rate (21, 65). Since total RNA is 98% rRNA and tRNA (17), this implies that *dnaA* mRNA is synthesized as a constant fraction of the stable RNA synthesis rate and that *dnaA* mRNA and rRNA synthesis are coregulated. Since stable RNA synthesis is under stringent control, mediated by the effector nucleotide ppGpp (5), one might expect that *dnaA* transcription is also under stringent control. In agreement with this expectation, it was found

that *dnaA* mRNA synthesis was inhibited during amino acid starvation of a *relA*$^+$ strain but stimulated in a *relA* strain (21).

Temperature control. The amount of DnaA protein as a fraction of total protein has been measured at different temperatures from 30 to 42°C (9). There is a sixfold decrease in the amount of DnaA protein over this range of temperatures. Over the same range of temperatures the initiation mass has been found to be approximately constant, although it increases approximately twofold between 23 and 30°C (29). In a *dnaA46* mutant there is a continuous increase in initiation mass between 23 and 35°C (29).

THEORY OF REPLICATION CONTROL

Definition and Significance of Terms

Replicon, replicator, and initiator. In analogy to the operon as a unit of transcription that is controlled at an operator site close to the transcription start, Jacob et al. (41) defined the replicon as a unit of DNA replication which is controlled at a replicator site near the replication start. These authors suggested that, in contrast to the negative control of transcription by a repressor, DNA replication might be controlled positively by an initiator. The initiator gene could be anywhere on the chromosome in the same way that a repressor gene does not have to be located close to the operator. The activity of the initiator was assumed to undergo cyclic changes, perhaps brought about by reactions occurring on the cell membrane. It was proposed that the DNA molecules of different replicons attach to the cell membrane near their origin, or replicator, and that upon initiation of replication, the membrane would grow in an equatorial zone between the replicated origins, thereby pulling the daughter replicons apart. It is not clear whether this idea is correct, since the mechanism of chromosome segregation is not yet known. It is also not known whether chromosome segregation is involved in the control of replication initiation.

Jacob et al. (41) left open the question of whether the synthesis, or only the activity, of the initiator is controlled and whether this control involves a special effector. Since they did not suggest a mechanism for the oscillator, the replicon hypothesis is not a model of replication control, although it is often referred to as such.

Oscillator. The cellular mechanism which generates cyclic initiation activity is provided by a structure that has yet to be defined biochemically and that is called here the oscillator. All models of replication control which have been proposed assume that this structure has multiple binding sites for a controlling factor which may act positively or negatively. Thus, the term oscillator is analogous to replicator or operator, which are also binding sites for control factors. The oscillator may or may not be located on the chromosome at *oriC*. Wherever it is located, it must produce a cyclic signal acting at *oriC*. The oscillator, together with the output apparatus producing the initiation signal, will be termed the oscillator system. The terms replicator (41) and oscillator differ as follows: the replicator is, by definition, located at the chromosome origin, whereas the oscillator may be located elsewhere; the replicator receives the cyclic signal, whereas the oscillator generates it.

To generate cyclic replication, an oscillator must (i) sense the growth of the cell so that it knows when to fire in intervals of one mass doubling time; (ii) generate an output signal that leads to initiation; and (iii) sense when initiation of replication has occurred so that it can be reset for repetition of the cycle.

The sensor for the growth of the cell could be a set of multiple binding sites for a specific control protein that is constitutively synthesized. For example, in the DnaA model of initiation control, the sensors for growth are the DnaA-binding sites at *oriC*. As the cell grows, more and more DnaA molecules accumulate at or around these binding sites to trigger initiation of replication. This hypothetical set of binding sites for a specific control protein that generates periodic initiations of replication is the essential feature of the oscillator. The control factor that binds to the oscillator could be either a positive factor that accumulates at these sites as the cell grows or a repressor whose concentration is diluted by growth. For a negative factor, one has to assume cooperative binding of the control factor molecules, which generates the all-or-none response of the oscillator as a result of the gradual growth process. If the oscillator is on the DNA, it might be a special kind of operator that generates the cyclic activity of the initiator gene. Since, as discussed below, the number of factor-binding sites must be on the order of 100 to produce the observed regularity of consecutive initiation events, and since an operator with 100 cooperatively acting binding sites requires a new, as yet undefined mechanism to function in a cyclic fashion, the notion that the oscillator is a special kind of operator seems unlikely. A more plausible idea, suggested by Hansen et al. (34), is that the oscillator is a titration mechanism which binds and sequesters the control factor, thus preventing initiation until the control factor accumulates to a sufficiently high concentration. In this case, the oscillator need not be confined to a single site but could consist of many sites dispersed along the DNA (34).

If the oscillator involves *oriC*, the factor that binds to the oscillator might also be called an initiator, as it fits the original definition as a positive factor controlling initiation at a site near the replication origin (41). The output of the oscillator could then be a change in DNA conformation that facilitates either DnaA binding or a step following DnaA binding. If the oscillator is not part of *oriC*, the cyclic output of the oscillator would be the initiator, in accordance with the definition of Jacob et al. (41). The initiator could be RNA, protein, or something else, such as a specific DNA structure, and it could function either as an activator or as a factor that removes an initiation repressor.

The sensor for the occurrence of initiation could be a binding site for a feedback signal generated by the initiation of replication, but it would be simpler if the oscillator structure sensed the initiation event through its own duplication. Structures duplicating after initiation would be *oriC*, a site on the DNA close to *oriC* but not included in *oriC* plasmids, or perhaps a membrane structure. Ward and Glaser (87) inhibited cell division by using a sublethal dose of penicillin and observed the growth rate of the resulting filaments. The rate of length increase doubled stepwise at about the time of initiation. This suggests a doubling of a membrane structure which might be involved in the oscillator mechanism.

Most replication control models that have been proposed operate so that the oscillator not only produces the cyclic signal for initiation, but also determines the initiation mass or number of *oriC* sites per cell mass. This is because these models assume that the oscillator is part of *oriC*. However, generation of a cyclic initiation signal and control of initiation mass need not be coupled. If the oscillator is not part of *oriC*, as in the initiator titration model described below,

generation of the initiation signal and control of the initiation mass are uncoupled. This raises the question of whether the initiation mass is controlled and, if so, how.

Initiation mass and origin concentration. Donachie (26) defined the initiation mass as the cell mass at the time of initiation of a round of replication, divided by the number of chromosomal *oriC* sites present at that time. It can be shown that the initiation mass is proportional (factor ln 2) to the total cell mass in a unit of volume of nonsynchronous exponential culture divided by the total number of replication origins in that volume of culture (M_0 = mass/origin) (16). In the following, the ratio mass/origin in exponential cultures will also be referred to as the initiation mass.

Sometimes, the cell mass at initiation (without being divided by the number of origins) is incorrectly referred to as initiation mass. Cooper and Helmstetter (25) defined the *C*-period as the time interval between initiation and termination of a round of replication. The reciprocal of *C* is the velocity of the replication forks. The *D*-period is the time between termination of a round of replication and the next cell division, which, in fast-growing bacteria, is approximately 20 min (25). The cell mass at initiation (M_i, i.e., not per origin) increases in a step function with increasing growth rate (16): $M_i = (1/\ln 2) \times M_0 \times 2^n$, where n is the next lower integer of $(C + D)/\tau$; i.e., twofold steps occur at doubling times for which $(C + D)/\tau$ has integer values (26). Assuming that $C + D = 60$ min, these steps occur at $\tau = 60$, 30, and 20 min, corresponding to growth rates of one, two, or three doublings per h, respectively.

The reciprocal of initiation mass, origins/mass, measures the cytoplasmic origin concentration. For plasmids, the origin concentration is equivalent to the plasmid copy number per cell mass. Thus, initiation mass, mass per origin, and origin concentration (origins/mass) all reflect the same parameter. The initiation mass expresses the protein requirement for initiation, i.e., the link between growth and replication initiation. We propose that the relevant parameter is mass/oscillator. If the oscillator doubles on initiation (see above), then mass/oscillator and mass/*oriC* (in the absence of *oriC* plasmids) are equivalent.

If the oscillator is not part of *oriC*, there may be no need for a mechanism to control the initiation mass. As described above, *oriC* plasmids increase in number because of random partitioning at division, combined with the elimination of plasmid-free cells by selection. Since the chromosome cannot be lost, there can be no change in the average chromosome number per cell in a population as long as replication is cyclic and equipartitioning of chromosomes occurs at division. Therefore the chromosome number per cell or cell mass, and thus the mass per chromosomal *oriC* site, must remain constant even in the absence of a specific control of the initiation mass.

There are some indications that the chromosome number, and hence the initiation mass, may not be controlled. If a *dnaA167* mutant is shifted from 42 to 30°C, the amount of DNA per cell is halved (28). Conversely, if a *dnaA46* mutant is shifted from 30 to 42°C with simultaneous overproduction of wild-type DnaA protein, the amount of DNA per cell is doubled (88), apparently producing diploid cells. Such diploidy could be conserved in the absence of a specific control of initiation mass if the two copies replicated in synchrony in a similar way to *oriC* plasmids. If it could be confirmed that the initiation mass is not controlled, this would strongly suggest that the oscillator is not located on the chromosome.

Growth rate invariance of initiation mass. It is sometimes assumed that the significance of the initiation mass lies in its constancy (growth rate invariance). However, the observation that initiation occurs when a certain amount of protein per origin has accumulated is implicit in the cyclic nature of initiation. The particular amount of protein per origin reflects the protein requirement of initiation, not any growth rate invariance of the initiation mass. If the synthesis of an initiation factor were subject to growth rate control, the initiation mass would vary with growth rate, as, in fact, it does at low growth rates. Cyclic initiation control does not depend on the growth rate invariance of the initiation mass.

The growth rate invariance of the initiation mass becomes important when considering a particular potential initiation factor, like DnaA, whose growth rate-dependent synthesis is known. In that case the question arises of whether the observed growth rate dependence of DnaA synthesis can generate the observed growth rate invariance of the initiation mass. For DnaA, this poses a problem.

Initiation age. Initiation age (a_i) is the time between cell division and the following initiation of a round of replication, measured in units of mass doubling time. For example, a_i = 0.5 means that rounds of replication are initiated halfway between two divisions separated by the average division interval. The average initiation age is a function of *C*, *D*, and τ (16): $a_i = n - (C + D)/\tau$, where n is the next higher integer of $(C + D)/\tau$ (25). Changes in *C*, *D*, or τ which alter the initiation age do not affect the initiation mass, and, conversely, changes in the initiation mass do not affect the initiation age (23). Thus, although it may seem counterintuitive, the initiation age is not relevant to the control of replication.

During continuous overexpression of DnaA, initiations were found to occur earlier in the cell cycle, i.e., at a lower initiation age (54). This seems to contradict the conclusion above, that the control of initiation does not affect the initiation age. However, in these experiments, an increased *C*-period was also observed under these conditions (see Chromosome Replication, above). As the formula for the initiation age (above) shows, an increase in *C* causes a decreased a_i. Thus, the altered initiation age during overexpression of DnaA is the result of a longer *C*-period rather than of a lower initiation mass. It is not clear why the *C*-period changed in those experiments. When using a non-temperature-sensitive *dnaA* mutation that produced an increased initiation mass, the *C*-period and initiation age were found to be unaltered (23).

That altered control of replication initiation does not affect the initiation age indicates a coupling between replication and division. If replication from *oriC* begins at a cell mass that is smaller or greater than normal as a result of an altered control of initiation, and if the *C*- and *D*-periods do not change as a result of the altered control of initiation, division is expected to occur accordingly earlier or later, so that the initiation age does not change. This coupling apparently does not occur when the chromosome is replicated from an integrated plasmid origin (6).

Models of Replication Control

Physical oscillators. Physical oscillators serve as intuitively plausible analogies for biochemical oscillators. One example is based on the siphon mechanism: a steady stream of water into a jar causes the water level in the overflow tube in the shape of an inverted U to rise until the level goes over the apex of the tube; then the whole content of the jar is suddenly emptied (i.e., siphoned out), while filling continues. The cycle then repeats. This system has features in

common with a growing cell: the continuous filling would be analogous to the growth of a cell, and the periodic emptying would be analogous to cyclic initiation of replication. The strict correlation between cell growth and cyclic initiation of replication is represented in the siphon model by a similarly strict correlation between a steady rate of filling and the frequency of periodic emptying. The faster the filling (growth), the greater the frequency of emptying (initiation) events. It is also noted that the emptying resets the device so that the cycle can begin anew. The ability to reset is an important property of all oscillator mechanisms and must have its analogy in biochemical oscillators.

An electrical analogy would be a capacitor with a glow discharge lamp in parallel. Charging of the capacitor at a steady rate causes a sudden discharge when the ignition voltage of the lamp is reached. The device produces periodic flashes of light. Here a sawtooth-shaped potential curve is generated by a steady flow of electricity. Again, the oscillation frequency is strictly correlated with a given rate of charging. In the cell, we are looking for some biochemical mechanism that produces similar cyclic events as a result of the continuous growth process.

Biochemical oscillators: positive control. (i) *oriC* model. A model for a positively controlled oscillator is obtained by assuming an initiator (positive factor) that is synthesized constitutively at a rate corresponding to a constant fraction of total protein synthesis and whose targets are multiple binding sites at the replication origin. The initiator accumulates at these sites throughout the cell cycle, and when N such sites have bound an initiator, this triggers initiation. The greater is N, the greater is the synchrony of initiation at multiple origins and the greater is the precision of the cycle period.

One may or may not assume that the initiator is consumed (or inactivated) in the process of initiation; the oscillator would work with either assumption. After initiation, it would always take one generation time to accumulate N initiators per origin, no matter how fast the growth or how much initiation factor is synthesized per total protein.

This model has not been spelled out in such detail in the literature, but it underlies the "$I + C + D$" hypothesis of Helmstetter et al. (37). How this model works is best illustrated in a computer simulation. The simulation generates an exponential increase in the amount of protein and stepwise doublings of origin and cell numbers every τ min (Fig. 2a; see Appendix for details). For $\tau = 25$ min, the number of origins per cell fluctuates between 4 and 8 and initiation occurs 15 min after division (Fig. 2b), in agreement with observed data for $\tau = 25$ min.

When the growth rate is decreased while all other parameters are being held the same, the initiation frequency changes accordingly, i.e., with $1/\tau$. The initiation mass (protein/origin at initiation) remains constant (Fig. 3a), but the cell size (protein/cell) changes (Fig. 3b). Again, these simulated changes agree with observations not only qualitatively but also quantitatively. Thus, the model correctly generates several observed features of the cell cycle.

The steady-state conditions achieved by the simulation are independent of the arbitrary starting conditions. If these were changed, for example by increasing the protein content of the starting cell 10-fold, one would observe more rapid initiations initially until the equilibrium situation seen in Fig. 3 and 4 was reached (Fig. 4b, lower curves). Changing the fractional synthesis of initiation factor, α_i, causes a change in the initiation mass and cell size, but other features of the control remain unchanged. If the assumption is dropped that the initiator is inactivated at initiation, the initiation mass and average cell size would be 50% reduced, but otherwise the cell cycle would be generated in the same manner and with the same precision.

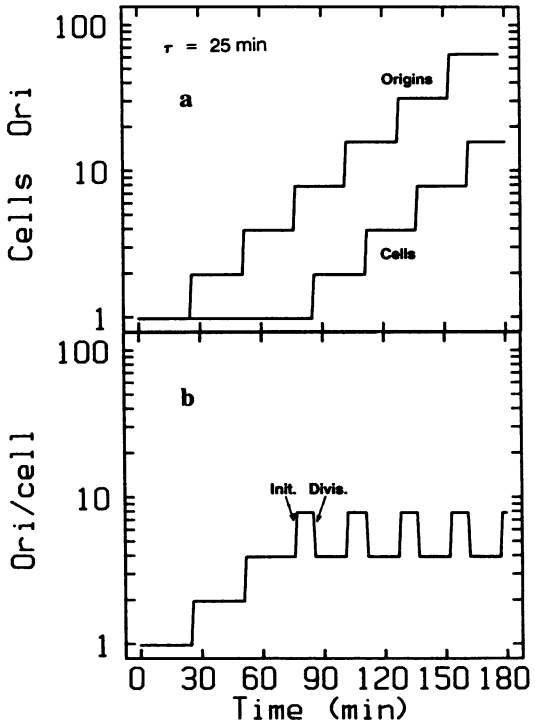

FIG. 2. Computer simulation of chromosome replication and cell division in a culture growing with a 25-min mass doubling time. The simulation was based on the positive control model described in the text. (a) Origin number (per unit of volume of culture) doubles at 25-min intervals (cyclic initiation); cell divisions occur 60 min after initiation (25). (b) Same simulation, but plotting origins per cell (quotient of curves in panel a). Origins/cell alternates between 4 and 8, and initiation occurs 15 min before division, in agreement with observed data (36).

In these simulations, random fluctuations of the number of initiators bound to the origin were not considered; therefore, the synchrony and precision of the cycle period are always perfect in the simulation and are independent of the number of binding sites (N). It would not be difficult to include randomness in both the synthesis of initiator molecules and the binding of initiator to the origins.

(a) DnaA protein as the initiator. The positive *oriC* model has been specifically tailored for DnaA protein as the positive initiation factor (55). Properties of *dnaA* gene expression were taken into account, including autoregulation and stringent control. This model is a special case of the basic model above, with one major addition: a refractory period after initiation was assumed during which the DnaA protein cannot bind to the replicator. Therefore, active initiator (DnaA-ATP) would be liberated as soon as initiation had occurred at one origin. This extra initiator would increase the amount of free initiator and thus cause an avalanche of further initiations at other origins in the same cell, since each origin liberates more active DnaA, which accelerates the process. This feature (which could be applied to any initiator, not only to DnaA) produces a synchrony of initiation in the computer simulation comparable to the observed synchrony.

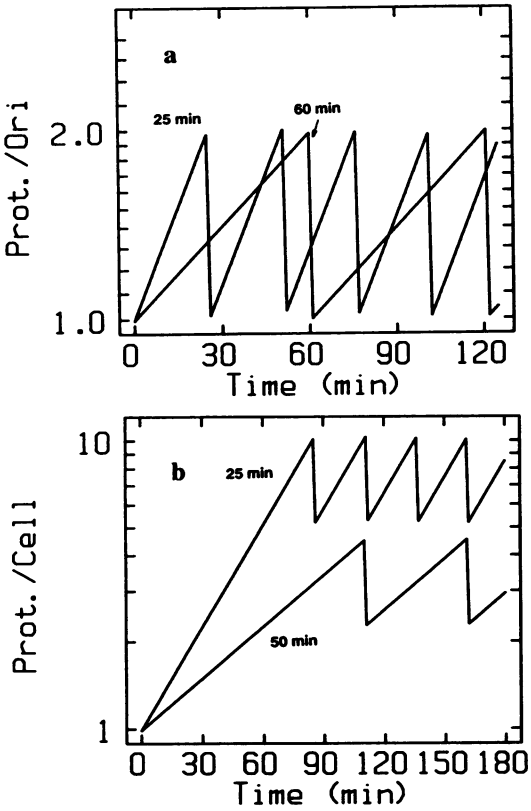

FIG. 3. Computer simulation of chromosome replication and cell division; same simulation as in Fig. 2 (τ = 25 min) and simulation with longer mass doubling times (60 or 50 min; otherwise, the same parameter values). (a) Protein/origin plotted. It is seen that protein/origin at initiation (initiation mass) is independent of the growth rate. (b) Protein/cell plotted. It is seen that fast-growing cells are bigger than slow-growing cells. The simulated relationships agree quantitatively with observations by Helmstetter and Cooper (36) and Schaechter et al. (72).

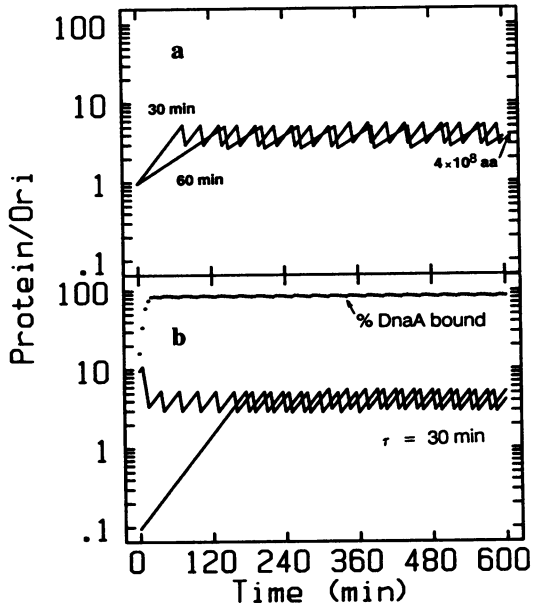

FIG. 4. Computer simulation of chromosome replication, assuming tight binding of DnaA initiator molecules to binding sites near the origin (see the text for details). (a) Initiation mass (protein/origin) is independent of growth rate, on average 4×10^8 amino acid residues in cellular protein per origin. (b) Steady-state initiation mass is reached within 3 h no matter whether one starts with a 10-fold smaller or 100-fold-larger cell. The dotted curve (percentage of initiator bound to origins) shows that about 90% of total initiator present is always bound to origins under the assumed conditions.

For DnaA, α_i is between 0.009 and 0.035%, increasing with the growth rate (20). This corresponds to 100 to 400 DnaA monomers per origin. This is an excess over what is needed, especially at higher growth rates, since only 20 to 40 DnaA monomers are expected to bind at each origin. There may be additional DnaA-binding sites on the DNA outside the origin region, but since the amount of total chromosomal DNA per origin decreases with increasing growth rate, the excess DnaA at higher growth rates cannot be expected to be all absorbed by binding to these other sites on the DNA outside the *oriC* region. It might be thought that autoregulation of DnaA synthesis could reduce the excess of DnaA, but the observed values of DnaA synthesis are the result of all regulation to which DnaA is subject, including autoregulation. The question therefore arises of how a constant initiation mass of the observed magnitude can be achieved with this excess DnaA and with the observed growth rate dependence of DnaA synthesis. Either much of the DnaA must always be free, i.e., if *oriC* binding or activation of DnaA is slow, or there are additional binding sites in the cell, not on DNA, which sequester the excess DnaA. The first possibility was explored by computer simulation; the second possibility is discussed further below.

The basic *oriC* model was modified to include a simulation of the binding of the DnaA initiator to its sites near the origin of replication (see Appendix). First, tight binding is considered. In this case the results should be the same as for the unmodified model (Fig. 2 and 3), except that the simulation now gives absolute values for the initiation mass corresponding to the observed value of 4×10^8 amino acid residues in the protein per origin, independent of the culture doubling time (Fig. 4a). The equilibrium initiation mass is approached within a few hours, no matter whether one starts with too small a cell of 0.1×10^8 amino acid residues in the protein or too large a cell of 10×10^8 amino acid residues in the protein (Fig. 4b).

Assuming a doubling time of 60 min and 30 binding sites per origin, and assuming also that binding to the origin is the rate-limiting step, then 30 DnaA monomers must bind to each origin during one generation, corresponding to a rate of 0.5 monomer per min per origin. At twice the growth rate (τ = 30 min), the binding would have to be twice as fast, i.e., 1 monomer per min per origin. To double the rate of binding, the concentration of free DnaA should double. Thus, excess total DnaA at high growth rates might be necessary to generate the extra free DnaA required to drive the binding reaction at the required speed.

To simulate each concentration-dependent DnaA binding, an initial set of values was chosen so that with τ = 60 min and the observed value of α_i = 0.015%, the correct initiation mass of 4×10^8 amino acid residues per origin was obtained (Fig. 5a, middle solid curve); this results in 17% of the total DnaA bound and 83% free (Fig. 5a, upper dotted curve).

If τ is now decreased to 30 min without changing α_i, the initiation mass (protein/origin) rises (Fig. 5a, upper solid curve) while the fraction of bound initiator declines without

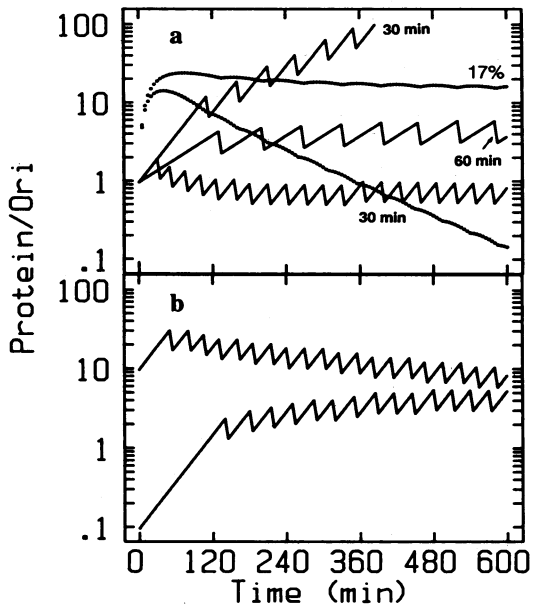

FIG. 5. Computer simulation of chromosome replication assuming reversible binding of initiator to binding sites near the origin; effect of varying τ (60 and 30 min, respectively), and synthesis of initiator protein (fraction, α_i, of total protein [see text for details]). (a) Protein at $t = 0$ set at 1×10^8 amino acid residues. For the solid curves, the ordinate gives the protein/origin; for dotted curves, the ordinate gives the percentage of total initiation factor bound to origins. For the middle solid and lower dotted curve, $\tau = 60$ min and $\alpha_i = 0.015\%$. The average protein/origin ratio (initiation mass) is seen to approach 4×10^8 amino acid residues; 17% of initiator is bound, and 83% is free. In the upper solid and lower dotted curves, changing the mass doubling time to 30 min while leaving other parameters unchanged destabilizes the system; the initiation mass increases (origin concentration decreases), and the fraction of bound initiator decreases without ever reaching a steady state (i.e., replication cannot keep up with growth). In the lower solid curve, the system is restabilized by increasing α_i to 0.035%. (b) $\tau = 30$ min, $\alpha_i = 0.035\%$; same as lower solid curve in panel a, but after changing the cell size in the starting conditions (10×10^8 or 0.1×10^8 amino acid residues in protein). It is seen that under the assumed conditions, it would take more than 10 h to reach steady-state growth, which does not happen in reality.

ever reaching an equilibrium level (Fig. 5a, lower dotted curve). That is, an increase in the growth rate destabilizes the system; replication does not keep up with growth, and DNA is being diluted out. The situation is restabilized by increasing α_i to the observed value for DnaA at $\tau = 30$ min, 0.035%. Now, however, the initiation mass decreases sixfold to a value which is too low (0.7×10^8 amino acids per origin [Fig. 5a, lower solid curve]) and is at variance with the observed constancy of the initiation mass. Furthermore, it takes more than 10 h to reach an equilibrium (Fig. 5b); this does not happen in reality.

These simulations show that, with a concentration-driven system, the concentration, and therefore the fractional synthesis, of initiation factor must increase with growth rate in order to achieve a growth rate invariance of the initiation mass, but the increase must be less than that actually observed for DnaA. Thus, if the system is to work for DnaA, some refinements must be added.

Since only a DnaA-ATP complex binds to its *oriC* binding sites, the pool of free DnaA should be divided into active (ATP-bound) and inactive DnaA. Because it is not known how much active and inactive DnaA is in the cell, how the relative proportions vary with growth rate, and how fast these forms of DnaA are converted into each other, different assumptions about these parameters can be made. Furthermore, the number of additional chromosomal binding sites for DnaA and their binding properties are not known; this gives even more freedom of choice in the parameter values. Therefore, it is always possible to obtain the correct initiation mass, using observed values of α_i for DnaA at a particular growth rate, by assuming a special growth rate dependence of one or more of these additional parameters (55).

(ii) Non-*oriC* model. The observation that *oriC* plasmids are compatible with the chromosome suggests that *oriC* is not an essential part of the oscillator. A model for a positively controlled oscillator located outside *oriC* has been proposed by Hansen et al. (34). In this model, termed the initiator titration model, the oscillator consists of approximately 75 high-affinity DnaA-binding sites dispersed on the DNA but concentrated near *oriC*. During most of the cell cycle, these sites are gradually filled with DnaA protein. Once they are filled, further DnaA synthesis is expected to lead to a rapid rise in the concentration of free DnaA, which then causes binding of DnaA to lower-affinity sites at *oriC*, resulting in initiation. The ensuing chromosome replication creates new high-affinity sites, which reset the oscillator for a new cycle.

Additional features of the model are similar to those described above for the DnaA model. In particular, it is assumed that free, active DnaA molecules are liberated from *oriC* following initiation; this is responsible for the synchrony of initiation at multiple origins. The model also assumes that *oriC* DNA is unavailable for DnaA binding for some time after *oriC* replication. This creates a refractory period that prevents multiple initiations at a single origin.

In this model, DnaA protein has two functions, one as initiator and the other as control factor for the oscillator. This distinguishes this model from the DnaA model discussed above. The model can account for the excess of DnaA protein over what is needed for initiation, and, since the high-affinity sites are not included in *oriC* plasmids, it can qualitatively account for the compatibility of *oriC* plasmids with the chromosome.

Because of its assumption that DnaA is the control factor for the oscillator, there are difficulties with this model. First of all, there is as yet no evidence that the postulated dispersed high-affinity binding sites exist. A second objection is that autoregulation of *dnaA* gene expression might suppress DnaA synthesis when the DnaA concentration rises after the high-affinity sites are filled, just at a time when further DnaA synthesis is required for initiation. However, this objection can be overcome if autoregulation is negligible at physiological concentrations of DnaA (65). Third, the model requires that the synthesis of DnaA protein not vary with growth rate in order to explain the growth rate invariance of the initiation mass. However, according to published results, DnaA synthesis as a fraction of total protein synthesis increases with increasing growth rate (20), which is consistent with the reported stringent control of *dnaA* gene expression (21). Moreover, the small (54) or even undetectable (24, 63) effects of continuous overproduction of DnaA on the initiation mass are at variance with this model. Finally, because of the high copy number (8 to 10 per chromosomal *oriC*) and the large number (20 to 40) of DnaA molecules per *oriC* required for initiation, the presence of

oriC plasmids should still significantly delay or desynchronize initiation, which is not observed (82, 83).

Because of these objections, it seems unlikely that the initiator titration model as postulated is correct. However, the idea of a non-*oriC* oscillator which titrates a control factor deserves serious consideration. If the factor were not DnaA, but some other protein whose synthesis is growth rate invariant and which is required only in small numbers to trigger initiation, such a model could explain all known observations of the control of bacterial chromosome replication. The model would also work if the oscillator were a membrane structure with multiple binding sites, provided that the structure doubled upon initiation.

(iii) Sompayrac and Maaloe version. The expression of a constitutive initiator gene cannot be expected to be entirely constant in time or growth rate invariant, since it is subject to metabolic control after changes in the growth medium and to gene dosage effects after replication of the gene during the cell cycle. Although a constant rate of initiator gene expression is not required to generate cyclic initiations (as long as the changes in gene expression are subject to the same cycle period), Sompayrac and Maaloe (78) proposed a mechanism which might produce an exactly constant rate of gene expression, independent of the growth rate and of gene dosage. The idea was to couple the initiator gene in an operon with a gene encoding an autorepressor for that operon. Whenever the expression of the operon deviated from its equilibrium value, the concentration of the autorepressor would deviate in the same manner to bring about an adjustment toward the controlled equilibrium. As a result, both repressor and initiator would be synthesized at a constant rate. Even after replication of that operon, when the gene output would normally double, the rate of initiator gene expression changed only by 3% in the computer simulation of Sompayrac and Maaloe. The small variation was based on an assumed relationship, relative derepression versus relative concentration of autorepressor, a curve to which the authors refer as a free parameter. A mechanism to generate this curve was not discussed.

It is not known whether a system as proposed by Sompayrac and Maaloe (78) exists, but, more importantly, it could not work as proposed. At the protein level, the expression of any gene depends on the amount of its mRNA relative to total mRNA because ribosomes compete for mRNA. Total mRNA synthesis is subject to growth rate control. Therefore, to keep the concentration of a particular protein constant, the rate of its mRNA synthesis must vary according to the varying rate of bulk mRNA synthesis. Thus, even if the kind of tight autoregulation assumed by Sompayrac and Maaloe could be produced by some mechanism, their system could not keep gene expression constant after changes in the growth medium.

(iv) Relationship between the model of Sompayrac and Maaloe and the DnaA model. Since DnaA is autoregulated, it has been suggested that the proposal of Sompayrac and Maaloe (78) applies to DnaA, i.e., that initiator and autorepressor are the same protein (55). Apart from our conclusions above that DnaA is not normally autoregulated and that the model of Sompayrac and Maaloe could not work as proposed, there is another difficulty with this idea. The positive initiation factor would work best if it bound tightly to its target sites so that the amount of factor accumulated, not its concentration, determined the timing of initiation. This is consistent with observation that inhibition of protein or RNA synthesis immediately inhibits initiation. On the other hand, the autorepressor can work only if its concentration is held constant and in excess over the concentration of binding sites. If the initiator and autorepressor were the same, the binding of initiator to the origin would reduce the repressor concentration and lead to an increasingly higher, rather than constant, rate of initiator synthesis.

It might seem that this difficulty could be overcome by proposing a concentration-dependent binding of initiator to its target sites. In that case a constant concentration of initiator and repressor would be desirable for a given rate of growth. However, then the concentration of initiator would have to increase with increasing growth rate to achieve the faster initiations required during faster growth. Therefore, the model for a constant repressor concentration would not be applied to different growth rates.

Negative control. (i) Inhibitor dilution model. A potential mechanism for negative control, known as the inhibitor dilution model, was proposed by Pritchard et al. (66). A stable initiation repressor was assumed to be made in bursts immediately after initiation of each round of replication. The repressor concentration would thus be maximal immediately after initiation and then drop as a result of further cell growth in the absence of repressor synthesis. The repressor gene was assumed to be near the replication origin, perhaps temporarily activated during its replication. The model assumed that a repressor concentration equal to half the maximum concentration would represent a threshold at which initiation is no longer repressed; then another round of replication would be initiated one generation after the previous one, causing another burst of repressor synthesis, and so forth. The required all-or-none response with only twofold differences in repressor concentration was assumed to be due to cooperative repressor binding: the greater the cooperativity, the greater the precision and synchrony of initiation.

In this model, a change in repressor synthesis, e.g., due to mutation, that led to a change in repressor gene activity would change the initiation mass but not the repressor concentration or initiation frequency. For example, if the amount of repressor synthesized during the burst were reduced, the next initiation would occur earlier when the cells were smaller than normal. The reduced burst at the reduced cell volume would again produce the normal peak concentration of repressor, and it would again take one mass-doubling time to dilute the repressor to the threshold concentration, equal to half the peak concentration.

(ii) Unstable repressor model. The inhibitor dilution model was subsequently modified by proposing an unstable replication repressor, constitutively expressed from a gene close to the replication origin (27). With this modification, there was no need to assume bursts of repressor synthesis immediately after initiation. Since replication causes a doubling of the repressor gene dosage, the concentration of the unstable repressor would double after initiation and thereby prevent further initiations. This model also produces cyclic twofold variations in repressor concentration in a sawtooth-shaped fashion, which must produce the all-or-none response with respect to initiation, again assumed to be due to cooperative repressor binding at the origin.

If, in this model, the condition of multiple binding sites at the origin (including cooperativity) is dropped, i.e., assuming only a single binding site, the result would be random replication. Such a model seems, indeed, to apply to ColE1 plasmid replication, in which an unstable replication repressor exists in the form of a small RNA molecule, called RNAI (81). The ColE1 RNAI does not bind to a replicator at the plasmid replication origin; rather, it binds to the preprimer

transcript, RNAII. The effect on the control and its mathematical description, however, are similar to those of the unstable repressor model (18).

Comparison of positive and negative control. Both the positive- and negative-control models described above generate a cycle frequency that is strictly coupled to the rate of cell growth. Initiations occur at intervals of one mass doubling time independent of the value of the growth rate. The control of synthesis of the control factor, initiator or repressor, affects the initiation mass and thereby the average cell size, but not the cycle period. We do not yet have an answer to the question of whether chromosome replication is controlled negatively or positively.

Pritchard et al. (66) have argued that a positive mechanism would not produce a constant initiation mass. For example, if initiation were occurring abnormally late in a particular cell as a result of fluctuations in the number of initiator molecules bound to the origin, the next initiation in the daughter cell should occur earlier, so that the average cell mass at initiation is kept constant. However, these authors asserted that this adjustment could not be achieved by positive control because of a lack of negative feedback. For that reason, they did not discuss further positive control and considered only the implications of negative control.

A stronger argument against positive control seems to be the following. Assuming that it were possible to introduce another chromosome into a cell, this would double both the origin concentration and the synthesis of a positive initiation factor because of the doubling of the gene dosage for the factor. As a result, initiation factor would accumulate twice as fast, but, owing to the doubling of the binding sites for the factor, it would also be consumed twice as fast, so that initiation would occur at the normal time and cell mass. Since the number of origins has doubled, but the cell mass at initiation is expected to remain normal, the origin/mass ratio would double. Therefore, the initiation mass would not be controlled.

Negative control, however, seems to be capable of controlling the initiation mass: under the same conditions, i.e., after a doubling of the chromosome number, the rate of repressor synthesis would double owing to gene dosage; therefore, the cells would have to grow to twice the normal size to dilute the repressor sufficiently for initiation to occur. Twice the number of origins at twice the cell size at initiation would give the normal origin per mass ratio; i.e., the initiation mass would be kept constant, as observed.

Although intuitively plausible, the above arguments are based on misconceptions. First, positive control of initiation would readily correct fluctuations in the origin/mass ratio because larger cells have more ribosomes and thus produce more initiator for a given amount of mRNA. Second, the gene dosage argument does not hold. During a doubling of the whole chromosome number, the rate of total transcription remains unchanged because transcription is limited by the concentration of RNA polymerase, not by the concentration of DNA (22). The concentrations of RNA polymerase and ribosomes also remain unchanged under these conditions (22). In other words, the doubled number of initiator genes must compete for RNA polymerase with the doubled number of all other genes. Therefore, each gene is transcribed at half the normal rate and so the total synthesis of each RNA species remains unchanged. As a result, initiation factor would accumulate at the normal rate in the case of positive control, but since it has to serve twice the number of origins, it would take twice the normal mass accumulation to get initiation. Twice the normal mass at twice the number of origins at initiation gives a constant initiation mass. Thus, positive control does produce a constant initiation mass.

If the notion of unchanged total transcription under conditions of excess DNA is applied to negative control, it is seen that negative control would not keep the initiation mass constant since the repressor synthesis would not be changed at a doubling of the chromosome number, contrary to the assumptions above which led Pritchard et al. (66) to accept only negative control.

In summary, positive but not negative control is suited to maintaining a constant initiation mass. Interestingly, negative control works for plasmids since plasmid DNA constitutes only a minor fraction of total cellular DNA. Therefore, a doubling of the plasmid copy number without simultaneous doubling of chromosomes would lead to a doubling of the synthesis of plasmid-encoded replication repressor and thus reduce the plasmid copy number again to the controlled value.

Other models. (i) More than one limiting factor. Every conceivable model of replication control can be classified as either positive or negative control. However, the actual mechanism of control can be more complex than was envisioned in the models described above. For positive initiation control as described above, the initiation factor was also the limiting factor; i.e., any change in the rate of factor synthesis was expected to cause a corresponding change in the rate of initiation. Conversely, if a limiting factor has been experimentally identified, such as DnaA, it might seem justified to conclude that this limiting factor must be the controlling factor of the oscillator. This, however, is not necessarily the case since more than one factor might be limiting. For example, the timing of initiation might be determined by a reaction preceding the DnaA step. If the DnaA step follows and requires a length of time that is not negligible in comparison with the generation time, the concentration of active DnaA could be limiting even if it is not involved in the oscillator. Therefore, a mutational defect in DnaA, e.g., a *dnaA46* strain grown at the permissive temperature, could lead to delayed and randomized initiation (77), despite a precise clock mechanism, i.e., if the mutant DnaA takes a longer than normal and variable time to form a functional DnaA cluster at the origin prior to initiation (39).

(ii) Removal of a repressor. Another possibility would be that the formation of a functional DnaA cluster at the origin is prevented most of the time by a repressor that blocks the DnaA-binding sites. An oscillator which is based on positive control as described above, but which lies outside the origin and does not involve DnaA, might then generate in cyclic bursts a positive factor capable of removing the repressor. Thus, initiation would be controlled negatively, but the positively controlled oscillator generates a positive factor which removes the repressor. Two possibilities exist for such a repressor. First, membrane proteins which protect specific regions at *oriC* have been described (42, 43). Second, a soluble protein has been characterized which binds to the 13-mer repeats within *oriC* and inhibits replication in vitro (40). However, overproduction of this protein, or inactivation of its gene, has only a minor effect on DNA replication during exponential growth (8), and so its significance remains questionable.

Concentration- versus amount-driven systems. If the control factor for the oscillator binds tightly to its multiple target sites, only the amount of factor synthesized determines how fast the sites are filled and when initiation occurs. However, if the binding is slow or highly reversible, the concentration, rather than the amount, of factor determines the rate of

filling of the target sites. The positive control described above was amount driven, whereas the negative control was concentration driven. Negative control must be concentration driven since, at a certain lower threshold concentration, all repressor should come off its binding sites. Positively controlled oscillators could also be concentration driven.

If binding of the positive factor to the target sites were slow, the concentration of factor must adjust to the rate of growth. This affects the conditions required to generate the observed growth rate invariance of the initiation mass: for the amount-driven system, synthesis of factor per total protein should be constant, whereas for the concentration-driven system, synthesis of factor per total protein should increase in proportion to the growth rate.

ALTERNATIVE TO CONTROL BY DnaA

The assembly of about 30 DnaA proteins at the origin as a first step for initiation seems to be ideally suited to producing a positively controlled oscillator. No other known feature of the origin suggests itself as part of a mechanism for cyclic control. Although this seems to argue for the correctness of the DnaA model, implications of this idea are at variance with other observations and make it necessary to consider alternative models, despite the absence of experimental support for such alternatives. In our view, the most serious objection to all DnaA models is the observation that initiation continues with perfect synchrony in the presence of excess DnaA protein (54), implying that some other limiting factor is providing the oscillator.

The following proposal could explain several observations of DnaA overexpression and *oriC* plasmid replication that are otherwise unexplained. It is not a model for an oscillator and has mainly heuristic value to stimulate further experiments and discussion.

The compatibility of *oriC* plasmids with the chromosome and the lack of copy number control of *oriC* plasmids suggest that *oriC* is not directly involved in the oscillator structure. In this case DnaA could not be the initiator as defined here, and the output of the oscillator would not be periodic initiation but periodic generation of an unstable initiation activator. This idea removes the difficulty with the excess of DnaA over what seems to be needed for initiation and its growth rate control despite the constancy of the initiation mass; it would further explain the synchrony of initiation, the observation that *oriC* plasmids do not affect the cell mass at initiation, the lack of copy number control of *oriC* plasmids, and the observation that during continued oversupply of DnaA, initiations occur with normal synchrony at only a slightly reduced initiation mass. These observations are difficult to reconcile with DnaA models.

The second important observation that led to the following proposal is the temporary effect of DnaA oversupply on chromosome and *oriC* plasmid replication: excess DnaA stimulates only a single round of replication (64, 76). This means that after a single stimulated round of replication, extra DnaA can no longer produce another initiation. Thus, either a repressor inhibits further replication or a positive factor that was present before the induction of extra DnaA is no longer active. Since it is difficult to see why a repressor present after replication should be absent before the induction of extra DnaA, a positive factor seems more likely. It is therefore proposed that all *oriC* sequences are provided with a factor required for the next initiation every time a round of replication is initiated. This factor facilitates initiation in the presence of an unphysiological excess of DnaA and can be used only once. This idea implies that the stimulation of an extra round of replication by excess DnaA should be followed by a period of reduced initiations since the stimulation only causes initiations to occur earlier than scheduled. Such a period of reduced initiations after stimulation of extra initiations has, indeed, been observed (64).

If a positive factor assists in initiation induced by an excess of DnaA, the question arises of how initiation starts at physiological concentrations of DnaA. We propose that the factor must be further activated by another factor that reduces the threshold concentration of DnaA required for initiation. This other (unstable) factor is also provided only once during the cell cycle and either is made in excess over the number of origins or acts catalytically, so that it can produce synchronous initiation at a large number of origins (which may be as many as 200 per cell).

Together, these considerations have led to the following proposal. (i) At physiological concentrations of DnaA, the binding and function of DnaA at the origins require two additional activating factors, A and B, which are unstable and are provided only once during a short period of the cell cycle by an oscillator system that does not involve *oriC* or DnaA and that has yet to be identified. The activator A might remove a repressor for initiation bound at *oriC* (40). B might be an effector that activates A. (ii) Factor A stays with the origin after initiation in preparation for the next round of initiations. In the presence of A alone, an excess of DnaA can cause initiation of a single round of replication without activator B, especially under conditions of reduced protein synthesis and elevated temperature. Further initiations require new factor A, generated only once during the cell cycle. (iii) The oscillator system that periodically generates factors A and B requires the continued synthesis of some protein. It could involve sites on the chromosome downstream from the origin which are not included in *oriC* plasmids, or it might be located on a cell membrane structure that doubles at initiation. (iv) A refractory period after initiation prevents multiple initiations until unused factors A and B have decayed. (v) The time required for the DnaA step following the activation by factors A and B is not negligible at physiological DnaA concentrations and especially not for temperature-sensitive *dnaA* mutants grown at the permissive temperature. This causes the loss of synchrony of initiations and increased initiation mass in such mutants.

In making such a proposal, we must also explain why hypothetical factors such as A and B have not been identified despite so much effort expended on the study of replication initiation. Our understanding of the control of initiation of chromosome replication is based on the identification of factors by genetic approaches. There is every reason to believe that mutations which specifically affect the control of initiation may not have easily detectable phenotypes. Cells grow when the normal replication control is completely removed, i.e., when replication is initiated at an integrated plasmid origin (49). There have been very few attempts to identify mutations which directly affect replication control, for example by screening for mutations which affect cell size, and it seems that there is a good possibility of making progress in this direction as well as in pursuing biochemical studies (40).

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APPENDIX

Computer Simulation of Replication Control

Positive *oriC* model. In the following, the control of replication implied in the model based on a positive factor acting at *oriC* is visualized by computer simulation. For this purpose, one may assume an arbitrary starting condition of one cell (Cells = 1) containing one unit of protein ($P = 1$), one origin of replication (*Ori* = 1), and zero initiator ($I = 0$). Furthermore, protein concentration increases exponentially (doubling time, τ), initiator is synthesized as a constant fraction (α_i) of total protein, and there are N initiator-binding sites at each origin that must be filled for initiation to occur. At initiation, the number of origins doubles (*Ori* = 2 × *Ori*) and all initiator becomes inactivated (value reset to $I = 0$); cell division always occurs 60 min after initiation of replication (25). These assumptions are written as a series of relationships, each corresponding to a step (steps 1 to 5) in a loop that must be recalculated for every differential time increment dt (dt = 1 min), beginning at $t = 0$:

$$dP = P \times \ln 2/\tau \times dt; \quad P = P + dP \tag{1}$$

$$dI = \alpha_i \times dP; \quad I = I + dI \tag{2}$$

$$\text{If } I/Ori = N \text{ then } Ori = 2 \times Ori; \quad I = 0 \tag{3}$$

$$\text{Cells} = Ori_{(t\,-\,60)} \tag{4}$$

$$t = t + dt \tag{5}$$

Step 3 sets the condition for initiation and resets the initiation factor to zero. The stimulation generates an exponential increase in protein and stepwise doublings of origin and cell numbers every τ min (Fig. 2).

Reversible binding of initiator to the target sites. In this case, one must distinguish between free and bound initiator (I_f and I_b) and include the following relationship for the rate of binding in the simulation:

$$dI_b/dt = (k_1 \times [I_f] - k_2) \times Ori$$

where the association constant k_1 is the number of initiator monomers binding to each origin per minute per unit of concentration of I. The unit of concentration will be defined as 1 monomer per 10^8 amino acid residues in total protein. The dissociation constant k_2 is the reciprocal of the average lifetime of the complex. The values of k_1 and k_2 are assumed to be independent of the number of monomers already bound to a given origin, an unrealistic assumption made for simplicity.

The following further relationships must be included:

$I_{tot} = I_f + I_b$ (total factor = free + bound)

$[I_f] = I_f/P$ (concentration of free factor as monomers per total protein)

$I_{tot} = (\alpha_i \times P)/MW_I$ (molecular weight of I [MW_I] as amino acid residues)

DnaA protein as the initiator. To simulate concentration-dependent DnaA binding on the computer, k_2 (dissociation of complex) was kept at 0.2/min. With τ = 60 min and the observed value of α_i = 0.015%, the only parameter left to choose was the binding constant k_1. The correct initiation mass of 4×10^8 amino acid residues per origin was then obtained by setting $k_1 = 0.018$ (Fig. 5a, middle solid curve).

REFERENCES

1. **Atlung, T., E. S. Clausen, and F. G. Hansen.** 1985. Autoregulation of the *dnaA* gene of *Escherichia coli* K12. Mol. Gen. Genet. **200**:442–450.
2. **Atlung, T., A. Løbner Olesen, and F. G. Hansen.** 1987. Overproduction of DnaA protein stimulates initiation of chromosome and minichromosome replication in *Escherichia coli*. Mol. Gen. Genet. **206**:51–59.
3. **Baker, T. A., and A. Kornberg.** 1988. Transcriptional activation of initiation of replication from the *E. coli* chromosomal origin: an RNA-DNA hybrid near *oriC*. Cell **55**:113–123.
4. **Bakker, A., and D. W. Smith.** 1989. Methylation of GATC sites is required for precise timing between rounds of DNA replication in *Escherichia coli*. J. Bacteriol. **171**:5738–5742.
5. **Baracchini, E., and H. Bremer.** 1988. Stringent and growth control of rRNA synthesis in *Escherichia coli* are both mediated by ppGpp. J. Biol. Chem. **263**:2597–2602.
6. **Bernander, R., and K. Nordström.** 1990. Chromosome replication does not trigger cell division in *E. coli*. Cell **60**:365–374.
7. **Bird, R. E., J. Louarn, J. Martuscelli, and L. Caro.** 1972. Origin and sequence of chromosome replication in *Escherichia coli*. J. Mol. Biol. **70**:549–566.
8. **Boye, E.** Personal communication.
9. **Boye, E., and A. Løbner-Olesen.** 1990. The role of *dam* methyltransferase in the control of DNA replication in *E. coli*. Cell **62**:981–989.
10. **Boye, E., A. Løbner-Olesen, and K. Skarstad.** 1988. Timing of chromosomal replication in *Escherichia coli*. Biochim. Biophys. Acta **951**:359–364.
11. **Bramhill, D., and A. Kornberg.** 1988. A model for initiation at origins of DNA replication. Cell **54**:915–918.
12. **Braun, R. E., K. O'Day, and A. Wright.** 1985. Autoregulation of the DNA replication gene *dnaA* in *E. coli* K-12. Cell **40**:159–169.
13. **Braun, R. E., K. O'Day, and A. Wright.** 1987. Cloning and characterization of *dnaA*(Cs), a mutation which leads to over-initiation of DNA replication in *Escherichia coli* K-12. J. Bacteriol. **169**:3898–3903.
14. **Bremer, H., and G. Churchward.** 1977. Deoxyribonucleic acid synthesis after inhibition of initiation of rounds of replication in *Escherichia coli* B/r. J. Bacteriol. **130**:692–697.
15. **Bremer, H., and G. Churchward.** 1985. Initiation of chromosome replication in *Escherichia coli* after induction of *dnaA* gene expression from a *lac* promoter. J. Bacteriol. **164**:922–924.
16. **Bremer, H., G. Churchward, and R. Young.** 1979. Relation between growth and replication in bacteria. J. Theor. Biol. **81**:533–545.
17. **Bremer, H., and P. P. Dennis.** 1987. Modulation of chemical composition and other parameters of the cell by growth rate, p. 1527–1542. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
18. **Bremer, H., and S. Lin Chao.** 1986. Analysis of the physiological control of replication of ColE1-type plasmids. J. Theor. Biol. **123**:453–470.
19. **Campbell, J. L., and N. Kleckner.** 1990. *E. coli oriC* and the *dnaA* gene promoter are sequestered from *dam* methyltransferase following the passage of the chromosomal replication fork. Cell **62**:967–979.
20. **Chiaramello, A. E., and J. W. Zyskind.** 1989. Expression of *Escherichia coli dnaA* and *mioC* genes as a function of growth rate. J. Bacteriol. **171**:4272–4280.
21. **Chiaramello, A. E., and J. W. Zyskind.** 1990. Coupling of DNA replication to growth rate in *Escherichia coli*: a possible role for guanosine tetraphosphate. J. Bacteriol. **172**:2013–2019.
22. **Churchward, G., H. Bremer, and R. Y. Young.** 1982. Transcription in bacteria at different DNA concentrations. J. Bacteriol. **150**:572–581.
23. **Churchward, G., E. Estiva, and H. Bremer.** 1981. Growth rate-dependent control of chromosome replication initiation in *Escherichia coli*. J. Bacteriol. **145**:1232–1238.
24. **Churchward, G., P. Holmans, and H. Bremer.** 1983. Increased expression of the *dnaA* gene has no effect on DNA replication in a dnaA+ strain of *Escherichia coli*. Mol. Gen. Genet. **192**:506–508.
25. **Cooper, S., and C. E. Helmstetter.** 1968. Chromosome replication and the division cycle of *Escherichia coli* B/r. J. Mol. Biol. **31**:519–540.
26. **Donachie, W.** 1968. Relation between cell size and time of initiation of DNA replication. Nature (London) **219**:1077–1079.
27. **Fantes, P. A., W. D. Grant, R. H. Pritchard, P. E. Sudbery, and**

A. E. Wheals. 1975. The regulation of cell size and the control of mitosis. J. Theor. Biol. 50:213–244.
28. Fralick, J. A. 1978. Studies on the regulation of initiation of chromosome replication in *Escherichia coli*. J. Mol. Biol. 122: 271–286.
29. Frey, J., M. Chandler, and L. Caro. 1981. The initiation of chromosome replication in a *dnaAts46* and a *dnaA*+ strain at various temperatures. Mol. Gen. Genet. 182:364–366.
30. Fuller, R. S., B. E. Funnell, and A. Kornberg. 1984. The *dnaA* protein complex with the *E. coli* chromosomal replication origin (oriC) and other DNA sites. Cell 38:889–900.
31. Georgopoulos, C. 1989. The *E. coli* DnaA initiation protein: a protein for all seasons. Trends Genet. 5:319–321.
32. Guzman, E. C., A. Jimenez Sanchez, E. Orr, and R. H. Pritchard. 1988. Heat stress in the presence of low RNA polymerase activity increases chromosome copy number of *Escherichia coli*. Mol. Gen. Genet. 212:203–206.
33. Hanna, M. H., and P. L. Carl. 1975. Reinitiation of deoxyribonucleic acid synthesis by deoxyribonucleic acid initiation mutants of *Escherichia coli*: role of ribonucleic acid synthesis, protein synthesis, and cell division. J. Bacteriol. 121:219–226.
34. Hansen, F. G., B. B. Christensen, and T. Atlung. 1991. The initiator titration model: computer simulation of chromosome and minichromosome control. Res. Microbiol. 142:161–167.
35. Hansen, F. G., E. B. Hansen, and T. Atlung. 1982. The nucleotide sequence of the *dnaA* gene promoter and of the adjacent *rpmH* gene, coding for the ribosomal protein L34, of *Escherichia coli*. EMBO J. 1:1043–1048.
36. Helmstetter, C. E., and S. Cooper. 1968. DNA synthesis during the division cycle of rapidly growing *Escherichia coli* B/r. J. Mol. Biol. 31:507–518.
37. Helmstetter, C. E., O. Pierucci, M. Weinberger, M. Holmes, and M.-S. Tang. 1979. Control of cell division in *Escherichia coli*, p. 517–579. *In* J. R. Sokatch and L. N. Ornston (ed.), The bacteria, vol. VII. Academic Press, Inc., New York.
38. Hughes, P., A. Landoulsi, and M. Kohiyama. 1988. A novel role for cAMP in the control of the activity of the *E. coli* chromosome replication initiator protein, DnaA. Cell 55:343–350.
39. Hwang, D. S., and J. M. Kaguni. 1988. Purification and characterization of the *dnaA46* gene product. J. Biol. Chem. 263: 10625–10632.
40. Hwang, D. S., and A. Kornberg. 1990. A novel protein binds a key origin sequence to block replication of an *E. coli* minichromosome. Cell 63:325–331.
41. Jacob, F., S. Brenner, and F. Cuzin. 1963. On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. 28:329–348.
42. Jacq, A., R. Kern, A. Tsugita, and M. Kohiyama. 1989. Purification and characterization of a low-molecular-weight membrane protein with affinity for the *Escherichia coli* origin of replication. J. Bacteriol. 171:1409–1416.
43. Jacq, A., M. Kohiyama, H. Lother, and W. Messer. 1983. Recognition sites for a membrane derived DNA binding protein preparation in the *E. coli* replication origin. Mol. Gen. Genet. 191:460–465.
44. Jensen, M. R., A. Løbner-Olesen, and K. V. Rasmussen. 1990. *Escherichia coli* minichromosomes: random segregation and absence of copy number control. J. Mol. Biol. 215:257–265.
45. Junker, D. E., Jr., L. A. Rokeach, D. Ganea, A. Chiaramello, and J. W. Zyskind. 1986. Transcription termination within the *Escherichia coli* origin of DNA replication, *oriC*. Mol. Gen. Genet. 203:101–109.
46. Keasling, J. D., B. O. Palsson, and S. Cooper. 1991. Cell-cycle-specific F plasmid replication: regulation by cell size control of initiation. J. Bacteriol. 173:2673–2680.
47. Kellenberger-Gujer, G., A. J. Podhajska, and L. Caro. 1978. A cold sensitive *dnaA* mutant of *E. coli* which overinitiates chromosome replication at low temperature. Mol. Gen. Genet. 162:9–16.
48. Kohara, Y., N. Todoh, X. Jiang, and T. Okazaki. 1985. The distribution and properties of RNA primed initiation sites of DNA synthesis at the replication origin of *Escherichia coli* chromosome. Nucleic Acids Res. 13:6847–6866.
49. Koppes, L., and K. Nordström. 1986. Insertion of an R1 plasmid into the origin of replication of the *E coli* chromosome: random timing of replication of the hybrid chromosome. Cell 44:117–124.
50. Koppes, L. J. H., C. L. Woldringh, and N. Nanninga. 1978. Size variations and correlation of different cell cycle events in slow-growing bacteria. J. Bacteriol. 134:423–433.
51. Kucherer, C., H. Lother, R. Kolling, M. A. Schauzu, and W. Messer. 1986. Regulation of transcription of the chromosomal *dnaA* gene of *Escherichia coli*. Mol. Gen. Genet. 205:115–121.
52. Leonard, A. C., and C. E. Helmstetter. 1986. Cell cycle-specific replication of *Escherichia coli* minichromosomes. Proc. Natl. Acad. Sci. USA 83:5101–5105.
53. Løbner-Olesen, A., T. Atlung, and K. V. Rasmussen. 1987. Stability and replication control of *Escherichia coli* minichromosomes. J. Bacteriol. 169:2835–2842.
54. Løbner-Olesen, A., K. Skarstad, F. G. Hansen, K. von Meyenburg, and E. Boye. 1989. The DnaA protein determines the initiation mass of *Escherichia coli* K-12. Cell 57:881–889.
55. Mahaffey, J. M., and J. W. Zyskind. 1989. A model for the initiation of replication in *Escherichia coli*. J. Theor. Biol. 140:453–477.
56. Masters, M., and P. Broda. 1971. Evidence for bidirectional replication of the *Escherichia coli* chromosome. Nature (London) New Biol. 232:137–140.
57. McMacken, R., L. Silver, and C. Georgopoulos. 1987. DNA replication, p. 564–612. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
58. Meselson, M., and F. Stahl. 1958. The replication of DNA in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 44:671–682.
59. Messer, W. 1987. Initiation of DNA replication in *Escherichia coli*. J. Bacteriol. 169:3395–3399.
60. Messer, W., H. E. N. Bergmans, M. Meijer, J. E. Womack, F. G. Hansen, and K. von Meyenberg. 1978. Mini-chromosomes: plasmids which carry the *E. coli* replication origin. Mol. Gen. Genet. 162:269–275.
61. Ogawa, T., T. A. Baker, A. van der Ende, and A. Kornberg. 1985. Initiation of enzymatic replication at the origin of the *Escherichia coli* chromosome: contributions of RNA polymerase and primase. Proc. Natl. Acad. Sci. USA 82:3562–3566.
62. Ogden, G. B., M. J. Pratt, and M. Schaechter. 1988. The replicative origin of the *E. coli* chromosome binds to cell membranes only when hemimethylated. Cell 54:127–135.
63. Pierucci, O., C. E. Helmstetter, M. Rickert, M. Weinberger, and A. C. Leonard. 1987. Overexpression of the *dnaA* gene in *Escherichia coli* B/r: chromosome and minichromosome replication in the presence of rifampin. J. Bacteriol. 169:1871–1877.
64. Pierucci, O., M. Rickert, and C. E. Helmstetter. 1989. DnaA overproduction abolishes cell cycle specificity of DNA replication from *oriC* in *Escherichia coli*. J. Bacteriol. 171:3760–3766.
65. Polaczek, P., and A. Wright. 1990. Regulation of expression of the *dnaA* gene in *Escherichia coli*: role of the two promoters and the DnaA box. New Biol. 2:574–582.
66. Pritchard, R. H., P. T. Barth, and J. Collins. 1969. Control of DNA synthesis in bacteria. Symp. Soc. Gen. Microbiol. 19:263–297.
67. Pritchard, R. H., and A. Zaritsky. 1970. Effect of thymine concentration on the replication velocity of DNA in a thymineless mutant of *Escherichia coli*. Nature (London) 226:126–131.
68. Rokeach, L. A., and J. W. Zyskind. 1986. RNA terminating within the *E. coli* origin of replication: stringent regulation and control by DnaA protein. Cell 46:763–771.
69. Russell, D. W., and N. D. Zinder. 1987. Hemimethylation prevents DNA replication in *Escherichia coli*. Cell 50:1071–1079.
70. Ryals, J., R. Little, and H. Bremer. 1982. Control of RNA synthesis in *Escherichia coli* after a shift to higher temperature. J. Bacteriol. 151:1425–1432.
71. Ryals, J., R. Little, and H. Bremer. 1982. Temperature dependence of RNA synthesis parameters in *Escherichia coli*. J.

Bacteriol. **151**:879–887.
72. **Schaechter, M., O. Maaloe, and N. O. Kjeldgaard.** 1958. Dependency on medium and cell size and chemical composition during balanced growth of *Salmonella typhimurium*. J. Gen. Microbiol. **19**:592–606.
73. **Sekimizu, K., D. Bramhill, and A. Kornberg.** 1987. ATP activates *dnaA* protein in initiating replication of plasmids bearing the origin of the *E. coli* chromosome. Cell **50**:259–265.
74. **Sekimizu, K., and A. Kornberg.** 1988. Cardiolipin activation of *dnaA* protein, the initiation protein of replication in *Escherichia coli*. J. Biol. Chem. **263**:7131–7135.
75. **Skarstad, K., E. Boye, and H. B. Steen.** 1986. Timing of initiation of chromosome replication in individual *Escherichia coli* cells. EMBO J. **5**:1711–1717.
76. **Skarstad, K., A. Løbner-Olesen, T. Atlung, K. von Meyenburg, and E. Boye.** 1989. Initiation of DNA replication in *Escherichia coli* after overproduction of the DnaA protein. Mol. Gen. Genet. **218**:50–56.
77. **Skarstad, K., K. von Meyenburg, F. G. Hansen, and E. Boye.** 1988. Coordination of chromosome replication initiation in *Escherichia coli*: effects of different *dnaA* alleles. J. Bacteriol. **170**:852–858.
78. **Sompayrac, L., and O. Maaloe.** 1973. Autorepressor model for control of DNA replication. Nature (London) New Biol. **241**: 133–135.
79. **Stuitje, A. R., N. de Wind, J. C. van der Spek, T. H. Pors, and M. Meijer.** 1986. Dissection of promoter sequences involved in transcriptional activation of the *Escherichia coli* replication origin. Nucleic Acids Res. **14**:2333–2344.
80. **Tanaka, M., and S. Hiraga.** 1985. Negative control of *oriC* plasmid replication by transcription of the *oriC* region. Mol. Gen. Genet. **200**:21–26.
81. **Tomizawa, J.** 1984. Control of ColE1 plasmid replication: the process of binding of RNA I to the primer transcript. Cell **38**:861–870.
82. **von Meyenburg, K., and F. G. Hansen.** 1987. Regulation of chromosome replication, p. 1555–1577. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
83. **von Meyenburg, K., F. G. Hansen, E. Riise, H. E. Bergmans, M. Meijer, and W. Messer.** 1979. Origin of replication, *oriC*, of the *Escherichia coli* K12 chromosome: genetic mapping and minichromosome replication. Cold Spring Harbor Symp. Quant. Biol. **43**:1-P 121-8.
84. **Wahle, E., R. S. Lasken, and A. Kornberg.** 1989. The *dnaB-dnaC* replication protein complex of *Escherichia coli*. I. Formation and properties. J. Biol. Chem. **264**:2463–2468.
85. **Wahle, E., R. S. Lasken, and A. Kornberg.** 1989. The *dnaB-dnaC* replication protein complex of *Escherichia coli*. II. Role of the complex in mobilizing *dnaB* function. J. Biol. Chem. **264**: 2469–2475.
86. **Wang, Q. P., and J. M. Kaguni.** 1987. Transcriptional repression of the *dnaA* gene of *Escherichia coli* by *dnaA* protein. Mol. Gen. Genet. **209**:518–525.
87. **Ward, C. B., and D. A. Glaser.** 1971. Correlation between rate of cell growth and rate of DNA synthesis in *Escherichia coli* B/r. Proc. Natl. Acad. Sci. USA **68**:1061–1064.
88. **Xu, Y. C., and H. Bremer.** 1988. Chromosome replication in *Escherichia coli* induced by oversupply of DnaA. Mol. Gen. Genet. **211**:138–142.
89. **Yasuda, S., and Y. Hirota.** 1977. Cloning and mapping of the replication origin of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **74**:5458–5462.
90. **Yoshimoto, M., H. Kambe-Honjoh, K. Nagai, and G. Tamura.** 1986. Early replicative intermediates of *E. coli* chromosome isolated from a membrane complex. EMBO J. **5**:787–791.
91. **Zaritsky, A.** 1975. Rate stimulation of deoxyribonucleic acid synthesis after inhibition. J. Bacteriol. **122**:841–846.
92. **Zyskind, J. W., and D. W. Smith.** 1986. The bacterial origin of replication, *oriC*. Cell **46**:489–490.