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Coordinated Replication and Sequestration of *oriC* and *dnaA* Are Required for Maintaining Controlled Once-per-Cell-Cycle Initiation in *Escherichia coli*

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Escherichia coli **cells were constructed in which the *dnaA* gene was moved to a location opposite *oriC* on the circular chromosome. In these cells the *dnaA* gene was replicated with significant delay relative to the origin. Consequently, the period where the newly replicated and hemimethylated *oriC* was sequestered no longer coincided with the period where the *dnaA* gene promoter was sequestered. DnaA protein synthesis was therefore expected to continue during origin sequestration. Despite a normal length of the sequestration period in such cells, they had increased origin content and also displayed asynchrony of initiation. This indicated that reinitiation occasionally occurred at some origins within the same cell cycle. The extra initiations took place in spite of a reduction in total DnaA protein concentration to about half of the wild-type level. We propose that this more efficient utilization of DnaA protein results from an increased availability at the end of the origin sequestration period. Therefore, coordinated sequestration of *oriC* and *dnaA* is required for maintaining controlled once-per-cell-cycle initiation.**

Initiation of chromosomal replication is a critical and highly regulated step in the *Escherichia coli* cell cycle. DNA replication is controlled in such a way that each replication origin, *oriC*, is initiated once and only once each cell cycle at a certain cell mass per origin, the initiation mass (15). The initiation mass is mainly controlled by accumulation of the initiator protein, DnaA (26). The DnaA protein binds to its recognition sites within *oriC*, and in the presence of architectural proteins the DNA duplex opens in an AT-rich region (for review see reference 24). Duplex opening is stabilized by the binding of DnaA protein associated with ATP to the single-stranded regions (43). Finally, the DnaA protein loads the DnaB helicase associated with DnaC to the single-stranded region (30), DnaC leaves, and the replisomes are assembled (24).

When a threshold level of DnaA protein is reached within the cell, all origins are initiated virtually simultaneously, i.e., in synchrony (40). Synchronous initiation is generally explained by sequestration (inactivation [12, 29, 46]) of newly replicated and hemimethylated origins by the SeqA protein. This directs successive initiations to "old origins" only (25). However, sequestration of newly replicated origins persists only for about one-third of the cell cycle and is therefore not sufficient for maintaining once-per-cell-cycle initiation at *oriC*. It is essential that the initiation potential is reduced by other means during sequestration. At least three mechanisms, all serving to lower the activity of the DnaA protein, are proposed to be important for lowering the initiation potential (reviewed in reference 7). These are (i) sequestration of the *dnaA* gene promoter, which prevents de novo synthesis of DnaA protein (12, 44); (ii) titration of DnaA protein to newly replicated chromosomal elements, which provides a sink for DnaA (21, 22, 33); and (iii) regulatory inactivation of DnaA (RIDA), which accelerates the hydrolysis of ATP-DnaA, the form active for initiation, to inactive ADP-DnaA (19).

In this work we describe the construction of cells carrying either the wild-type *dnaA* gene or the *dnaA46* gene in the λ attachment site (*attB*) located opposite *oriC* on the *E. coli* chromosome. Subsequent deletion of the original *dnaA* gene in these strains allowed us to evaluate the contribution of *dnaA* gene sequestration to timely once-per-cell-cycle initiation of replication.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains used were *Escherichia coli* K-12 and derived from CM735 (*metE46 trp-3 his-4 thi-1 galK2 lacY1* or *lacZ4 mtl-1 ara-9 tsx-3 ton-1 rps-8* or *rps-9 supE44* λ^- [18]). CM742 is CM735 *dnaA46* (17), ALO2465 is CM735 *dnaA850*::Tn*10 dnaA*::*attB* (this work), and ALO2073 is *dnaA850*::Tn*10 dnaA46*::*attB* (this work).

Plasmids pFH871 and pFH891 (2) served as sources for the *dnaA* and *dnaA46* genes, respectively. The *attP* cassette used for site-specific integration into *attB* came from plasmid pTAC3588 (5), whereas the λ Int protein was supplied from plasmid pTAC3422 (5). Plasmid pFH539 is a pBR322-derived plasmid containing the *dnaA* gene and *dnaA* gene promoters (47), and plasmid pdnaN is a pBR322-derived plasmid containing the *dnaN* gene under p*lac* control as well as the *lacI* gene (16). Plasmid pTAC4511 was previously described (3).

Growth conditions. Cells were grown in AB minimal medium (13) supplemented with 0.2% glucose, 0.2% glycerol or 0.5% acetate, and 10 μg/ml thiamine. When indicated, Casamino Acids were added to a final concentration of 0.5%. Tryptophan, histidine, and methionine were added to 50 μg/ml where indicated. Ampicillin, tetracycline, chloramphenicol, and kanamycin were added to final concentrations of 100, 10, 6, and 50 μg/ml, respectively, when required. Cell growth was monitored by measuring optical density at 450 nm (OD_{450}).

Flow cytometry. Exponentially growing cells (OD_{450} = 0.15 to 0.25) were treated with rifampin (300 μg/ml; Novartis Pharma Inc.) and cephalexin (36 μg/ml; Sigma Chemical Co.) to inhibit initiation of DNA replication and cell division, respectively (6, 40). Incubation continued for 4 hours to complete ongoing rounds of replication. When initiation of DNA replication is inhibited but ongoing rounds of replication are allowed to finish, the number of fully replicated chromosomes per cell measured by flow cytometry will represent the number of origins in each cell at the time of drug addition (40). In a culture of cells with synchronous initiation, the integral number of chromosomes is two,

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four, or eight (i.e., 2^n), but with asynchronous initiation, additional peaks representing cells with three, five, six, or seven chromosomes (i.e., $\neq 2^n$) appear. Cells were fixed in 70% ethanol and stained with 90 μg/ml mithramycin (Acros Organics Inc.) and 20 μg/ml ethidium bromide (Merck Inc.) in a buffer containing 10 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$ (26). Finally flow cytometry was performed as described previously (26) using an Apogee A10 instrument (Apogee Inc.). Numbers of origins per cell and relative cell mass were determined as described previously (26).

Southern blot analysis. Total cellular DNA was prepared from exponentially growing cells according to the method of reference 27. DNA was digested with HindIII, and fragments were separated on an 0.7% agarose gel, transferred by capillary transfer to a Hybond-N^+ membrane (Amersham Pharmacia Biotech), and probed with a 1,051-bp *terC* fragment and a 1,196-bp *oriC* fragment, which hybridize to 4.1-kb and 2.1-kb chromosomal HindIII fragments, respectively. Probes were prepared as described in reference 3 and labeled with [α-^{33}P]dATP (Amersham Pharmacia) using the Random Primer system (Prime-a-gene; Promega Inc.).

The *oriC*/*terC* ratio was determined by measuring the intensities of hybridization to the 2.14- and the 4.1-kb fragments using ImageQuant version 5.2 software (Molecular Dynamics Inc.). Hybridization signals were normalized to the signals of control plasmid pTAC4511 (3) included on the gel, where *oriC* and *terC* bands are present in a 1:1 ratio.

Microscopy. Cultures were grown in glucose-Casamino Acids medium to an OD_{450} of 0.25, and cells were fixed in 70% ethanol. Cells were applied to glass slides coated with a 0.1% solution of poly-L-lysine (Sigma). To visualize the nucleoid content, cells were stained with a 4′,6-diamidino-2-phenylindole (DAPI)-containing buffer (Pierce Biotechnology Inc.) at a final concentration of 1 μg/ml.

Microscopy was performed using a Leica DM5000B phase-contrast/fluorescence microscope (Leica Microsystems A/S) equipped with a 100× PL Fluotar numerical aperture 1.3 objective. Pictures were taken using a DC 480 camera (Leica Microsystems A/S) that was connected to a computerized image analysis system (IM 50; Leica Microsystems A/S).

Immunoblotting. Exponentially growing cells were harvested for Western blot analysis at an OD_{450} of 0.3 to 0.4. Cells were resuspended in 10 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, and the total protein content was determined using a colorimetric assay (28) in order to adjust all samples to the same protein level. Samples of 5 to 10 μg of protein were applied to the gel. Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Criterion-Precast Gel; 10 to 20% Tris-HCl; Bio-Rad Inc.) and transferred to a polyvinylidene difluoride membrane, 0.2 μm (Millipore), with a semidry blotting apparatus (Bio-Rad Inc.). All the following incubations were at room temperature. The membrane was blocked overnight in Tris-buffered saline (TBSa) plus 2% Tween as main blocking agent (2% Tween 20, 150 mM NaCl, 50 mM Tris-HCl, pH 10), rinsed with TBSa plus 0.05% Tween (0.05% Tween 20, 150 mM NaCl, 50 mM Tris-HCl, pH 10) for 5 min, incubated for 2 h with polyclonal rabbit anti-DnaA antiserum (obtained from K. Skarstad), and washed with TBSa plus 0.05% Tween. The membrane was further incubated for 1.5 h in the presence of porcine anti-rabbit immunoglobulin G antibody conjugated to alkaline phosphatase (DAKO A/S) and washed with TBSa plus 0.05% Tween. The membrane was scanned on a Storm 840 imaging system (Molecular Dynamics Inc.), and quantification was carried out using ImageQuant version 5.2 software (Molecular Dynamics Inc.).

Measurement of the replication and division periods. The replication time, C, for cells grown in rich medium was determined from the origin/terminus ratio (O/T) obtained from Southern blotting. For a culture having a doubling time τ, and a chromosomal replication time C, the stoichiometry between origins O and termini T is given by the formula $O/T = 2^{C/\tau}$ (10). After the C period and the number of origins per cell is determined, the time following termination of replication until cell division, D, can be calculated using the formula $ori/cell = 2^{(C+D)/\tau}$ (10). Note that these determinations of C and D periods are valid even for cells that initiate replication asynchronously (10).

For slow-growing cells the O/T-based method proved not to be sufficiently sensitive for an accurate determination of C and D periods. These periods were therefore determined by computer simulation of DNA histograms obtained by flow cytometry of exponentially growing cells (31).

Calculation of gene dosage. Gene dosage of the *dnaA* gene was calculated for wild-type cells and *dnaA850*::Tn*10* cells carrying the *dnaA* gene in *attB* using the following formula (10): $X_C = 2^{\{[C*(1-m)+D]/\tau\}}$, where m determines the location of the specific gene (X) relative to the origin of replication. As the distance between the *E. coli* origin and terminus is 2.32 Mb, $m_{wt} = 0.043/2.32 = 0.02$, for the wild-type *dnaA* gene located 43 kb from *oriC*. The *attB* region is located 1.51 Mb from *oriC*, and m_{attB} is 1.51/2.32 = 0.65. τ is the doubling time. C is the time of replication (from initiation to termination of the chromosome). C was determined as described above. D is the time following termination of replication until cell division. D was determined as described above. Note that this calculation of gene dosage is valid even for cells that initiate replication asynchronously (10).

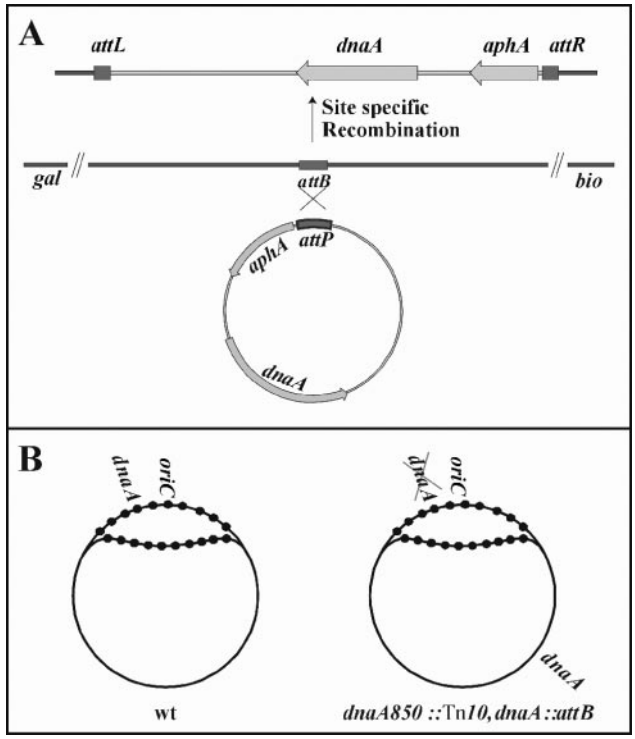

FIG. 1. Construction of strains with *dnaA* or *dnaA46* in the chromosomal *attB* site. (A) The *dnaA*/*dnaA46* gene was ligated to a nonreplicating *attP*-carrying cassette (bottom). Lambda Int protein-dependent site-specific recombination with the chromosomal *attB* site resulted in cells carrying an additional *dnaA* gene inserted in *attB*. Subsequently the *dnaA* gene was removed from its normal location. (B) In wild-type cells *dnaA* is replicated shortly after *oriC*. Consequently, both *oriC* and *dnaA* are hemimethylated and sequestered by the SeqA protein (●) in the same part of the cell cycle (left). In cells carrying a *dnaA* gene in *attB* only, this is replicated with significant delay relative to *oriC*. Consequently, the *dnaA* gene is not sequestered for the same part of the cell cycle as the origin (right).

RESULTS

Cells with the *dnaA* gene located at *attB*. In *E. coli* the *dnaA* gene is located about 43 kb away from the replication origin. Consequently the *dnaA* gene is replicated shortly (less than 1 minute) after initiation at *oriC* has taken place. *dnaA* and *oriC* are therefore hemimethylated and sequestered largely simultaneously (12) (Fig. 1B), and no or very little de novo DnaA protein synthesis takes place during origin sequestration (44). Furthermore both regions remain sequestered for a large fraction of the cell cycle (12). We constructed cells where only one functional copy of the *dnaA* gene was inserted into the lambda attachment site *attB* located about 1.5 Mb away from the origin. Briefly, DNA fragments containing the *dnaA* or *dnaA46* gene were ligated to a fragment containing the λ *attP* site in such a way that the resultant circular molecules did not contain replication origins (Fig. 1A). Site-specific recombination with the bacterial *attB* in the presence of lambda integrase (5)

TABLE 1. Cell cycle parameters for fast-growing cells

Strain	Growth medium	Doubling time (min)	No. of origins/cell	Relative cell mass	Relative cell mass/origin
CM735 (wt)	Glucose + Casamino Acids	38	4.9	1.0	1.0
ALO2465 (*dnaA850*::Tn*10* *dnaA*::*attB*)	Glucose + Casamino Acids	60	6.9	1.6	1.1
ALO2465/pFH539[a]	Glucose + Casamino Acids	41	ND[e]	ND	ND
ALO2465/pdnaN[b]	Glucose + Casamino Acids	59 (−IPTG), 77 (+IPTG)[d]	ND	ND	ND
CM735	Acetate	220	2.0	1.0	1.0
ALO2465	Acetate	219	2.2	1.0	0.9
CM742 (*dnaA46*)[c]	Glucose + Casamino Acids	58	4.2	1.0	1.2
ALO2073 (*dnaA850*::Tn*10* *dnaA46*::*attB*)[c]	Glucose + Casamino Acids	66	3.9	1.2	1.5
CM742[c]	Glycerol	107	2.0	1.0	1.0
ALO2073[c]	Glycerol	124	2.5	1.5	0.8

[a] pFH539 is a pBR322-derived plasmid containing the *dnaA* gene and *dnaA* gene promoters (47).
[b] pdnaN is a pBR322-derived plasmid containing the *dnaN* gene under p*lac* control as well as the *lacI* gene (16).
[c] Growth temperature was 30°C.
[d] IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM.
[e] ND, not determined.

resulted in cells containing the original *dnaA* gene at its normal position as well as a second copy of either *dnaA* or *dnaA46* in the *attB* site. The original *dnaA* gene was subsequently replaced by the *dnaA850*::Tn*10* allele by P1 transduction (Fig. 1B). In the resultant cells, *oriC* and *dnaA* are separated by 1.5 Mb. Because the distance between *oriC* and the *dnaA* gene now corresponds to approximately two-thirds of the distance from the origin to the terminus of replication, replication of the *dnaA* gene will be delayed with approximately two-thirds of a replication time relative to the origin at any given growth rate. Consequently, the origin region and the *dnaA* gene promoter are not expected to be sequestered simultaneously in these cells (Fig. 1B). Cells carrying the *dnaA* gene in the *attB* site grew slower than their wild-type counterparts (Table 1). The growth defect could be complemented by a plasmid carrying *dnaA* but not by a *dnaN*-carrying plasmid (Table 1) and therefore resulted from altered expression of the *dnaA* gene in its new location rather than polar effects of the *dnaA850*::Tn*10* on the *dnaN*, *recF*, and *gyrB* genes. Because the Tn*10* is inserted about 60 bp following the start codon of *dnaA* (23), the downstream genes were transcribed from promoters within or downstream of *dnaA* (38).

DnaA synthesis following initiation does not shorten the sequestration period. The minimal time between successive initiations was measured for strains CM742 (*dnaA46*) and ALO2073 (*dnaA850*::Tn*10* *dnaA46*::*attB*) as previously described (45). Cells were grown exponentially at a permissive temperature (30°C) for five to six generations, shifted to 42°C, and incubated at this nonpermissive temperature for 90 min before being shifted back to a permissive temperature (30°C). The average number of origins in both strains decreased during incubation at the nonpermissive temperature (Fig. 2), indicating that initiations ceased while cells continued to divide. After 90 min at 42°C, CM742 cells contained mainly one fully replicated chromosome, whereas a significant number of ALO2073 cells contained two chromosomes. Upon the shift back to 30°C, resulting in reactivation of the DnaA protein, all cells of CM742 initiated replication within the first 5 min and again after approximately 30 min. This period of 25 min thus represents the minimal interreplication time for these cells

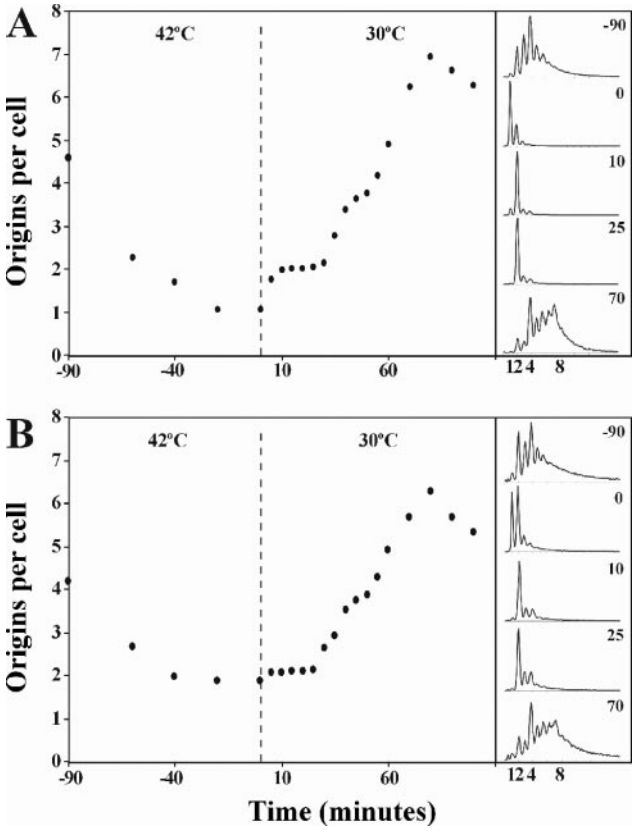

FIG. 2. Reinitiation in cells carrying *dnaA* in *attB*. Cells of strain CM742 (A) and CM742 *dnaA850*::Tn*10* *dnaA46*::*attB* (ALO2073) (B) were grown at 30°C in minimal medium supplemented with glucose, Casamino Acids, and tryptophan. At time $T = -90$ the culture was shifted to 42°C, kept at this nonpermissive temperature for 90 min, and shifted back to 30°C at time $T = 0$. At the times indicated cell samples were collected for treatment with rifampin and cephalexin for 4 h prior to flow cytometry analysis. The median (the value above and below which 50% of the distribution can be found) was used as a robust measure of the central tendency of individual cells (39) and is plotted as origins per cell. The panels on the right-hand side of the figure show selected DNA histograms for rifampin-treated cultures.

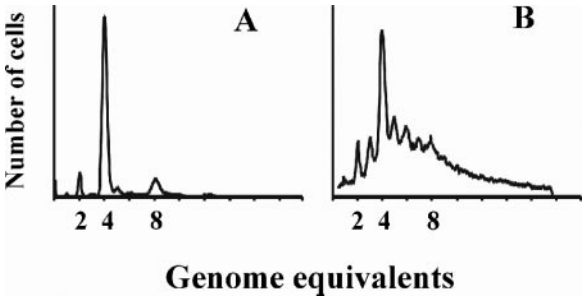

FIG. 3. Initiation of replication is affected by the *dnaA* gene location. DNA histograms of CM735 (wt) (A) and ALO2465 (*dnaA850*::Tn*10 dnaA*::*attB*) (B). Cells were grown at 37°C in minimal medium supplemented with glucose, Casamino Acids, and tryptophan and treated with rifampin and cephalexin prior to flow cytometry analysis. Distinct peaks represent the accumulation of cells with an integral number of chromosomes which correspond to the number of origins at the time of drug addition (40). The scale on the number of cells is arbitrary and represents a minimum of 60,000 cells in all panels.

(Fig. 2A). For strain ALO2073 the situation was similar. All cells containing one origin of replication initiated immediately after the shift back to permissive temperature, whereas cells containing two chromosomes for unknown reasons did not (Fig. 2B). A second round of initiation was observed after 30 min. The minimal time between successive initiations in these cells was therefore 25 min as well.

This experiment clearly demonstrates that the minimal time between initiations at the same origin is not shortened in ALO2073 cells which have continued DnaA protein synthesis during origin sequestration. The DnaA protein synthesized during incubation at nonpermissive temperature in these cells will reduce but not turn off the *dnaA* transcription when renatured (2, 9). It therefore seems that the duration of the sequestration period is insensitive to smaller changes in the DnaA protein concentration.

Occasional reinitiation of replication in cells carrying *dnaA* in *attB*. We analyzed the cell cycle of CM735 (wt) and CM742 (*dnaA46*) cells as well as their isogenic counterparts carrying either the wild-type *dnaA* gene inserted in *attB* (*dnaA850* ::Tn*10 dnaA*::*attB*; ALO2465) or the *dnaA46* gene inserted in *attB* (*dnaA850*::Tn*10 dnaA46*::*attB*; ALO2073). When analyzed by flow cytometry, wild-type cells contained mainly four origins of replication while some contained two and some contained eight origins (Fig. 3A). This indicates that origins were initiated in synchrony (40). Cells of ALO2465 carrying the *dnaA* gene in *attB* also contained mainly four fully replicated origins, but in addition a number of cells had three, five, six, and seven origins (Fig. 3B), indicating that initiations occurred asynchronously in these cells. The number of origins per cell was increased from an average of 4.9 for the wild type to 6.9 in cells carrying *dnaA* in *attB* (Table 1). Because the cell size was also somewhat increased for the latter, the cell mass per origin was about the same for the two cell types (Table 1). The asynchrony observed for strain ALO2465 did not result from polar effects of the *dnaA850*::Tn*10* on *dnaN*, *recF*, or *gyrB* gene expression because cells in which the normal *dnaA* gene was inactivated by a nonpolar *dnaA* allele were similar to ALO2465 cells with respect to synchrony (not shown). Similarly, strain ALO2465 continued to initiate asynchronously when additional DnaN protein was supplied from a plasmid (not shown).

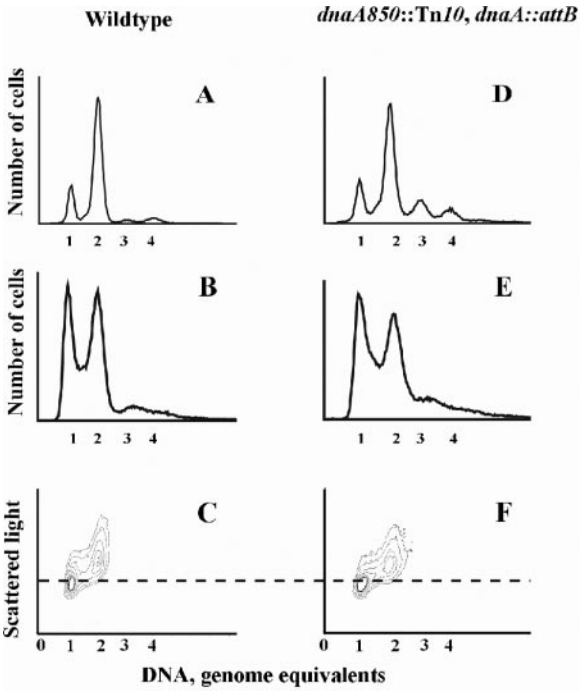

FIG. 4. The initiation mass of slow-growing cells carrying *dnaA* in *attB*. Flow cytometry histograms of wild-type (CM735) and *dnaA850*::Tn*10* cells carrying *dnaA* in *attB* (ALO2465) grown under steady-state conditions at 37°C in minimal medium supplemented with acetate, tryptophan, histidine, and methionine. The parameters measured were number of origins in rifampin- and cephalexin-treated cells (A and D) and DNA content of exponentially growing cells (B and E). Three-parameter contour plots of exponentially growing cells are shown in panels C and F. The isocontour lines are drawn through points with the same number of cells. Thus, the collection of almost concentric rings at the one- or two-chromosome position represents a "mountain" of cells. The dashed line represents the cell mass at which initiation takes place (8). The scale on number of cells and scattered light is arbitrary and represents a minimum of 100,000 cells in all panels.

When grown with acetate as sole carbon source, ALO2465 cells grew with the same doubling time as wild-type cells (~220 min) but contained more origins per cell, although the difference was not as pronounced as that observed at fast growth (Fig. 4; Table 1). The average cell mass was similar to the mass of wild-type cells, and it therefore contained an increased number of origins per mass. Because initiations took place on one origin only, the cell mass at initiation can be read off the three-parameter contour plots (Fig. 4C and F) (8). Wild-type cells and ALO2465 cells carrying *dnaA* in *attB* had a similar initiation mass.

We suggest that continued synthesis of wild-type DnaA protein during origin sequestration led to reinitiation at the same origin in some cells. These extra initiations caused an increase in origin content per cell. It is conceivable that the extra initiations occurred at the end of the sequestration period, as this was not affected by continued DnaA synthesis.

The increased number of origins in cells carrying *dnaA* in *attB* was not a consequence of initiations becoming asynchro-

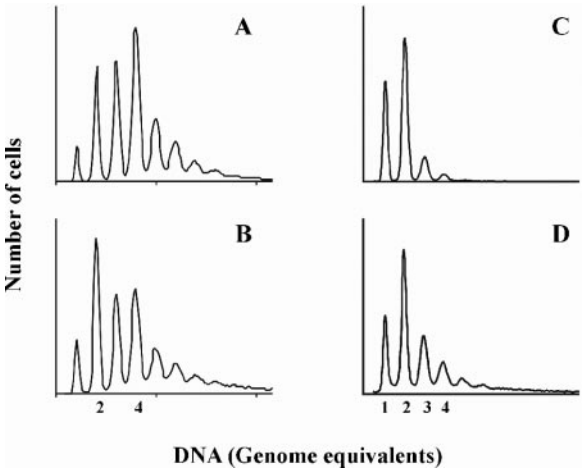

FIG. 5. Initiation of replication is affected by the *dnaA46* gene location at slow growth. DNA histograms of CM742 (*dnaA46*) (A and C) and ALO2073 (*dnaA850*::Tn*10 dnaA46*::*attB*) (B and D) cells. Cells were grown at 30°C in minimal medium supplemented with glucose, Casamino Acids, and tryptophan (A and B) or with glycerol, tryptophan, histidine, and methionine (C and D) and treated with rifampin and cephalexin prior to flow cytometry analysis. Distinct peaks represent the accumulation of cells with an integral number of chromosomes which correspond to the number of origins at the time of drug addition (40).The scale on number of cells is arbitrary and represents a minimum of 60,000 cells in all panels.

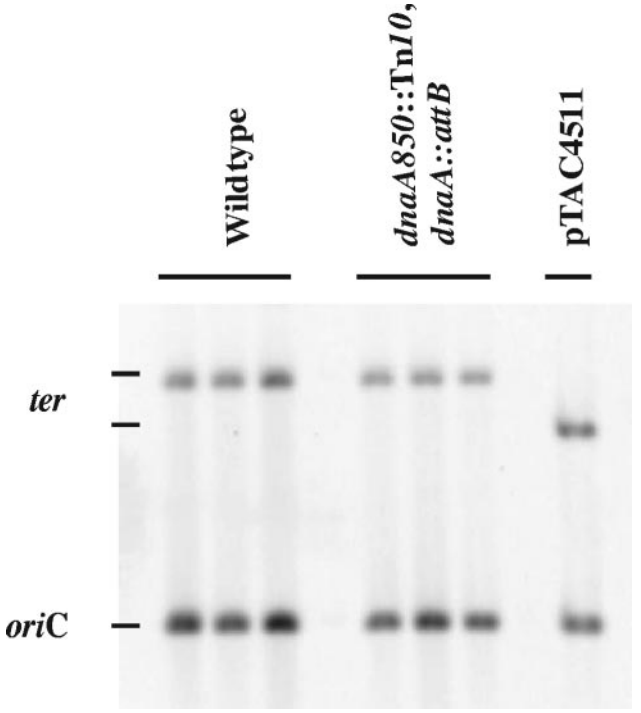

FIG. 6. The *ori*/*ter* ratio is increased in cells carrying *dnaA* in *attB*. Three individual cultures of wild-type (CM735) and *dnaA850*::Tn*10 dnaA*::*attB* (ALO2465) cells were grown exponentially at 37°C in minimal medium supplemented with glucose, Casamino Acids, and tryptophan. DNA was extracted and digested with HindIII, and Southern hybridization was performed with an *oriC* probe and a *ter* probe (Materials and Methods). A sample of plasmid pTAC4511 digested with HindIII was also included on the blot. This sample contains *oriC* and *terC* in a 1:1 proportion.

nous per se because asynchronous *dnaA46* mutant cells grown in rich medium (Fig. 5) had their origin content reduced relative to wild-type cells (Table 1). Cells carrying the *dnaA46* gene in *attB* were also asynchronous (Fig. 5) but contained slightly fewer origins than cells carrying the *dnaA46* gene in its normal location (compare Fig. 5A and B; Table 1). Cell size was increased which resulted in an increased cell mass/origin ratio, indicating an increased initiation mass in these cells. When strain CM742 (*dnaA46*) was grown in glycerol minimal medium with a doubling time of 107 min, cells contained mainly one or two replication origins (Fig. 5C; Table 1), indicating that initiations took place only once on a single copy of *oriC*. Cells having the *dnaA46* gene in *attB* (ALO2073) grew slower, and a considerable fraction of cells contained three or four replication origins. Therefore, some origins were reinitiated in the same cell cycle under slow growth. Because the cell size was also increased, the cell mass per origin was about the same as for wild-type cells.

Cell cycle periods of cells carrying *dnaA* in *attB*. The cellular number of origins is a function of the replication and division periods (C and D) as well as cellular growth rate. The increase in number of origins in cells carrying the wild-type *dnaA* gene in *attB* could therefore result from an increase in the C or D period of these cells or both (10).

We determined the origin-to-terminus ratio for fast-growing wild-type cells and *dnaA850*::Tn*10* cells carrying the *dnaA* gene in *attB* by Southern blot hybridization (Fig. 6). We included a sample of plasmid pTAC4511 containing both *oriC* and *terC* on the blot in order to correct for differential labeling efficiencies of the *oriC* and *terC* probes (3). In wild-type cells the *ori*/*ter* ratio was found to be 2.5. Because this ratio depends only on the cellular growth rate and length of the C period, the latter could be determined to be approximately 45 min (Table 2). Knowing the number of origins per cell (Fig. 3; Table 1), we could determine the D period to be approximately 42 min. These durations of C and D periods are in good agreement with what was previously determined for this strain (1). Strain

TABLE 2. Replication and division periods for cells carrying *dnaA* in *attB*

Strain	Growth medium	Doubling time (min)	*ori*/*ter* ratio	*C* period (min)	D period (min)
CM735 (wt)	Glucose + Casamino Acids	38	2.5	45 ± 8	42
ALO2465 (*dnaA850*::Tn*10 dnaA*::*attB*)	Glucose + Casamino Acids	60	3.0	90 ± 13	77
CM735 (wt)	Acetate	220	ND[a]	79	103
ALO2465 (*dnaA850*::Tn*10 dnaA*::*attB*)	Acetate	219	ND	92	90

[a] ND, not determined.

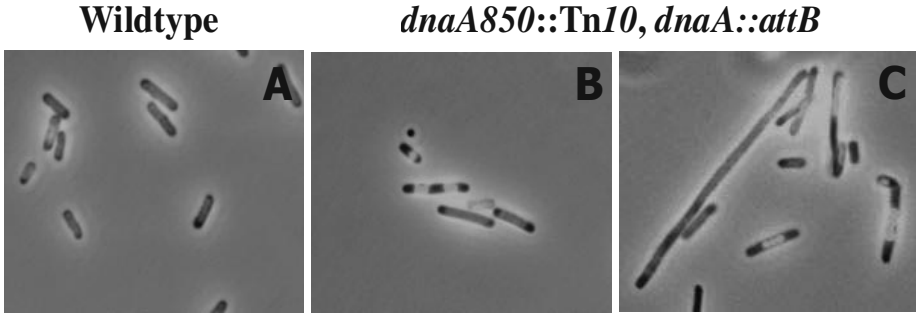

FIG. 7. Cells carrying *dnaA* in *attB* are heterogeneous with respect to cell size and nucleoid positioning. Strains CM735 (wt) (A) and CM735 *dnaA850*::Tn*10* *dnaA*::*attB* (ALO2465) (B and C). Panel B shows the morphology of the majority of ALO2465 cells, whereas the less frequent filamentous cells are shown in panel C. Cells were grown at 37°C in minimal medium supplemented with glucose, Casamino Acids, and tryptophan. Cells were fixed and stained prior to microscopic analysis as described in Materials and Methods.

ALO2465 carrying the *dnaA* gene in *attB* grew slower than its wild-type counterpart but had a slightly higher *ori*/*ter* ratio of 3.0. The C and D periods of this strain were 90 and 77 min, respectively (Table 2), i.e., both were extended considerably relative to wild-type cells. A microscopic inspection revealed that cells carrying the *dnaA* gene in *attB* were larger than wild type, heterogeneous, and frequently filamentous with irregular positioning of the nucleoids (Fig. 7).

For slow-growing cells, the cell cycle periods were determined from simulations of the DNA histograms of exponentially growing cells (31). Under these growth conditions wild-type cells had C and D periods of 79 and 103 min, respectively, whereas *dnaA850*::Tn*10* cells carrying the *dnaA* gene in *attB* had C and D periods of 92 and 90 min, respectively (Table 2). Microscopic inspection of slow-growing cells revealed no gross differences between wild-type cells and *dnaA850*::Tn*10* cells carrying the *dnaA* gene in *attB* (not shown).

We may conclude that, in fast-growing *dnaA850*::Tn*10* cells carrying the *dnaA* gene in *attB*, the increased number of origins per cell is accompanied by an increase in duration of both C and D periods. Because cells are quite heterogeneous, these average numbers are likely to cover large cell-to-cell differences, and individual cells in such a culture presumably do not undergo identical cell cycles. The increased C period could result from overinitiation at *oriC* such as previously observed (4, 26, 34, 42) or from a slight shortage of DnaN protein due to the Tn*10* insertion in *dnaA*. The *dnaN* gene encodes the ring-shaped β-subunit dimer of the polymerase III holoenzyme—the so-called sliding clamp—which stabilizes the binding of polymerase III to DNA strands to enable processive replication over lengths of several kilobases (36, 48). At slow growth differences between wild-type cells and *dnaA850*::Tn*10* cells carrying *dnaA* in *attB* were smaller, with less than 15% difference in the duration of their C and D periods.

The level of DnaA protein is decreased in cells carrying the *dnaA* gene in *attB*. The increase in origin content for cells carrying the wild-type *dnaA* gene in *attB* might result from an increased level of DnaA protein relative to wild-type cells. Such differences in expression of the same gene in different chromosomal contexts have previously been observed (37).

We therefore quantified the cellular content of DnaA protein in wild-type (CM735) cells and cells carrying the *dnaA* gene in *attB* only (ALO2465). The immunoblot revealed that the concentration of DnaA protein in cells carrying the *dnaA* gene in *attB* was reduced to about half of the wild-type level during growth in rich medium (Fig. 8A), whereas less change relative to wild type was observed when growth was slow (Fig. 8B). Because *attB* is located fairly close to the chromosomal terminus of replication, the gene dosage of *dnaA*, when inserted here, must be lower than the gene dosage of the *dnaA* gene at its normal origin-proximal location. We calculated the gene dosage of *dnaA* (10) in the two different strains, using the previously determined C and D periods. In rich medium the *dnaA* gene dosage for wild-type cells was found to be 4.8, which is in good agreement with the average number of origins per cell (Table 1), whereas it was reduced to 3.5 in cells carrying *dnaA* in *attB*. During slow growth the *dnaA* gene dosage was reduced from 1.8 in wild-type cells to 1.5 in cells carrying *dnaA* in *attB* (Fig. 8).

We can conclude that the additional initiations observed for cells carrying the *dnaA* gene in the *attB* site did not result from an increased level of DnaA protein. In contrast, the DnaA protein level of these cells was reduced relative to the wild type primarily at fast growth (Fig. 8). Because the *dnaA* gene dosage in ALO2465 cells was also reduced relative to wild type, the *dnaA* gene was expressed in a gene dosage-dependent manner. This may indicate that autoregulation plays little role for *dnaA* gene expression in normally growing cells such as previously described (4) or that *dnaA* gene expression, despite being autoregulated, shows a dampened dependence on gene dosage (14).

DISCUSSION

We have constructed cells where the *dnaA* gene was moved from its normal location adjacent to *oriC* to the *attB* locus, 1.5 Mb away from the origin. In these cells the *dnaA* gene is replicated, hemimethylated, and sequestered with significant delay relative to the origin. Consequently, synthesis of DnaA protein is assumed to continue during origin sequestration in these cells. This led to occasional reinitiation at some origins within the same cell cycle, manifested as an increased number of replication origins per cell and asynchronous initiation.

Timely sequestration of the *dnaA* gene is required for controlled initiation of replication. In *E. coli*, the *dnaA* gene promoter is sequestered by a mechanism similar to that of *oriC*

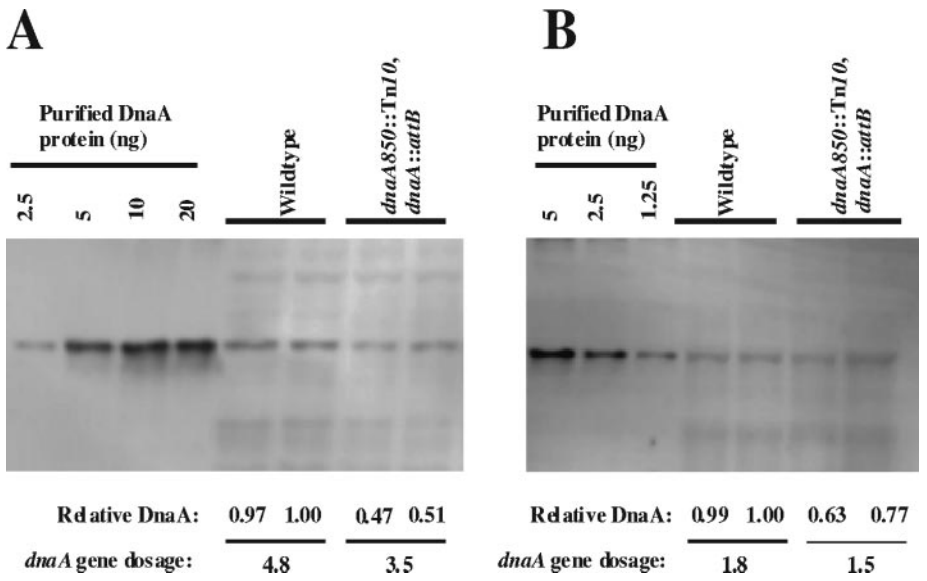

FIG. 8. Cellular DnaA protein content. Strains CM735 (wt) and CM735 *dnaA850*::Tn*10 dnaA*::*attB* (ALO2465) were grown at 37°C in minimal medium supplemented with glucose, Casamino Acids, and tryptophan (A) or in minimal medium supplemented with acetate, tryptophan, histidine, and methionine (B). Samples were taken, and the total protein content was adjusted to the same amount using a Lowry analysis prior to loading on a sodium dodecyl sulfate-polyacrylamide gel. For fast-growing cells 8 μg of total protein was applied to the gel (A) whereas only 5 μg was used for slow-growing cells (B). Following blotting, the filter was probed with a polyclonal DnaA antibody. The control lanes contain the indicated amounts of purified DnaA protein. The relative amount of DnaA protein was quantified for each sample and normalized to wild-type cells. The gene dosage of *dnaA* was calculated as described in Materials and Methods.

and for the same fraction of the cell cycle (12). Sequestration of the *dnaA* gene promoter prevents de novo DnaA protein synthesis (44), and this mechanism was proposed to reduce the amount of the initiator DnaA protein to a level that does not sustain any further initiations within the same cell cycle (reviewed in reference 7).

In wild-type cells the initiation mass is normally set by accumulation of DnaA protein (26). It was therefore surprising to find that fast-growing cells carrying their only copy of the *dnaA* gene at *attB* had cell mass per origin similar to that of wild-type cells, despite a 50% reduction in DnaA protein concentration. In order for cells to accumulate sufficient DnaA protein for initiation to take place, they must grow to a larger size before they initiate replication at the same amount of DnaA per origin as wild-type cells, which explains the observed increase in cell size and in generation time. However, once initiation(s) occurs at a given origin it is not limited to once per cell cycle, which explains why cells that carry the *dnaA* gene at *attB* had increased origin content and initiated asynchronously. Such occasional reinitiation at an origin within the same cell cycle results in a culture where individual cells undergo different cell cycles (32). In agreement with this, cells carrying *dnaA* in *attB* were quite heterogeneous with respect to both cell size and nucleoid positioning.

There are at least two scenarios for how origins may be reinitiated in cells carrying *dnaA* in *attB*. First, the activity of the DnaA protein itself could be increased, i.e., by having a larger fraction in the ATP-bound form. This is not likely since cells are wild type with respect to the RIDA system (19, 20). Second, the timing of DnaA protein synthesis within the cell cycle could be altered in such a way that the protein becomes available for initiation in a part of the cell cycle where initiations do not normally occur. In the period following initiation at *oriC*, the origin is hemimethylated and sequestered whereas the *dnaA* gene, now located in *attB* and therefore replicated with a delay of two-thirds of the C period, remains fully methylated and continues to synthesize DnaA protein. Some of this newly synthesized DnaA protein is expected to be in the ATP-bound form and active for initiation. At the end of the *oriC* sequestration period, where origins again become available for initiation, the level of DnaA protein is elevated, and we suggest that this leads to reinitiation at some origins within the same cell cycle. The net result of such occasional reinitiation is a culture of cells that are asynchronous and contain an elevated number of origins per cell (Fig. 3B; Table 1). The increased replication time in cells carrying *dnaA* in *attB* may well be a consequence of occasional reinitiation at *oriC*; *seqA* mutant cells reinitiate replication at an origin within the same cell cycle due to lack of sequestration (29), and DnaA protein-overproducing cells reinitiate at the end of the sequestration period (34). In both cases untimely reinitiation at an origin leads to a considerable increase in the replication period.

In *dnaA46* mutant cells initiations occur throughout most of or the entire cell cycle, and some origins are reinitiated while others are not initiated at all (41). Therefore, in fast-growing cells containing multiple origins of replication, initiation at one origin only leads to replication and sequestration of the *dnaA46* gene located near the initiated origin, whereas the remainder of the *dnaA46* genes carried within the same cell are not sequestered. Consequently, DnaA46 protein synthesis occurs continuously throughout the cell cycle even when the gene is in its normal position. Therefore, there is little effect of moving the *dnaA46* gene to the *attB* position with respect to initiation synchrony. The lower *dnaA46* gene dosage is, how-

ever, reflected in a slightly reduced number of origins per cell and increase in initiation mass (Fig. 5A and B; Table 1). In slow-growing *dnaA46* cells initiations take place at a single origin, and this leads to sequestration of *oriC* as well as the *dnaA46* gene, i.e., the gene is expressed in a cell-cycle-specific manner. This explains why we observe that cells carrying *dnaA46* in *attB* overinitiate replication at slow growth (Fig. 5; Table 1); DnaA46 protein synthesis continues through origin sequestration so that origins are frequently reinitiated in the cell cycle.

It is likely that cells carrying *dnaA* in *attB* sequester their *dnaA* gene following passage of the replication fork, i.e., continue to express *dnaA* in a cell-cycle-specific manner that is altered relative to wild type. This poses the main difference between this and a previous report showing that cell-cycle-specific expression of *dnaA* is not important for once-per-cell-cycle initiation (26). In the earlier report, the *dnaA* gene was expressed from a *lac* promoter on a plasmid, whereas the chromosome carried a *dnaA46* mutation. At elevated temperature active DnaA protein was presumably expressed from the plasmid only in a non-cell-cycle-specific manner, yet initiations were synchronous. It is not clear to us why our data differ from this report. The data presented here are on the other hand in agreement with several recent reports indicating that constitutive and elevated DnaA protein expression, from a plasmid, leads to asynchronous initiations (4, 34, 41).

Two mechanisms were previously proposed to lower the amount of the initiator DnaA protein during origin sequestration. RIDA converts DnaA associated with ATP to the ADP-associated form inactive for initiation (19, 20), and replication generates new DnaA protein binding sites that serve to bind (titrate) free DnaA protein away from the origin (21, 22). The relative contribution of these two mechanisms to maintaining once-per-cell-cycle initiation has been assessed by preventing RIDA from taking place (11) and by deletion of the *datA* region (22). In both cases, reinitiation at origins within the same cell cycle was observed, resulting in initiation asynchrony and an elevated number of origins per cell (11, 22). However, it has recently been reported that deletion of *datA* mainly results in early initiation at a reduced mass per origin (35), suggesting that this locus mainly controls the accumulation of DnaA protein necessary for an initiation event. Our data add the timely sequestration of the *dnaA* gene promoter to RIDA and possibly *datA* as necessary elements for maintaining once-per-cell-cycle initiation of replication.

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