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# DnaA Boxes Are Important Elements in Setting the Initiation Mass of *Escherichia coli*

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**The binding of DnaA protein to its DNA binding sites—DnaA boxes—in the chromosomal *oriC* region is essential for initiation of chromosome replication. In this report, we show that additional DnaA boxes affect chromosome initiation control, i.e., increase the initiation mass. The cellular DnaA box concentration was increased by introducing pBR322-derived plasmids carrying DnaA boxes from the *oriC* region into *Escherichia coli* and by growing the strains at different generation times to obtain different plasmid copy numbers. In fast-growing cells, where the DnaA box plasmid copy number per *oriC* locus was low, the presence of extra DnaA boxes caused only a moderate increase in the initiation mass. In slowly growing cells, where the DnaA box plasmid copy number per *oriC* locus was higher, we observed more pronounced increases in the initiation mass. Our data clearly show that the presence of extra DnaA boxes increases the initiation mass, supporting the idea that the initiation mass is determined by the normal complement of DnaA protein binding sites in *E. coli* cells.**

The DnaA protein is an essential factor for initiation of duplication of the bacterial chromosome from a specific site, the origin of replication, *oriC*. The DnaA protein binds to DnaA boxes in *oriC*, and in vitro studies indicate that initiation takes place when sufficient DnaA protein—approximately 20 monomers—has been bound to *oriC* (8). The formation of this so-called initial complex leads to the opening of a region in *oriC* containing AT-rich 13-mers and allows the entry of DnaB and DnaC proteins to form the pre-prepriming complex, which is followed by several other stages, as detailed by Sekimizu et al. (30). The DnaA protein binding sites—the DnaA boxes—have the consensus sequence $TT^{A}_{T}TNCACA$ (29). There are 308 consensus DnaA boxes on the *E. coli* chromosome, three of which are located within the minimal *oriC* locus. Using the DNA binding domain of the DnaA protein to isolate restriction fragments from a digest of total chromosomal DNA, only a handful of fragments could be isolated and characterized as containing high-affinity DnaA protein binding motifs in vitro (27).

Initiation of chromosome replication is a complex process which, besides *oriC* and the DnaA protein, involves a number of accessory factors. In the period between one initiation and the next, *oriC* will undergo a number of structural changes to be prepared for the new initiation. The newly replicated (and hemimethylated) GATC Dam methylation sites in *oriC* facilitate membrane binding (24). In this state, *oriC* is inaccessible for initiation and it is conceivable that the eclipse period could be defined as the period during which *oriC* is sequestered (6). Also, other factors have roles in the initiation of chromosome replication (31). Many details of the initiation process are quite well understood due to the impressive work at Arthur Kornberg's laboratory (17). However, how the bacterial cell senses when initiation is supposed to take place in the cell cycle is still under debate.

Phenomenologically, the time of initiation is coupled to the mass increase such that initiation occurs at a critical mass per origin—the initiation mass (9). The initiation mass, expressed as units of optical density per amount of DNA, appears to be nearly constant at different growth rates (4). Initiation can be thought to occur either when the mass per origin has increased sufficiently or when the number of origins per unit of mass has decreased sufficiently at a given growth rate. These views were reflected in the autorepressor model (33) and the inhibitor dilution model (25), respectively.

It has previously been shown that changing the DnaA protein concentration or activity changes the initiation mass. Overproduction of DnaA protein decreases the initiation mass (3, 20), i.e., increases the origin-to-mass ratio. Conversely, cells, which have lower DnaA protein activity than normal have a higher initiation mass. A lower DnaA protein concentration or activity has been obtained, for example, in *dnaA*(Ts) strains grown at nonpermissive temperatures, at which suboptimal DnaA protein concentrations were achieved by induction of normal DnaA protein from a plasmid system (20). Also, a *dnaA*(Ts) strain which was grown at semipermissive temperatures, at which it was expected that the activity of the DnaA protein would decrease (13), showed increasing initiation masses at increasing growth temperatures. These changes in initiation mass were paralleled by changes in *dnaA* gene expression; i.e., low DnaA protein activity led to derepression of the *dnaA* promoter, and high DnaA protein activity led to repression of the *dnaA* promoter (2, 5).

The initiator titration model (11) combines the views of the two models mentioned above. The essence of this model is that there is a long period of the cell cycle where newly synthesized DnaA proteins are titrated by binding to high-affinity binding sites. When the cell cannot titrate any more DnaA protein, there will be free DnaA protein molecules which can participate in a postulated lower-affinity reaction, namely, making the initiation complex. Thus, the high-affinity binding sites are postulated to be inhibitor elements preventing the DnaA protein from forming the initiation complex. In addition, the ini-

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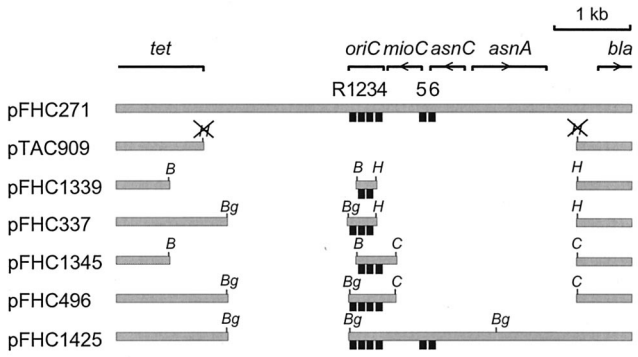


FIG. 1. Plasmids carrying DNA from the *oriC* region of *E. coli*. The relevant genes are indicated above the map of plasmid pFHC271, which is a chimeric plasmid carrying *oriC* and neighboring sequences cloned into the *Hin*dIII site of pBR322; the positions of the *tet* and *bla* genes are shown. Plasmid pTAC909 (2) is identical to pBR322, except that the *Hin*dIII site has been destroyed, rendering the *tet* gene inactive. The remaining plasmids are deletion derivatives of pFHC271 and carry different numbers of DnaA boxes (represented by black boxes below the respective plasmids) from the *oriC* region. The restriction enzyme sites used to make the deletions are indicated at the different junctions: *B*, *Bam*HI; *Bg*, *Bgl*II; *C*, *Cla*I; *H*, *Hin*dIII. The chromosomal *oriC* locus is inactivated on all of the plasmids except pFHC271. Plasmids pFHC496 and pFHC1425 carry all of the DnaA boxes of the minimal *oriC* locus but lack the small *Bgl*II fragment, which carries sequences from the 13-mer region of *oriC*, that is essential for *oriC* function.

tiator titration model postulates that DnaA protein released at the time of initiation from one origin will increase the free-DnaA-to-origin ratio, thereby increasing the probability of initiation of the remaining origins. This will result in the observed synchronous initiation at multiple origins in fast-growing cells.

The experimental basis of the formulation of the initiator titration model was a study in which *dnaA* gene expression was determined in cells carrying additional DnaA boxes (12). In the present study, we have extended this analysis to further characterize the regulatory role of the DnaA boxes in the control of initiation of chromosomal replication. We introduced additional DnaA boxes carried on pBR322-derived plasmids into cells and studied their effect on initiation mass, cell size, and DNA content in balanced bacterial cultures growing at different rates. We have also investigated the effect of mutating one or more of the DnaA boxes in *oriC* with respect to the titrating ability by using the mutations described by Holz et al. (14).

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The *E. coli* BBC119 is an LJ24 derivative (*thi-1 leu-6 lacY1 lacIZ*Δ(*Mlu*I) *supE44 tonA21 rpsL rfbD1*) (26) which has been lysogenized with λRB1 (5) that carries a *dnaA′-′lacZ* fusion. Southern blotting was used to check that the strain was a single lysogen of λRB1 (data not shown). The plasmids used (Fig. 1) are deletion derivatives of plasmid pFHC271 that contain a complete *oriC* region cloned into pBR322. None of the deletion derivatives have a functional *oriC* locus (see reference 12 for details). Plasmids with mutations in the *oriC* DnaA boxes were constructed by exchanging a *Cla*I-*Bgl*II fragment in plasmid pFHC496 with the same fragment from the mutated DnaA box plasmid (14).

**Bacterial growth experiments.** The host strain and its plasmid-containing derivatives were kept in balanced growth for more than 10 mass doublings in A+B medium (7) supplemented with 1% Casamino Acids–0.2% glucose, 0.2% glucose, 0.2% glycerol, or 0.4% succinate. Thiamine was always present at 2 μg/ml, and leucine was present in the minimal medium at 20 μg/ml.

**Flow cytometric procedures.** Samples were prepared and flow cytometry was performed as summarized in reference 32. Average cell mass was determined as average light scatter, and the average amount of DNA per unit of mass was determined as fluorescence per unit of light scatter of samples taken directly from exponentially grown cultures. The average number of origins per cell was determined from parallel samples incubated for more than 3 h with rifampin (300 μg/ml) to block initiation of replication and cephalexin (36 μg/ml) to block cell division (20). This treatment normally results in fully replicated chromosomes, which will be equivalent to the number of origins per cell at the time of drug addition and can be visualized directly by flow cytometry. The percentage of asynchronous cells was calculated as $100*N_{async}/(N_{total} - N_1)$, where $N_{async}$ is the sum of all cells which do not contain $2^n$ origins ($n = 0, 1, 2, 3, 4$, etc.), $N_{total}$ is the total number of analyzed cells, and $N_1$ is the number of cells carrying 1 origin. The number of cells that have one chromosome is subtracted from the total number of cells because we cannot say anything about synchrony of initiation in such cells.

**Enzyme measurements.** Cell extracts prepared by treatment with toluene were used to determine β-galactosidase activity as previously described (23).

**Determination of plasmid copy number per *oriC* locus by Southern blotting.** Total (plasmid and chromosome) DNA was prepared as previously described (10) with the modifications described previously (3). The DNA was restricted with *Eco*RI and *Hin*dIII, and Southern blot analysis was carried out as previously described (3) by using a [$^{35}$S]dATP-labeled probe mixture. Probes for hybridization were prepared by labeling DNA with [$^{35}$S]dATP using DNA polymerase I Klenow fragment and hexanucleotide random priming. We used probes, one made from a PCR-derived fragment of 295 bp from the *tet* gene of pBR322 (from position 723 to position 1017), and another prepared from a PCR-derived fragment of 1,197 bp, which will hybridize to the 2.1-kb *Hin*dIII fragment carrying most of the *gidA* gene from the *oriC* region, to estimate copy numbers per *oriC* locus in Southern hybridization experiments. The Southern blots were quantified by using the Instant Imager (Packard). We included samples of plasmid pFHC271 digested with *Hin*dIII to obtain exact plasmid copy numbers per *oriC* locus by normalizing the plasmid and chromosomal hybridization signals of the total DNA samples from the experiment to the hybridization signals of pFHC271 where the fragments representing the plasmid part and the chromosomal part are present at a 1:1 ratio.

## RESULTS

It was previously shown that the presence of extra DnaA boxes in strains carrying pBR322-derived plasmids with different combinations of the DnaA boxes from the *oriC* region (Fig. 1) would titrate DnaA protein to various degrees, depending on the number, and apparently also the quality, of the DnaA boxes (12). This titration was measured as the derepression of the autoregulated *dnaA* promoter of a *dnaA′-′lacZ* fusion gene positioned at the λ*att* site. In the present study, we have investigated how the introduction of additional DnaA boxes affects initiation of chromosome replication.

**Mutations in DnaA boxes decrease titration and decrease changes in cell size.** The plasmids we used in previous studies were different in structure and contained different parts of chromosomal DNA from the *oriC* region. Therefore, to prove that the effects we observed were a consequence of the presence of the DnaA boxes on the plasmids, we constructed a number of plasmids which had the same structure as plasmid pFHC496 (Fig. 1) but had mutations in the different DnaA boxes. Strains carrying these plasmids and control strains carrying plasmids with *oriC* fragments containing no, two, three, or four DnaA boxes were used to determine DnaA–β-galactosidase activity (Fig. 2A) as a measure of the derepression of the *dnaA* gene. We also determined cell size by determining the average light scatter of cells by flow cytometry (Fig. 2B). Changes in cell size should be expected if addition of extra DnaA boxes to cells changes the initiation mass.

A strain carrying plasmid pFHC496, which contained all of the DnaA boxes from the *oriC* region, showed a 1.45-fold increase in DnaA–β-galactosidase activity and a 1.15-fold increase in cell size compared to the strain with control plasmid pTAC909. Similar increases in DnaA–β-galactosidase activity and cell size were observed for the strains containing plasmid pBBC166 or pBBC168. The mutation in plasmid pBBC166 does not abolish DnaA box activity (14); thus, we expected results similar to those obtained with plasmid pFHC496. In the case in which the R3 DnaA box was mutated (plasmid pBBC168), we also got similar results, as expected, because the R3 DnaA box shows no DnaA protein binding in vivo (28).

In contrast, mutations in DnaA boxes which decreased the DnaA box quality to zero (14) also decreased the titration

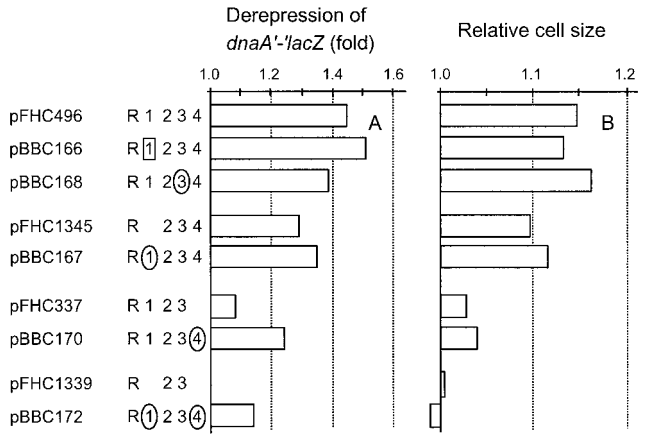


FIG. 2. Mutations in DnaA boxes cause phenotypes which are similar to the phenotypes caused by plasmids in which the corresponding DnaA boxes are absent. Expression of the *dnaA'-'lacZ* gene (measured as DnaA–β-galactosidase activity) is shown in panel A, and cell size estimated as light scatter in the flow cytometer is shown in panel B. These parameters are expressed relative to the values obtained in the control strain carrying plasmid pTAC909. The pBBC plasmids with mutations in different DnaA boxes are derivatives of plasmid pFHC496 (Fig. 1). The DnaA boxes present on the different plasmids (Fig. 1) are indicated to the left. The R1 box in plasmid pBBC166 (boxed number) contains a neutral change of the DnaA box from the R1 and R4 type (TTATCCACA) to the R2 type (TTATACACA), which has the same in vitro binding affinity as the R1 type (14). The remaining mutant DnaA boxes (circled numbers) all had the sequence TTTCCCACA. This sequence shows no affinity for DnaA protein in in vitro binding assays (14). Cultures of strains carrying the respective plasmids were grown in glucose minimal medium. In this growth medium, changes in relative cell size caused by the DnaA box plasmids were more readily observed than on a richer growth medium (see Fig. 3).

efficiency of the plasmid, i.e., the derepression of the *dnaA* gene, and decreased the cell size relative to that of the strain with plasmid pFHC496 (Fig. 2). A plasmid with a particular mutation in a DnaA box gave a result comparable to that obtained with a plasmid in which this DnaA box was absent. Compare, for example, the results obtained with plasmids pFHC337 and pBBC170, plasmids pFHC1345 and pBBC167, and plasmids pFHC1339 and pBBC172. However, plasmids pBBC167, pBBC170, and pBBC172 reproducibly derepress a little bit more than the plasmid in which the corresponding DnaA boxes were not present. We suggest that these plasmids, which, except for the DnaA box mutations were identical to pFHC496, still contained sequences contributing to the structural organization of *oriC* and therefore resulted in more titration.

From these experiments, we conclude that the DnaA boxes are required for efficient titration of DnaA protein by the plasmids carrying *oriC* and that the effects on derepression of *dnaA* gene expression are paralleled by an increase in cell size, indicating an increased initiation mass.

**Cell size, origins, and total chromosomal DNA at different growth rates.** To study how the addition of extra DnaA boxes affects the control of initiation of chromosome replication in more detail, we extended the flow cytometric analysis. We varied the intracellular DnaA box concentration by using strains carrying plasmids with different DnaA titration activities (pTAC909, pFHC496, and pFHC1425) and by growing these strains in media which would give different plasmid copy numbers due to the pBR322 copy number increase seen with a decreasing growth rate (1, 19).

Table 1 shows the generation times, the plasmid copy number per *oriC* locus, and the plasmid copy number per unit of mass (light scatter) obtained for the three strains with the different generation times. The relative copy numbers per unit of mass of the different plasmids at a given growth rate were similar, indicating that the presence of extra DnaA boxes (and DNA) on the plasmids had little (or no) effect on the replication control of plasmid pBR322. Thus, the increase in absolute plasmid copy number per *oriC* locus observed in the strain containing pFHC1425 grown in glucose minimal medium and in the strains containing pFHC496 and pFHC1425 grown in glycerol minimal medium could just as well be considered a decrease in chromosomal *oriC* copy number. In the glycerol minimal medium, DnaA box plasmids pFHC496 and pFHC1425 also slowed growth.

TABLE 1. Copy numbers of DnaA box plasmids

| Growth medium | Plasmid | Doubling time (min) | No of copies *oriC*[a] | No. of copies/LS[b] |
|---|---|---|---|---|
| Glucose + Casamino Acids | pTAC909 | 34 | 14 | 1.0 |
| | PFHC496 | 34 | 13 | 0.8 |
| | PFHC1425 | 34 | 13 | 0.8 |
| Glucose | pTAC909 | 72 | 42 | 1.0 |
| | PFHC496 | 74 | 37 | 0.8 |
| | PFHC1425 | 74 | 51 | 0.9 |
| Glycerol | pTAC909 | 98 | 43 | 1.0 |
| | PFHC496 | 112 | 57 | 1.1 |
| | PFHC1425 | 116 | 67 | 1.0 |

[a] Plasmid copy number per *oriC* locus was determined by Southern blotting as described in Materials and Methods.

[b] Relative plasmid copy number per unit of mass (LS = light scatter) was calculated from the number of copies per *oriC* locus and flow cytometry data on the number of origins per unit of light scatter. The data presented are relative to those of strain BBC119 carrying plasmid pTAC909 grown on the respective growth media.

Figure 3 shows the flow cytometric analysis of the DnaA box plasmid-containing strains. In the fast-growing cultures (Fig. 3A), the DnaA box plasmids only caused small relative increases in cell light scatter. In the more slowly growing cultures (Fig. 3C and E), where the DnaA box-to-*oriC* locus ratio was higher due to the higher copy number of the DnaA box plasmids, we observed more pronounced changes in relative cell size. In contrast, the average DNA content per cell and the DNA distribution of the population of cells at one particular growth rate were similar for the three strains. Thus, the data show that the DNA concentration decreased in cells containing extra DnaA boxes, especially with the long generation times. It should be noted that plasmid pFHC1425, which caused the most marked changes in the flow cytometric distributions, also caused the highest derepression of *dnaA* gene expression at all growth rates; e.g., in glucose-Casamino Acids, we found 1.59-fold derepression for pFHC1425 versus 1.29-fold derepression for pFHC496. The fluorescence and light scatter distributions (as well as the DnaA–β-galactosidase activity) obtained for our background strain (BBC119) without a plasmid and containing plasmid pTAC909 were identical and very similar to the distributions obtained for several other *E. coli* K-12 strains. Unfortunately, the cells of our strain had a strong tendency to stick together during preparation for flow cytometry. Analysis of the same cell samples by microscopy (examples are shown in Fig. 4) showed that the apparent presence of very big cells in most cases could be explained as two (or more) cells sticking together. However, in the strain carrying pFHC1425 grown in glycerol minimal medium, the long cells were real (Fig. 4) and constituted approximately 30% of the population. These big

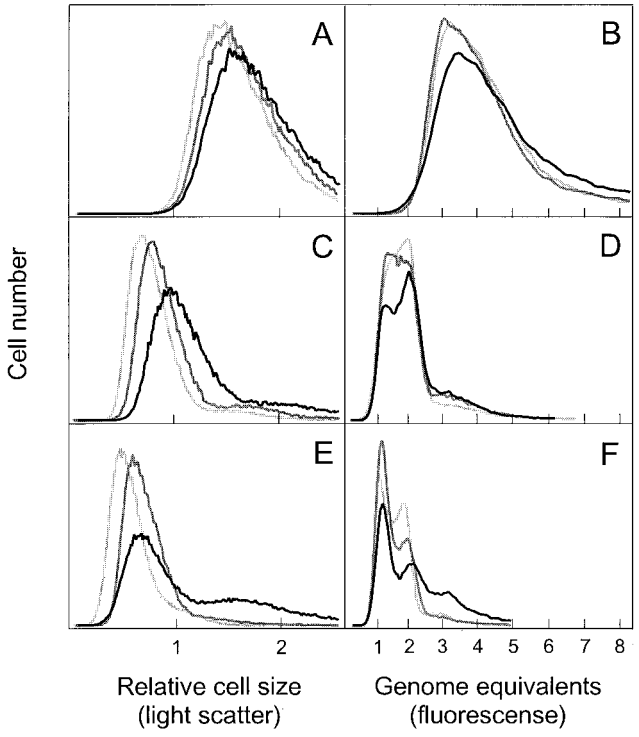


FIG. 3. Cell size and DNA distribution are changed in strains carrying plasmids supplying extra DnaA boxes. Cell size and DNA distribution were determined by flow cytometry as the light scatter and fluorescence distributions, respectively, of cell populations. Cell number is normalized to the total number of cells analyzed. Strains carrying plasmids pTAC909, pFHC496, and pFHC1425 were grown in minimal growth medium supplemented with glucose-Casamino Acids (A and B), glucose (C and D), or glycerol (E and F). The light scatter and fluorescence distributions obtained for strains carrying the three plasmids are shown in different colors as follows: pTAC909, light gray; pFHC496, dark gray; pFHC1425, black.

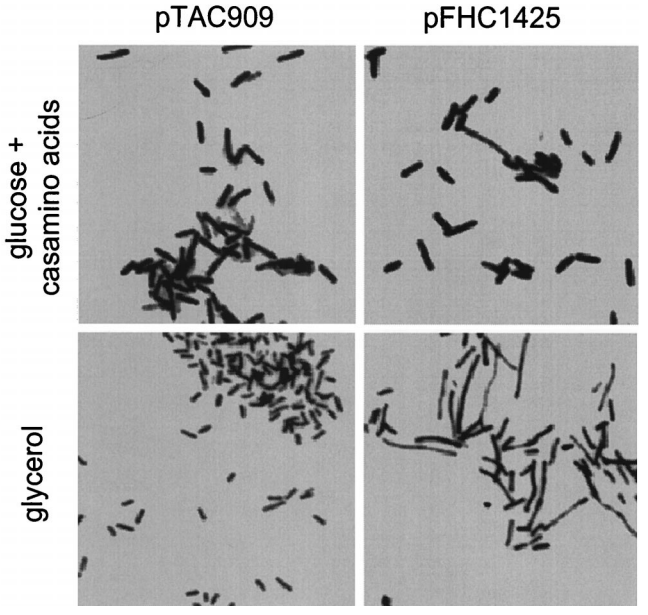


FIG. 4. Fluorescence microscopy of plasmid-carrying strains without (pTAC909) and with additional (pFHC1425) DnaA boxes. The different growth media used are indicated. The same ethanol-fixed cells which were used for flow cytometry in Fig. 3 were stained with a fluorescent probe hybridizing to the 16S rRNA. Computer images of the fluorescent cells were collected via a charge-coupled device camera.

cells represent a fraction in which the presence of a high number of extra DnaA boxes had also affected cell division, as the large majority of these cells contained more than two genome equivalents (Fig. 5). This analysis showed that the presence of extra DnaA boxes, especially in slowly growing bacteria, increased the average cell size and, in the extreme case, resulted in a very heterogeneous cell size distribution.

We also determined the average number of origins per cell by using a flow cytometer and used this value to calculate the origin-to-mass (fluorescence/light scatter) ratio. The results are presented in Fig. 6 and show that the origin-to-mass ratio, which is inversely proportional to the initiation mass, decreased as a result of the presence of extra DnaA boxes. This result allowed us to conclude that the additional titration of DnaA protein caused by the extra DnaA boxes delayed initiation of chromosome replication and therefore caused an increase in initiation mass and, consequently, cell size.

**Additional DnaA boxes disturb the synchrony of initiation.** Finally, we studied the effect of the extra DnaA boxes on initiation synchrony by flow cytometry. Figure 7 shows the flow cytometric fluorescence distributions of rifampin-cephalexin-treated samples of strains carrying control plasmid pTAC909, which did not contribute extra DnaA boxes, and plasmids pFHC496 and pFHC1425, which did. These distributions showed a significant increase in initiation asynchrony caused by the plasmids carrying additional DnaA boxes. There was a correlation between the level of derepression of the *dnaA* promoter and the initiation asynchrony. Thus, the plasmids carrying the most effective sets of *oriC* DnaA boxes with respect to derepression of the *dnaA* promoter were also those which affected the initiation asynchrony most. The strain carrying plasmid pFHC1425 exhibits approximately 60% asynchronous cells, in contrast to the strain without a plasmid (or with plasmid pTAC909), which shows only 17% asynchronous cells (Fig. 7). The maximal asynchrony which can be obtained

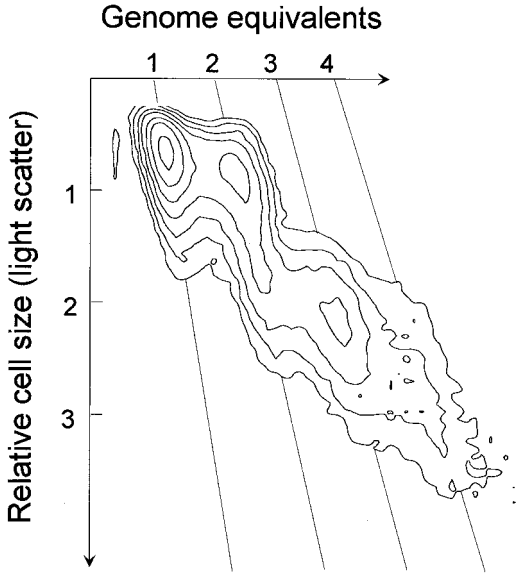


FIG. 5. Three-dimensional flow cytogram of the strain carrying plasmid pFHC1425 grown in glycerol minimal medium (the same sample as shown in Fig. 3E and F and 4).

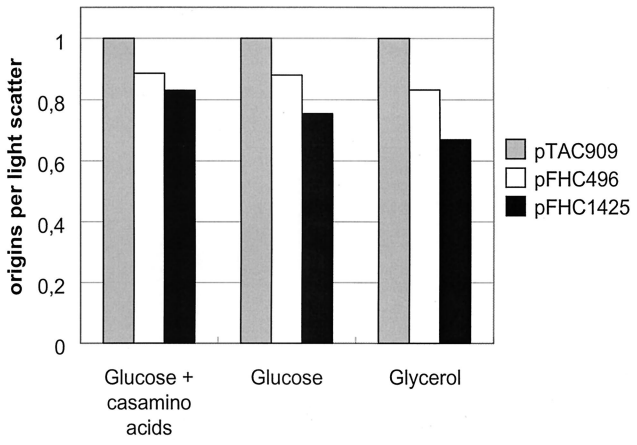


FIG. 6. Decrease in the origin/mass ratio with increasing load of additional DnaA boxes. The origin/mass ratio was determined as the fluorescence/light scatter ratio (in populations of cells treated with rifampin and cephalexin for a time sufficient to finish ongoing rounds of replication) and expressed relative to the origin/mass ratio obtained in the strain carrying pTAC909 grown in the different growth media.

in cells with the number of origins per cell obtained in this experiment is approximately 75%.

## DISCUSSION

Previous studies using *dnaA*(Ts) mutants showed that low DnaA protein activity or concentration increased the initiation mass and led to derepression of the *dnaA* promoter. Here we have studied how extra DnaA protein binding sites affect the initiation mass by using strains containing plasmids carrying DnaA boxes to compete for DnaA protein binding with the normal target DnaA binding sites of the cell. The main finding of this study is that the introduction of extra DnaA protein binding sites leads to an increase in initiation mass. In general, we found proportionality between the number of extra DnaA boxes and the effect on initiation mass. This, in turn, suggests that the DnaA protein binding sites situated on the chromosome are important elements in the setting of the initiation mass through titration of DnaA protein as proposed in our initiator titration model (11).

By using plasmids with mutations in different *oriC* DnaA boxes, we could show that the previously reported titration of DnaA protein and, thus, derepression of the *dnaA* gene (12), as well as changes in cell size, observed in this study, were caused by the increased cellular content of DnaA boxes.

The good correlation between the presence of DnaA boxes and cell size, indicating that titration of DnaA protein affected chromosome initiation control, prompted us to use the flow cytometer in a more thorough study to determine the DNA-to-mass ratio, as well as the origin-to-mass ratio, which is inversely proportional to the initiation mass. We varied the cellular DnaA box content by introducing plasmids carrying different numbers (and qualities) of DnaA boxes in our strain and by growing the DnaA box plasmid containing strains in media giving different generation times and, therefore, different plasmid copy numbers. In complete agreement with our expectations, we found that the higher the number of extra DnaA boxes, the greater was the effect on the origin-to-mass ratio and cell size.

We observed increasing asynchrony of initiation in individual cells containing increasing numbers of extra DnaA boxes. In all of these cases, the overall regulation of chromosome replication was relatively unaffected, as the initiation mass was only moderately changed. It has been proposed that when a fast-growing cell starts to initiate chromosome replication, initiation at the first *oriC* locus will release DnaA protein and thus increase the free DnaA protein concentration, making it more likely to initiate at the next *oriC* locus, etc. (11, 22). The initiation cascade also works in strains containing minichromosomes, which are present at 5 to 10 copies per chromosomal origin. Minichromosomal origins are initiated at the same time as the chromosomal origins (18) and do not disturb initiation synchrony (21). We suggest that the initiation asynchrony we observed was caused by partial interference with the initiation cascade, because the extra DnaA boxes were accumulated in concert with the replication of the pBR322-derived plasmids; i.e., they were accumulated during the cell cycle in proportion to the mass increase and, thus, independently from any cell cycle-related controls. This might present plasmid DnaA boxes at a time relative to chromosome initiation such that the initiation cascade is disrupted by the plasmid(s) titrating the DnaA protein released from the origins initiated first in the cascade.

The present work clearly shows that the introduction of extra DnaA binding sites from the high-affinity DnaA protein binding region *oriC* causes a significant increase in initiation mass. Our data complement previous studies in which changes in the intracellular concentration or activity of DnaA protein were shown to change the initiation mass. Recently, it was shown that plasmids carrying the *datA* locus, one of the other high-affinity DnaA protein binding regions on the chromosome (15), had very similar effects on chromosome initiation control (16). The authors of that report also showed that deletion of the *datA* locus from the chromosome caused overinitiation, i.e., a decrease in initiation mass.

It should be mentioned that the results from our work and the results of Kitagawa et al. (16) are in full agreement with

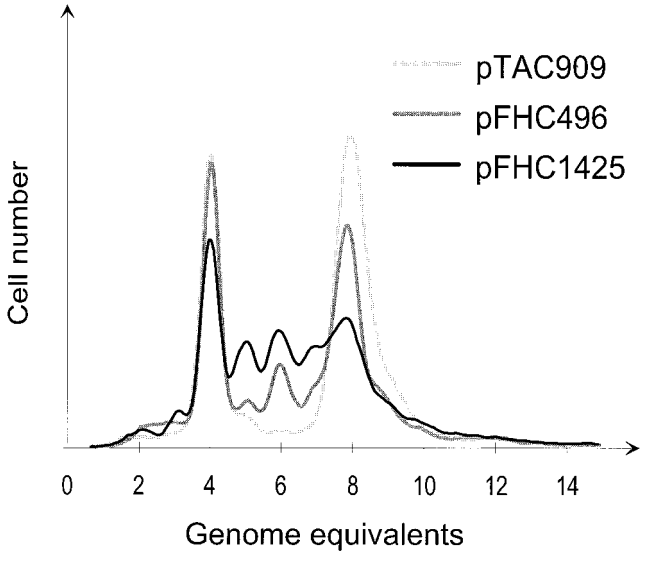


FIG. 7. Increase in initiation asynchrony due to the introduction of pBR322-derived plasmids carrying extra DnaA boxes into cells. Origin distributions were obtained as the fluorescence distribution of a population of cells grown in glucose-Casamino Acids medium and treated with rifampin and cephalexin for a time sufficient to finish ongoing rounds of replication. The light gray curve shows the control strain BBC119 carrying plasmid pTAC909. The other two curves demonstrate the increase in initiation asynchrony caused by plasmids which introduce extra DnaA boxes into the cells.

computer simulations of the initiator titration model (data not shown). We are fully aware that a number of other factors are also actors in the play of initiation of chromosome replication; e.g., there might be factors that alter DnaA protein activity or compete with DnaA protein binding to DnaA boxes. However, it is generally agreed that the DnaA protein is the main actor in initiation. Our data, which show that (extra) DnaA boxes are negatively acting elements which delay initiation, i.e., change the initiation mass, lend support to the initiator titration model which is based on very simple law of mass action considerations and where we postulate that the DnaA boxes in *oriC* and at other places on the chromosome are the main inhibitory elements defining the initiation mass.

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