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Inhibition and Restart of Initiation of Chromosome Replication: Effects on Exponentially Growing *Escherichia coli* Cells

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Escherichia coli **strains in which initiation of chromosome replication could be specifically blocked while other cellular processes continued uninhibited were constructed. Inhibition of replication resulted in a reduced growth rate and in inhibition of cell division after a time period roughly corresponding to the sum of the lengths of the C and D periods. The division inhibition was not mediated by the SOS regulon. The cells became elongated, and a majority contained a centrally located nucleoid with a fully replicated chromosome. The replication block was reversible, and restart of chromosome replication allowed cell division and rapid growth to resume after a time delay. After the resumption, the septum positions were nonrandomly distributed along the length axis of the cells, and a majority of the divisions resulted in at least one newborn cell of normal size and DNA content. With a transient temperature shift, a single synchronous round of chromosome replication and cell division could be induced in the population, making the constructed system useful for studies of cell cycle-specific events. The coordination between chromosome replication, nucleoid segregation, and cell division in *E. coli* is discussed.**

A central objective in bacterial cell cycle research is to understand how chromosome replication, nucleoid processing, and cell division are coordinated with each other and with cellular growth. A common approach used to study the interplay between chromosome replication and cell division in bacteria is to block replication and analyze the effects on division and cell morphology.

Chromosome replication can be inhibited by the addition of rifampin or chloramphenicol, both of which block initiation of replication but allow ongoing rounds to continue to termination (runout of replication; illustrated by flow cytometry in references 48 and 49). This approach has been used to study chromosome positioning and its dependence on replication (25). However, since the result of the drug additions, inhibition of transcription or translation, affects most processes in the cell, the specific effects of the blocked replication are obscured. In addition, cellular metabolism and growth rapidly stop, limiting the analysis to short-term effects. Replication runout is also observed when certain mutants in glycerol metabolism are deprived of glycerol, which results in inhibition of phospholipid synthesis (46), and when amino acid auxotrophs are deprived of amino acids (33). Again, the treatments have drastic effects on cellular growth and metabolism.

The elongation stage of chromosome replication can be blocked in thymine-requiring mutants by removal of thymine from the growth medium, and the effects on cell division may be studied (5, 15, 18, 23, 28). A disadvantage with this approach, and with other treatments or mutations that inhibit elongation, is that blockage of ongoing replication results in induction of the SOS response (38). This involves a diverse set of functions that affect different aspects of cellular physiology (reviewed in reference 52), among them initiation of cell division, which is blocked through the action of the SfiA (SulA) protein (27). Furthermore, nucleoids that contain chromosomes whose replication has been interrupted cannot be properly segregated during partition (discussed in reference 42), which complicates experiments in which the role of normally processed nucleoids is being investigated. Thus, the use of thymine-requiring mutants is limited by pleiotropic effects that may conceal the specific effects of inhibition of replication.

An alternative approach in cell cycle studies is to use conditional mutants affected in genes that are essential for ongoing chromosome replication (5, 28, 39). Examples of mutants in which replication rapidly stops after a shift to a nonpermissive temperature, quick-stop mutants (described in reference 32), are *dnaB* and *dnaE* mutants, in which DNA unwinding and polymerization, respectively, are blocked. As for the thymine-requiring mutants, the results with quick-stop mutants are affected by induction of the SOS response (52), and the incompletely replicated chromosomes cannot be properly segregated. Furthermore, inactivation of a temperature-sensitive gene product is usually achieved by shifting the growth temperature from 30 to 42°C. This induces the heat shock response, another set of functions that affect many cellular processes (reviewed in reference 40), including cell division.

A second class of mutants that can be used for cell cycle analysis are those that are affected in functions required only during initiation of replication, slow-stop mutants (reviewed in reference 32). This class mainly consists of mutants bearing alleles of the *dnaA* and *dnaC* genes that yield temperature-sensitive protein products. The blockage of initiation of chromosome replication does not induce the SOS response (38), and runout of replication occurs in the *dnaA* or *dnaC* mutants at the restrictive temperature, similar to when initiation is blocked by the addition of antibiotics (see above).

Temperature-sensitive *dnaA* mutants have been used for cell cycle analyses (26, 28, 39, 46). There are, however, some disadvantages with the use of conditional *dnaA* mutants. The DnaA protein binds to 9-mer sequences called DnaA boxes which are distributed throughout the chromosome. DnaA is a transcriptional repressor, and binding of DnaA affects a number of operons that contain DnaA boxes in the promoter region, including the operon encoding the *dnaA* gene itself and the *dnaN* gene. Thus, pleiotropic side effects are generated, further complicated by heat shock interference (see above)

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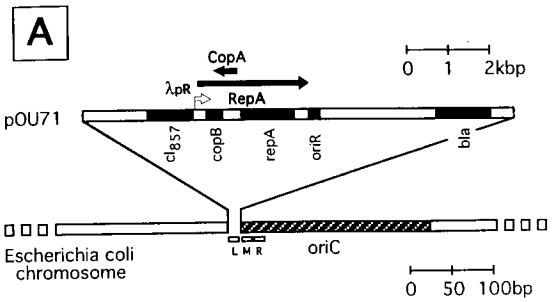
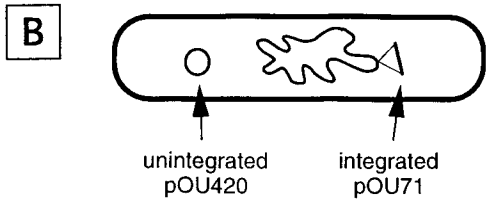
since large temperature shifts are also employed in the use of slow-stop mutants. Furthermore, the DnaA protein has been suggested to be the key element controlling the timing of initiation of chromosome replication in *Escherichia coli* (21, 36), and it is therefore desirable not to manipulate the *dnaA* gene or protein in studies of the coordination between chromosome replication and other processes.

The DnaC protein is involved in the early stages of initiation of chromosome replication (32). In certain *dnaC* mutants, initiation of chromosome replication does not take place at the restrictive temperature, which results in replication runout. Such *dnaC* mutants have been extensively used in different cell cycle studies (10, 44, 46, 50, 51). However, large temperature shifts are again required to inactivate the mutant protein in vivo. In addition, temperature-sensitive proteins do not always retain the wild-type properties even at the permissive temperature. As an example, initiation of chromosome replication in the temperature-sensitive *dnaA46* mutant shows an asynchrony phenotype at the permissive temperature in flow cytometric analysis (48), indicating that the timing of initiation during the cell cycle, or the coordination of multiple initiations in the cells, is deficient. Whether the properties of the mutant DnaC protein at the permissive temperature are similar to those of its wild-type counterpart is not known.

We wanted to create a situation in which initiation of chromosome replication could be specifically turned off without the use of mutants and without major side effects on cellular physiology. For this purpose, the *E. coli intR1* strains that have been constructed and characterized in our laboratory (41) were used. In *intR1* strains, various plasmid R1 derivatives are inserted into the origin of chromosome replication, *oriC*, such that *oriC* is inactivated; replication instead starts at the R1 origin, *oriR*, of the integrated plasmid (Fig. 1). Initiation of replication at *oriR* is negatively controlled by the antisense RNA CopA (43), and it is possible to shut off chromosome replication in the *intR1* strains by overproducing CopA (31). We reasoned that if the overproduction could be achieved with little other disturbance of the cellular physiology, it should be possible to analyze the specific effects of blocked chromosome replication on growth, nucleoid distribution, and cell division in actively growing populations of otherwise undisturbed *E. coli* cells.

Here, we report that after introduction into *intR1* strains of a plasmid (pOU420) from which CopA can be conditionally overproduced, initiation of chromosome replication can be specifically turned off by a minor reduction in growth temperature (3°C). This minimizes pleiotropic effects on cellular physiology from, for example, the SOS and heat shock regulons. The blockage of initiation of chromosome replication was found to be reversible, allowing an analysis of the cell division pattern after restart of replication. Furthermore, a single synchronous round of chromosome replication and cell division could be induced with a transient temperature shift. This allows studies of transcription, translation, and other activities as a function of different stages in the cell cycle. On the basis of the findings obtained, the rules that govern cell division and septum localization in *E. coli* are discussed.





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°C	RepA production from pOU71	CopA production from pOU420	Initiation of chromosome replication in EC::71CW(pOU420)
36	+	+++++++	Initiation blocked
37	++	++++++	Low efficiency
38	+++	+++++	Reduced efficiency
39	++++	++++	Close to normal level
40	+++++	+++	Close to normal level
41	++++++	++	Increased efficiency
42	+++++++	+	Over-initiation

FIG. 1. Strain EC::71CW containing plasmid pOU420. (A) The lower part shows the *oriC* region (hatched box) of the *E. coli* chromosome, into which the R1 miniplasmid pOU71 is integrated in the *intR1* strains, with DNA-unwinding elements shown as open boxes below *oriC*. The inserted pOU71 plasmid shown in the upper part replaces a 16-bp *Bgl*II fragment in *oriC*; this prevents initiation of replication from *oriC* by removing the leftmost of the three unwinding elements. Shown in pOU71 are also the CopB, RepA, and β-lactamase genes and the *c*I857 temperature-sensitive repressor allele (filled boxes), the RepA mRNA and the CopA antisense RNA (solid arrows), and the plasmid R1 origin (filled box). The position of the λ p_R promoter is shown as an open arrow. Note that different scales are used in the upper and lower parts. (B) Schematic drawing of a cell in strain EC::71CW(pOU420) containing plasmid pOU71 integrated into *oriC* in the chromosome and the unintegrated pOU420 plasmid. (C) The RepA protein, which is rate limiting for replication from the integrated pOU71 plasmid in the *intR1* strains, is produced from pOU71 in increased amounts at high growth temperatures. The antisense RNA CopA, which inhibits initiation of replication from pOU71, is produced from the unintegrated pOU420 plasmid in increased amounts at low growth temperatures. Increased production is schematically depicted by an increased number of plus signs. The production rates are idealized to illustrate the principle of regulation; i.e., they do not reflect experimentally determined rates. Thus, at 36 to 40°C, the change in the output of CopA from pOU420 probably dominates over the change in RepA production from pOU71 (see Materials and Methods). The combined effect on initiation of replication from the integrated pOU71 plasmid is indicated in the right-hand column.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids and strains used in this study are listed in Table 1. Plasmid pOU420 consists of a *Sal*I fragment from plasmid pOU82 (11) cloned into the *Sal*I site of pBR325 (18a). The fragment contains the λ p_R promoter and the λ *c*I857 temperature-sensitive repressor gene, cloned in front of the *copA* gene from plasmid R1. The two promoters are in opposite orientations, such that transcription from λ p_R enters the *copA* gene and interferes with transcription, and thus with production, of CopA RNA. Since the λ p_R promoter is controlled by the temperature-sensitive repressor, the promoter activity increases at higher temperatures, and the convergent transcription into the *copA* promoter therefore results in CopA RNA production gradually decreasing with increasing temperatures. At temperatures below 38°C, however, a high concentration of CopA is obtained within the cells, enhanced by the high copy number of the pBR325 vector replicon of pOU420 (Fig. 1). To introduce the origin region from an *intR1* strain into pOU420-containing strains (described below), selection for ampicillin resistance had to be used; therefore, the ampicillin resistance gene of pOU420 was inactivated. This was achieved by partial restriction cleavage with *Pvu*I, creation of blunt ends with T4 DNA polymerase, and self-ligation. The resulting Ap^s derivative of pOU420 was screened for temperature-dependent CopA synthesis by transformation into a strain contain-

TABLE 1. *E. coli* plasmids and strains used in this study

Plasmid or strain[a]	Parent	Relevant features[b]	Reference(s) or source
Plasmids			
pOU71	R1, phage λ, Tn*3*	R1 miniplasmid, Ap^r, replication Td	34
pOU420	pBR325 + pOU82	High-copy-number plasmid, Ap^r Cm^r, CopA production Td	Kenn Gerdes
pOU420Ap^s	pOU420	As pOU420, *bla* gene inactivated	This study
Strains			
EC1005		*metB1 nalA relA1 spoT1* λ^r F^-	19
EC::71CW	EC1005	EC1005 + Δ*oriC*::pOU71, Ap^r, replication Td, bidirectional chromosome replication	7
EC::71CW *dinD1*::*lac*	EC::71CW	EC::71CW + *dinD1*::*lac* Ap^r Km^r	This study
EC::71CW(pOU420)[c]	EC1005(pOU420)	EC1005(pOU420) + Δ*oriC*::pOU71, Ap^r Cm^r	This study
EC::71CW(pOU420)[c] *dinD1*::*lac*	EC::71CW(pOU420)	EC::71CW(pOU420) + *dinD1*::*lac*, Ap^r Cm^r Km^r	This study
EC::71CW(pOU420)[c] *sfiA*::Tn*5*	EC::71CW(pOU420)	EC::71CW(pOU420) + *sfiA*::Tn*5*, Ap^r Cm^r Km^r	This study
B/rA			24
MG1655			4, 29

[a] For further description of strains EC::71CW and EC::71CW(pOU420) and of plasmids pOU71 and pOU420, see Fig. 1, Materials and Methods, and Results. As for strain EC1005, *intR1* derivatives of strains B/rA and MG1655 were constructed and designated B/rA::71CW and MG::71CW. The nomenclature also follows that of the EC1005 series for derivatives of these strains containing plasmid pOU420, the *sfiA*::Tn*5* fusion, etc.

[b] Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; Td, temperature dependent.

[c] The Ap^s derivative of pOU420 was used.

ing plasmid pJL99 (35), which carries a *repA-lac* translational fusion. Since the CopA RNA inhibits translation of the *repA* mRNA, CopA production could be monitored by measuring β-galactosidase activity from the fusion. Thus, we chose isolates that gave dark blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates (37) at 42°C and light blue colonies at 30°C. Finally, plasmid pOU420Ap^s was transformed into strains EC1005, MG1655, and B/rA, selecting for chloramphenicol resistance. For simplicity, this Ap^s derivative is referred to hereafter as pOU420.

The construction and characterization of the *intR1* strain EC::71CW (Table 1; Fig. 1) has been described previously (7). In EC::71CW, the R1 miniplasmid pOU71 has been integrated into *oriC* such that *oriC* is inactivated and replication instead starts from pOU71. In this plasmid, the λ p_R promoter and the λ *c*I857 temperature-sensitive repressor gene are cloned in front of the promoter for the *repA* gene. With higher temperature, transcription from the λ p_R promoter increases, resulting in an increased production of the RepA protein. RepA is rate limiting for plasmid R1 replication, and therefore the frequency of initiation of chromosome replication in EC::71CW increases with temperature. Thus, chromosome replication in EC::71CW and CopA production in pOU420 (see above) are both controlled by the λ p_R promoter and the cI857 protein. This makes the replication initiation frequency in EC::71CW(pOU420) strongly temperature dependent, since in a temperature downshift there are both an increase in the production of the inhibitor of replication, CopA, and a decrease in the production of the positively acting RepA protein. In a temperature upshift, the opposite is true; i.e., the replication initiation frequency quickly increases as the inhibitor production is decreased while RepA production increases. Since the interaction between the CopA antisense RNA and the RepA mRNA follows apparent first-order kinetics (43), i.e., is primarily dependent only on the concentration of CopA unless the RepA mRNA is overproduced, the main effect at 36 to 40°C is probably due to the changes in the output of CopA from plasmid pOU420.

The origin region from an *intR1* strain containing pOU71 integrated into *oriC* was transferred by P1 transduction into the strains containing pOU420. The ampicillin resistance gene of pOU71 was used for selection, and the presence of pOU420 was ensured by concomitant selection for chloramphenicol resistance. The *dinD1*::*lac* fusion from strain DW8, originally from strain JH140 (22), and the *sfiA*::Tn*5* insertion from strain GC2508, originally from strain GC4540 (12), were also introduced into the various strains by P1 transduction, with selection for kanamycin resistance. Transformations and phage P1-mediated transductions were performed by standard techniques (37, 47).

Media, growth conditions, and optical density and CFU determinations. The bacterial strains were grown in M9 medium (47) supplemented with 0.2% (wt/vol) glucose. Unless otherwise stated, supplements were added at the following concentrations: ampicillin, 20 μg/ml; chloramphenicol, 50 μg/ml; kanamycin, 50 μg/ml; and methionine, 50 μg/ml. The cultures were incubated in thermostatically controlled water baths (Heto) with a maximum deviation of 0.2°C from the set temperatures or in incubation cupboards (Termaks) with similar precision in temperature control. Optical density was monitored with a Klett-Summerson colorimeter at 640 to 700 nm. The number of CFU per milliliter was determined by serial dilutions in prewarmed growth medium, followed by plating at 39°C on solid medium of the same composition as the liquid growth medium.

In all experiments in which exponentially growing cultures were required, initial overnight batch cultures were inoculated from solid media 2 days in advance. On the following day, serial dilutions from the overnight cultures were used to inoculate new liquid cultures. On the next morning, exponentially growing cultures with appropriate optical densities were chosen for shift experiments; dilution into fresh medium was avoided to obtain cultures as undisturbed as possible. Thus, before the shifts the cultures had normally been growing exponentially for more than 12 h.

In growth experiments in which the cultures were incubated for extensive time periods in the absence of chromosome replication (not shown), a subpopulation of small cells that rapidly outgrew the longer cells eventually appeared. The subpopulation consisted of cells that had escaped the replication inhibition, as judged by flow cytometric analysis. This population was therefore not further considered, but it should be pointed out that caution must be exercised in this type of experiment to avoid prolonged incubation or subcultivation, which may result in selection of populations with other properties than those of the original construct.

Measurement of SOS induction and β-galactosidase activity. Strains with or without the *dinD1*::*lac* fusion (see above) were harvested in exponential growth phase, transferred to petri dishes, and irradiated with UV light for 0, 30, or 90 s. β-Galactosidase activity was measured at different time points after the irradiation, and a substantial increase (see Fig. 5) was taken as evidence for expression of the fusion in response to induction of the SOS regulon after UV-induced DNA damage. Several independent isolates were tested and compared with controls lacking the fusion. Selected strains that showed increased β-galactosidase activity in response to irradiation were then used to monitor induction of the SOS regulon after temperature shifts (see the legend to Fig. 5). β-Galactosidase activity was determined essentially as outlined by Miller (37).

Microscopic studies. For photomicrography, 0.1- to 0.5-ml samples containing fixed cells were centrifuged, washed in 1 ml of 0.9% NaCl, and recentrifuged. Part of the supernatant was removed, leaving 20 to 50 μl in the tubes. The cells were resuspended in the remaining buffer, and 5- to 10-μl aliquots were spread on microscope slides covered with thin layers of 1% agar containing 0.9% NaCl and 0.5 μg of DAPI (4′,6-diamidino-2-phenylindole) per ml. With this staining technique, extensive nucleoid condensation is avoided, to minimize overestimation of the nucleoid-free areas within the cells (1).

The cells were analyzed in a Nikon Optiphot-2 combined phase and fluorescence microscope. The images were digitalized with a charge-coupled device (CCD) camera (Sony Instruments) connected to a computerized image analysis system (software and hardware from Bergströms Instruments). The digitalized images were printed with a Sony UP-860/CE video printer.

For cell length measurements, the cell areas were automatically measured with the image analysis system. Since the average cell width was nearly constant, about 0.8 μm (not shown), the average cell lengths were obtained by dividing the area measurements by 0.8. The proportion of septated cells (Fig. 3 and 6) was determined manually from the digitalized images, and the lengths of unseparated daughter cells in septated cells (Fig. 10) were determined with a length measurement tool provided with the software.

Flow cytometry. For flow cytometry (49), 0.15- to 0.30-ml samples were collected from the bacterial cultures, centrifuged, and resuspended in 0.3 ml of 10 mM Tris (pH 7.4). For fixation, 1 ml of 95% ethanol was added. Prior to

measurement, the cells were pelleted by centrifugation, the ethanol was removed, and the cells were washed in 1 ml of a solution containing 10 mM Tris (pH 7.4) and 10 mM $MgCl_2$. After centrifugation, all but 50 to 200 μl of the supernatant was removed, and the cells were resuspended in the remaining liquid. For staining, 30 μl of the cell suspension was mixed with an equal volume of a solution containing ethidium bromide (40 μg/ml) and mithramycin A (200 μg/ml) dissolved in the same buffer. In each sample, plastic calibration beads (Bio-Rad) with a diameter of 1.5 μm were included as an internal standard to standardize the scale for comparisons between samples and to monitor instrument performance. The beads were of uniform size and fluorescence, with coefficients of variation of <0.7% in diameter and <1.5% in fluorescence. The sample analysis was performed with an Argus flow cytometer (Skatron).

Runout of chromosome replication. To analyze the number of replication origins by flow cytometry, rifampin (300 μg/ml) and cephalexin (10 μg/ml) were added to 2-ml samples immediately after removal from cultures shifted between the different growth temperatures (Fig. 8; these drugs were not added in the analyses shown in Fig. 4, 7, and 9). Rifampin blocks initiation of chromosome replication from the origin of plasmid R1 (14), and cephalexin blocks further cell division that otherwise will result in an underestimation of the number of origins per cell (49). The number of chromosome equivalents per cell after runout is a measure of the number of replication origins that were present at the time of drug addition (further explained in Results). The samples were incubated at 39°C for 2 h to allow the replication forks to run to completion. Fixation, staining, and flow cytometry were performed as described above.

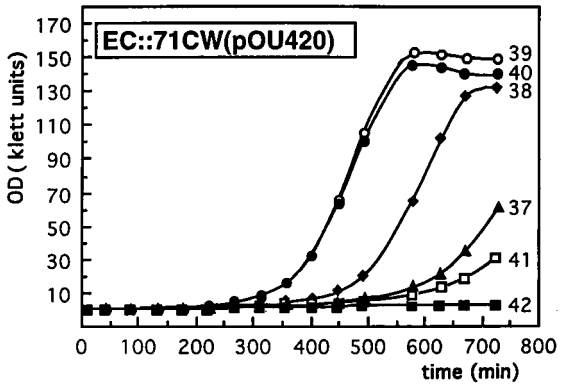

FIG. 2. Growth of strain EC::71CW(pOU420) at different temperatures. Optical density (OD) measurements of strain EC::71CW containing plasmid pOU420 (see Fig. 1). The strain was exponentially grown overnight (see Materials and Methods) in M9 medium at 39°C, with selection for plasmid pOU420. At time zero, the culture was split and shifted to the temperatures indicated.

RESULTS

Initial characterization of *E. coli* strains in which initiation of chromosome replication can be specifically blocked. In an *intR1* strain, a derivative of plasmid R1 is inserted into *oriC* in the chromosome (Fig. 1). After integration, chromosome replication is controlled by the inserted plasmid, while *oriC* no longer functions. When the integrated plasmid is the R1 mini-derivative pOU71, as in the *intR1* strain EC::71CW (7), the rate of initiation of chromosome replication is temperature dependent (Fig. 1A; Table 1). At temperatures below 35°C, the chromosome copy number is close to that of a wild-type strain replicating from *oriC*. With increasing temperature, the replication initiation frequency gradually increases because of more efficient production of the mRNA for the initiator protein RepA (further explained in Materials and Methods), which is rate limiting for initiation of replication from *oriR* (35). Thus, at 38°C, cellular growth and survival start to decline, and temperatures above 38°C are inhibitory to growth because of overreplication of the chromosome (7).

Initiation of replication from the R1 basic replicon is negatively controlled by the antisense RNA CopA which specifically binds to the RepA mRNA (Fig. 1A), thereby inhibiting initiation of replication by preventing translation of the RepA reading frame (43). In plasmid pOU420, derived from pBR325 (see Materials and Methods), CopA production can be controlled by temperature such that increasing amounts of CopA are produced as the temperature is lowered (Fig. 1C; Table 1). Thus, chromosome replication can be turned off in *intR1* strains with the use of plasmid pOU420, by overproduction of CopA (31). By combining strain EC::71CW with plasmid pOU420 (Fig. 1B), a double effect can be obtained. As the temperature is lowered, the RepA production from pOU71 decreases while simultaneously the CopA synthesis from pOU420 increases (Fig. 1C), with the latter effect probably dominating (see Materials and Methods). Thus, a large negative effect on initiation of chromosome replication can be obtained from a small temperature shift.

Plasmid pOU420 was first transformed into the strain to be used, whereafter the *intR1* origin region was introduced by P1 transduction. The transduction mixtures were plated at different temperatures, selecting for both the unintegrated pOU420 plasmid and the integrated pOU71 plasmid. Large numbers of transductants were obtained at temperatures ranging from 38 to 40°C, whereas lower or higher temperatures resulted in a drastically reduced transduction frequency (not shown). The growth characteristics of a transductant isolated at 39°C are shown in Fig. 2. With selection for both the unintegrated plasmid and the integrated plasmid, optimal growth was restricted to a temperature interval of only 2°C (39 and 40°C), with a doubling time of about 43 min. The culture shifted to 38°C required several hours more of incubation before stationary-growth phase was reached, and a shift to 37°C resulted in a further reduction in growth. At 41°C, growth was even less efficient than at 37°C, and at 42°C, little increase in optical density took place.

The results (Fig. 1C) were interpreted as follows. The reduced survival and growth at low temperatures was attributed to inhibition of chromosome replication through massive production of CopA from the high-copy-number plasmid pOU420. At 39 and 40°C, the CopA production from pOU420 was not sufficient to inhibit initiation of replication but still high enough to counteract the increased RepA mRNA production from pOU71 that results in overreplication in EC::71CW lacking pOU420. Thus, in the presence of pOU420, no growth inhibition was apparent at these previously lethal temperatures, presumably because of a new balance between the CopA antisense RNA and the RepA mRNA within the cells, such that a normal initiation frequency from the R1 basic replicon was obtained. At 42°C, the CopA production was insufficient to counteract the further increase in production of the RepA mRNA from pOU71, and as with EC::71CW lacking pOU420, this temperature was lethal because of overreplication of the chromosome.

The experiments demonstrated that growth of EC::71CW containing plasmid pOU420 was strongly affected when the growth temperature was lowered by only 1 to 2°C, presumably because of the combined effect of an increased CopA synthesis and a decreased RepA synthesis.

Effects of specific inhibition of initiation of chromosome replication in exponentially growing *E. coli*. The constructed system allowed a detailed analysis of the effects of a block in initiation of chromosome replication in exponentially growing *E. coli* in which other processes continue uninhibited. Various growth and cell parameters were therefore analyzed during a temperature downshift of 3°C.

When the growth temperature was lowered from 39 to 36°C, the growth rate was found to gradually slow from a doubling time of 43 min to a significantly lower rate, approaching a linear instead of exponential increase (Fig. 3A). The CFU

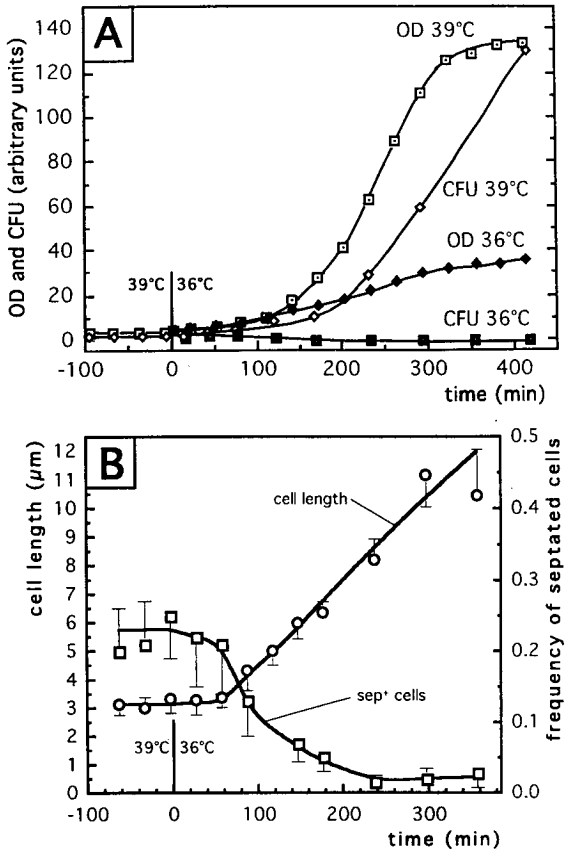

FIG. 3. Growth and cell characteristics of strain EC::71CW(pOU420) after a shift of the growth temperature from 39 to 36°C. Strain EC::71CW containing plasmid pOU420 was exponentially grown overnight (see Materials and Methods) in M9 medium at 39°C. At time zero (indicated by a vertical line), part of the culture was shifted to 36°C and the rest was further incubated at 39°C. (A) Optical density (OD) and CFU measurements from the two cultures. (B) Cell length and frequency of septated cells in the culture shifted to 36°C. Cell length and septation were determined by computer-aided image analysis as described in Materials and Methods.

values ceased to increase after about 45 min, as expected if no new rounds of chromosome replication were being initiated, and a high-resolution analysis (not illustrated) showed that after this a reduction in CFU took place. The reduction indicated that when initiation of replication had been blocked for more than 45 min, cell viability decreased, whereas the cells studied by microscopic analysis (described below) continued to grow, and in liquid culture the optical density continued to increase. Also, when the temperature shift was reversed (see below), most cells were capable of restarting chromosome replication. The reason for the discrepancy between the CFU data and the other analyses is not clear. One possibility is that the cells became more fragile during the period of growth in the absence of replication and thus more sensitive to the plate spreading procedure.

Microscopic analysis revealed that the average cell lengths remained constant until about 60 min after the downshift (Fig. 3B). Then, an increase in length of approximately 3 μm/100 min began. The proportion of chromosomeless cells was about 2%. Visible septation could be detected in about 20% of the cells at 39°C (Fig. 3B). After the shift, this proportion gradually decreased to a residual level of 2% after 240 min. These residual divisions gave rise to some chromosomeless cells, thereby increasing their proportion in the culture over time.

At 39°C, the cells displayed narrow size and DNA content distributions in flow cytometric analysis (Fig. 4A and B) and regular cell and nucleoid morphology when studied by phase-fluorescence microscopy (Fig. 4C). However, all distributions were somewhat broader than for a wild-type *E. coli* strain (not shown) because of the random initiation of chromosome replication during the cell cycle in *intR1* strains (31). A downshift in growth temperature to 36°C for 2 h, enough to allow ongoing rounds of replication to finish, had dramatic effects on all parameters studied. At this stage, most cells contained one or two fully replicated chromosomes (Fig. 4E), showing that runout of DNA replication indeed had occurred and that new initiations did not take place. This finding also shows that the R1 origin in the integrated pOU71 plasmid was the only functional origin; upon inhibition of initiation by overproduction of CopA, no other origin activity was detectable. The average DNA content decreased (compare Fig. 4E and B), indicating that cell division took place after the runout of the ongoing replication, thus reducing the DNA content per cell. After this division, most cells contained a single, centrally located nucleoid (Fig. 4F), although cells with more than one nucleoid and DNA-less cells could also be detected. The average cell size was greater than that in the unshifted population (compare Fig. 4D and F with Fig. 4A and C), showing that after completion of chromosome replication and subsequent division, further cell division was inhibited.

The experiment was repeated in other genetic backgrounds to investigate the generality of the observations (not shown). In *intR1* derivatives of strain MG1655, which is closely related to the original wild-type *E. coli* K-12 isolate (4; however, also see reference 29), the results were similar to those obtained with the EC1005-derived strains. This finding showed that the mutations carried by the EC1005 strains that might have influenced the results, particularly the *relA* mutation, had little effect on the outcome of the experiment. When the *E. coli* B/rA (24) genetic background was used, the doubling time at 39°C was longer than that for the K-12 derivatives, about 55 min instead of 43 min. The cell size and DNA content distributions were generally broader at all temperatures, and the proportion of cells with several chromosome equivalents and nucleoids was higher than in the K-12 derivatives. A shift to 36°C had, however, essentially the same effect as in the K-12 background. We attribute the general differences mainly to a considerably more disturbed coordination between replication and cell division in *intR1* derivatives of *E. coli* B/rA than in K-12 derivatives. The reason for this is not known, although the reported differences in the lengths of the cell cycle periods between various *E. coli* strains (3) may be important. Also, when the integrated R1 plasmid is introduced by P1 transduction, the region surrounding *oriC* in the *E. coli* K-12 donor strain replaces the corresponding region in the B/rA recipient strain, which might have adverse effects due to differences in the organization of this region between the strains.

The main conclusion from all experiments was, however, the same: cell division continued as long as ongoing rounds of chromosome replication were still being terminated in the population. After runout and subsequent division, however, further cell division was inhibited while cellular growth continued, resulting in an increased average cell size and a decrease in the number of cells with ongoing septation.

Role of the SOS response in cell division inhibition. When *E. coli* chromosome replication is interrupted or the DNA is damaged, the SOS system is induced (reviewed in reference 52). This results in synthesis of the SfiA (SulA) protein, which blocks cell division by preventing the formation of the ring of FtsZ molecules, an important early step in division (8). Several

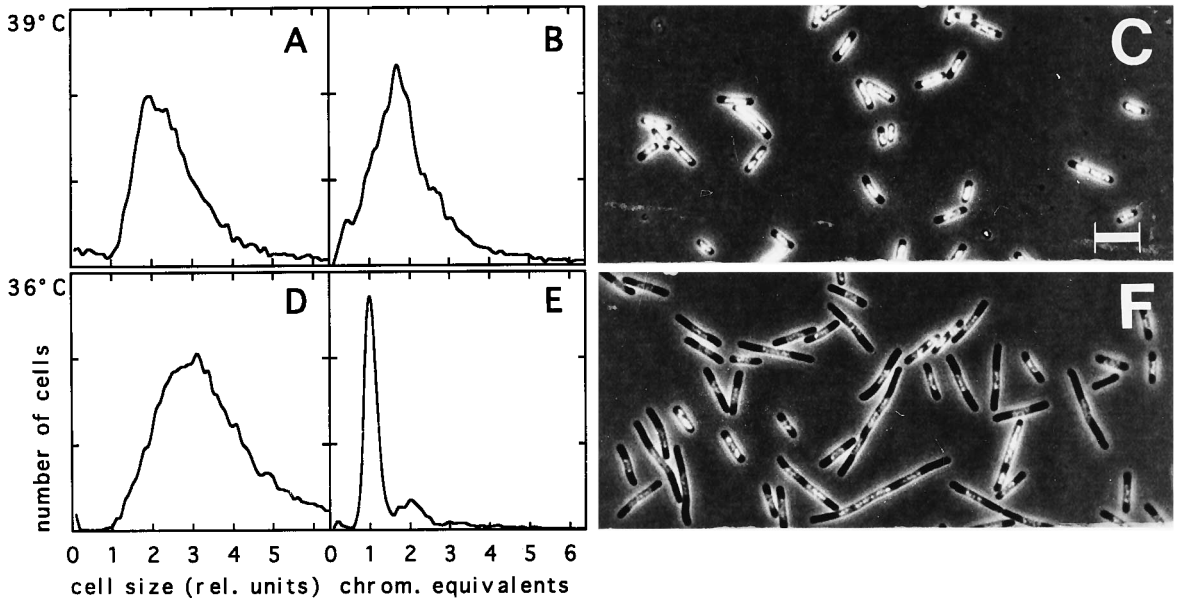

FIG. 4. Cell size, DNA content, cell morphology, and nucleoid distribution before and after shifting the growth temperature from 39 to 36°C. Strain EC::71CW(pOU420) was exponentially grown at 39°C overnight, whereafter it was shifted to 36°C and grown for an additional 2 h. The first two columns show flow cytometry measurements of cell size (light scatter; A and D) and DNA content (fluorescence; B and E) distributions, with the growth temperatures indicated at the left. DNA-less (nonfluorescent) cells were not recorded. (C and F) Photomicrographs of the same samples. The cell morphology and nucleoid distribution were visualized by simultaneous phase and fluorescence microscopy after DAPI staining (see Materials and Methods). The bar equals 4.8 μm.

experiments were performed to test whether the observed division inhibition was mediated by the SOS system.

An SOS-inducible *dinD1*::*lac* fusion was introduced into the *intR1* strains by P1 transduction, and irradiation with UV light confirmed that induction of the SOS response resulted in an increase in the β-galactosidase activity from the fusion (Fig. 5). A control strain lacking the fusion showed a low basal level of β-galactosidase activity, indicating that the chromosomal *lac* operon did not disturb the measurements. Strain EC::71CW (without plasmid pOU420) containing the fusion showed a higher level of β-galactosidase activity than the control without the fusion at 34°C, indicating that the SOS system is partially induced in this strain during exponential growth. A shift to 39°C resulted in a drastic increase in β-galactosidase activity, showing that overreplication resulted in significant induction of the SOS response, presumably through generation of complex unfinished replication structures when the cells filled up with chromosome DNA. The same strain containing plasmid pOU420 showed a low basal level at the permissive temperature, 39°C, and no difference was observed after a shift to 34°C. Thus, the SOS system was not measurably induced by the inhibition of initiation of DNA replication in this strain. Similar results were obtained when the experiments were repeated with independent clones and *intR1* strains derived from strain MG1655 (not shown). When an allele of the *sfiA* gene carrying a transposon insertion that prevents formation of functional SfiA protein was introduced into EC::71CW(pOU420), inhibition of chromosome replication resulted in a block of cell division that in microscopic analysis was indistinguishable from that obtained when the wild-type *sfiA* allele was present (not illustrated).

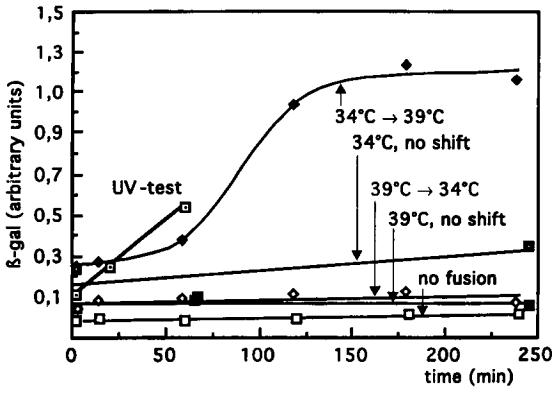

FIG. 5. Induction of the SOS response after temperature shifts. Strains containing a *dinD1*::*lac* fusion were grown in M9 medium at 34°C (strains without plasmid pOU420) or 39°C (strains containing pOU420). At time zero, part of each culture was shifted to the reciprocal temperature, and the rest was further incubated at the original temperature. At different times after the temperature shifts, β-galactosidase activity was measured. Designations (see text for details): UV-test, control of induction of the SOS response by UV irradiation; 34°C→39°C, strain EC::71CW without plasmid pOU420, shifted from 34 to 39°C; 34°C, no shift, same strain kept at 34°C; 39°C→34°C, strain EC::71CW containing plasmid pOU420, shifted from 39 to 34°C; 39°C, no shift, same strain kept at 39°C; no fusion, same strain but lacking the *dinD1*::*lac* fusion, shifted from 39 to 34°C.

We conclude that SOS- and SfiA-mediated division inhibition was not the main reason for the cessation of division that was observed after runout of chromosome replication.

Effects of resumption of initiation of chromosome replication. After upshift to 39°C, the excess CopA produced at 36°C in strain EC::71CW(pOU420) is rapidly degraded since the in vivo half-life of the CopA RNA is short (1 to 2 min [49a]). Therefore, the CopA concentration quickly decreases and initiation of replication from the R1 origin of the integrated pOU71 plasmid can resume. This allowed an analysis of the effects of resumed chromosome replication and nucleoid processing on cell division and on septum localization. Therefore, cultures downshifted to 36°C were shifted back to 39°C. Again, initiation of chromosome replication was expected to be the

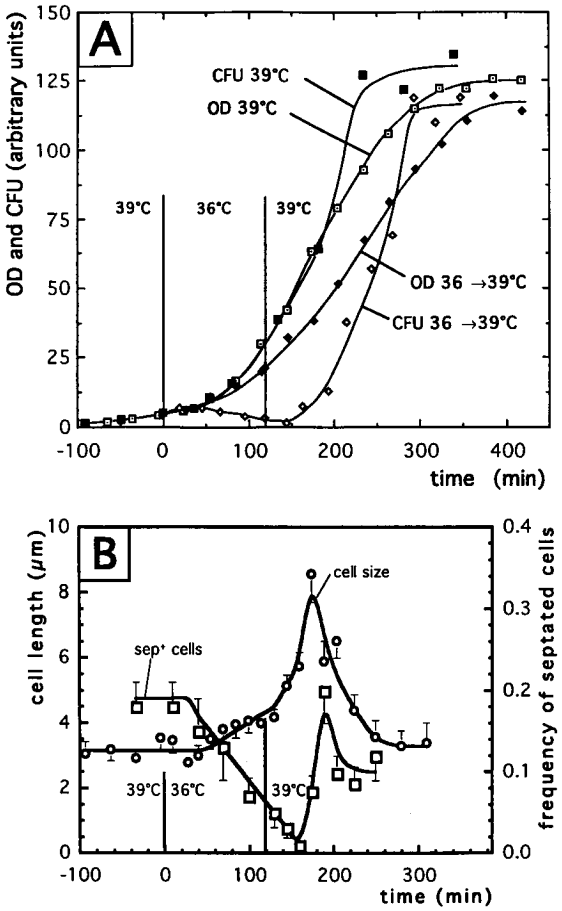

FIG. 6. Growth and cell characteristics of strain EC::71CW(pOU420) after temperature upshifts from 36 to 39°C. Strain EC::71CW containing plasmid pOU420 was exponentially grown overnight in M9 medium at 39°C, and at time zero part of the culture was shifted to 36°C (see legend to Fig. 3). After 120 min, part of the culture at 36°C was shifted back to 39°C. Temperature shift times are indicated by vertical lines. (A) Optical density (OD) and CFU measurements. (B) Cell length and frequency of septated cells. Designations: 39°C, culture grown at 39°C without temperature shift; 36°C→39°C, culture shifted to 36°C and then shifted back to 39°C after 120 min.

process mainly affected by the shift; the cellular physiology should not be largely altered by an increase in the growth temperature of 3°C.

Growth and CFU were measured as in the downshift experiment (see above). After removal of the replication initiation block by raising the growth temperature, rapid growth and the increase in CFU resumed after a time delay (Fig. 6A). In higher-resolution analysis (not shown), the CFU values initially increased more than could be accounted for by the increase in the number of genome equivalents (see below). Thus, the decrease in CFU that was observed after runout of replication in the downshift experiments (see above) was reversed, indicating that in the absence of chromosome replication, colony-forming ability declined more than could be explained by the absence of replication alone. The majority of the cells were, however, still viable, since colony-forming ability was restored when replication was restarted.

The kinetics of changes in cell size and septation frequency in the cultures were also analyzed (Fig. 6B). The initial part of the curve, i.e., the downshift part, was similar to that in the first downshift experiment (Fig. 3). After the return to 39°C, the cells continued to increase in length for about 60 min, whereafter a dramatic decrease took place. Also, the frequency of septated cells continued to decrease for 40 min after the upshift, whereafter the proportion rapidly increased; a virtual burst of septation could be observed at this stage.

The resumption of DNA replication and the effects on cell morphology and nucleoid distribution were studied by flow cytometry and phase-fluorescence microscopy. The DNA content of the cells increased after the upshift (Fig. 7B, E, H, and K), confirming that DNA replication had restarted. In the phase-fluorescence studies, a significant increase in DAPI fluorescence was apparent (Fig. 7C, F, I, and L), and the area that was occupied by DNA increased. The cell sizes continued to increase for about 60 min after the upshift to 39°C (Fig. 7A, C, D, F, G, and I). After this, however, a population of smaller cells appeared (Fig. 7J and L), showing that the septation increase detected after 40 min (Fig. 6B) had resulted in a corresponding increase in cell division. Most of the small cells had a DNA content of more than 1.5 genome equivalents (not shown), indicating that more than one round of chromosome replication had been initiated during the time elapsed since the temperature upshift.

The kinetics of initiation of DNA replication after the upshift to 39°C were analyzed in more detail. Samples were removed at different time points after the upshift, and cephalexin and rifampin, which inhibit septation and initiation of replication, respectively, were added. Replication was allowed to finish (runout; see the introduction), and the number of fully replicated chromosomes in each cell was then determined by flow cytometry. This number reflects the number of origins of replication per cell at the time of the drug additions, provided that the cells did not divide during the runout period. The initiation kinetics could thus be monitored by comparing the relative number of cells with different numbers of fully replicated chromosomes after the runout. Initiation was found to take place within 10 min after the upshift in most of the cells containing one chromosome (Fig. 8; compare relative heights of the peaks corresponding to one and two chromosome equivalents among the panels), despite the fact that initiation of replication in plasmid R1 is random during the cell cycle (20, 31). The cells were large after growth at 36°C (see above), with the result that the copy number of the single integrated R1 plasmid, measured as molecules per cell volume, was low. Therefore, the probability for initiation was high in all cells after the upshift, explaining the initiation synchrony. Further initiations occurred successively, increasing the relative height of the peaks corresponding to three or more chromosomes. Particularly, cells with four chromosomes, i.e., with four origins at the time of drug addition, gradually became more frequent than cells with three, five, or more origins.

Since initiation of chromosome replication was found to be essentially synchronous, an experiment was performed to obtain a population of cells going through a single synchronous round of chromosome replication and cell division. This was achieved by restricting the incubation at 39°C to 8 min only, whereafter the culture was returned to 36°C to inhibit further initiations. Flow cytometry revealed that this procedure indeed resulted in the DNA content in a majority of the cells containing one chromosome gradually increasing to two chromosomes over a time period of about 60 min (Fig. 9). The average size of the cells increased during the same time period, showing that cellular growth continued and that division still was inhibited. During this time period, a population of small cells gradually resolved from the elongated cells. This population was already present at the time of the shift, since little septation was detected in microscopic analysis during the first 40 min

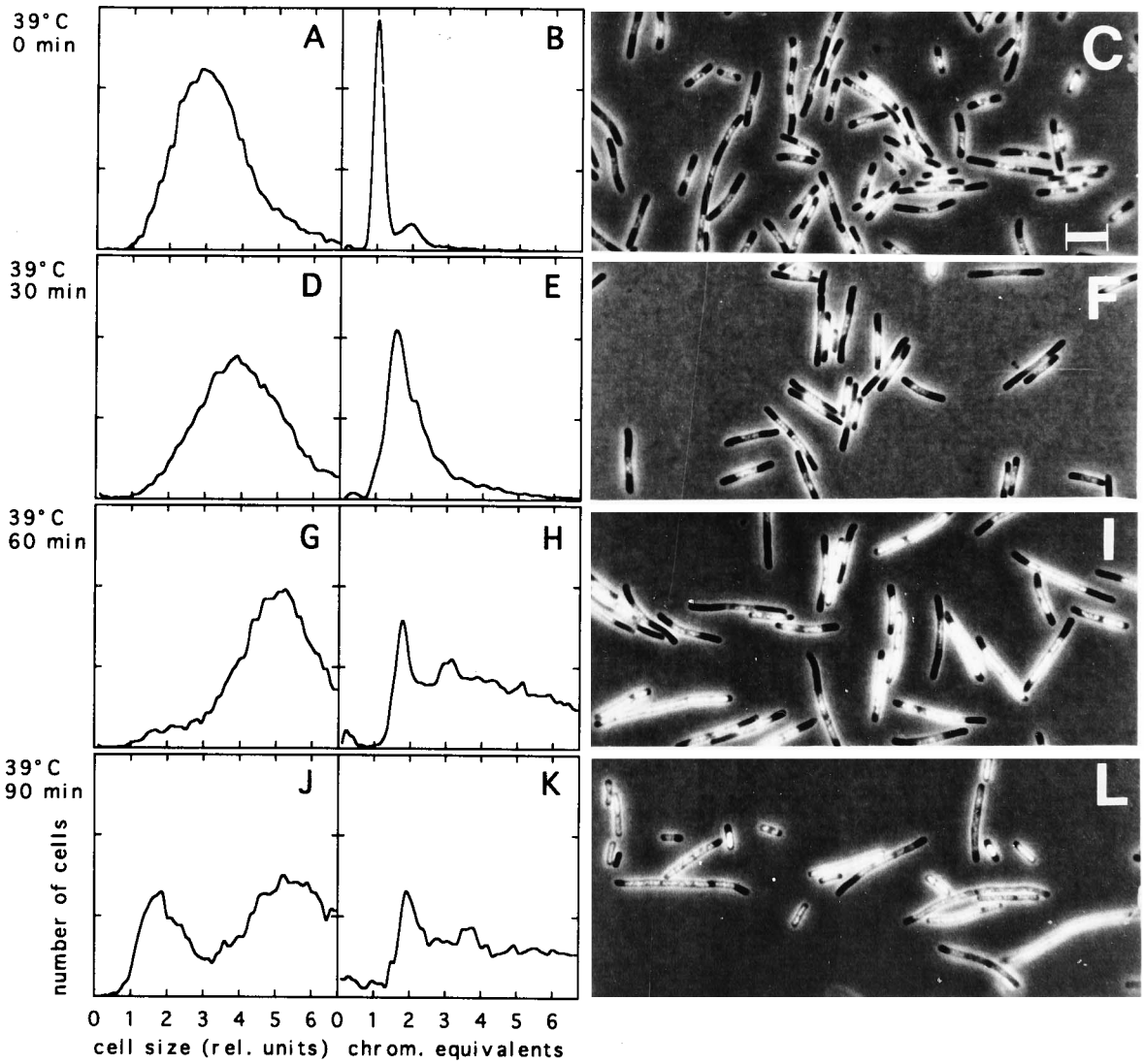

FIG. 7. Cell size, DNA content, cell morphology, and nucleoid distribution in strain EC::71CW(pOU420) during a temperature upshift from 36 to 39°C. Strain EC::71CW containing plasmid pOU420 was exponentially grown overnight in M9 medium at 39°C, shifted to 36°C, and shifted back to 39°C after 120 min at 36°C. Cell size and DNA content were analyzed by flow cytometry, and photomicrographs were taken with combined phase and fluorescence illumination (see legend to Fig. 4). (A to C) Samples collected after 120 min at 36°C (just before shifting the culture back to 39°C); (D to L) samples collected 30 (D to F), 60 (G to I), and 90 (J to L) min after the shift to 39°C. DNA-less (nonfluorescent) cells were not recorded in the flow cytometry analysis. The bar equals 4.8 μm.

after the shift (not shown; see Fig. 6B). In the 70-min sample, the population of small cells increased significantly, and in the DNA distribution, the peak corresponding to a single chromosome reappeared, showing that a round of cell division occurred around this time point. This population did not increase significantly at later time points, showing that the synchronous initiation of chromosome replication had resulted in a synchronous round of cell division after a time delay during which chromosome replication took place. Many cells remained at a DNA content corresponding to two chromosomes, however, showing that in these cells, a round of chromosome replication was not sufficient to enable the cells to carry out division or that they divided asymmetrically to produce one chromosomeless and one chromosome-containing cell. Cells containing two chromosomes at the time when replication was initiated (smaller peak to the right in the 0-min frame in Fig. 9) went through an increase in DNA content to about four chromosomes at 60 min, after which this peak decreased in relative height, indicating that part of this cell population also divided. To summarize, the transient shift to 39°C allowed a single synchronous round of chromosome replication and cell division to be initiated in the population of cells of different sizes.

Because of the inhibition of initiation of chromosome replication, the cells grown at 36°C had a drastically reduced DNA content, seen as areas without fluorescence in the micrographs (Fig. 4F and 7C). Consequently, large nucleoid-free stretches were available for division, and we reasoned that it should be possible to determine whether there were preferred sites for division within these stretches after restart of replication. The position of newly forming septa was scored during the burst of divisions that took place about 60 to 70 min after restart of replication, using phase-fluorescence microscopy and image analysis (nucleoid condensation is avoided in the staining technique; see Materials and Methods). As observed previously (Fig. 7), the nucleoids became extended before division took place, thereby reducing the sizes of the available DNA-free

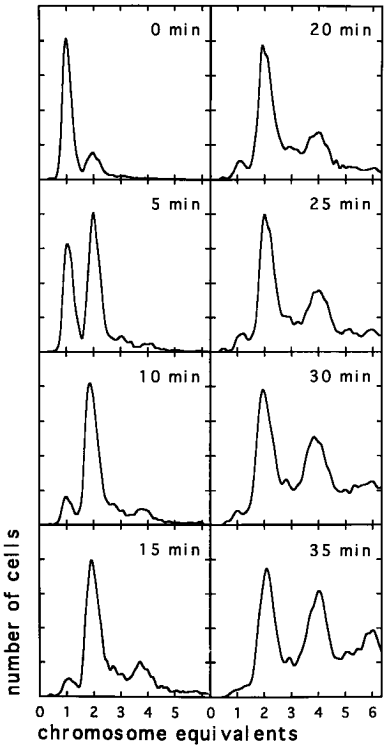

FIG. 8. Kinetics of restart of chromosome replication after a temperature upshift from 36 to 39°C. Strain EC::71CW containing plasmid pOU420 was grown exponentially overnight in M9 medium at 39°C, shifted to 36°C, and shifted back to 39°C after 120 min at 36°C. Samples were removed at different time points, treated with rifampin and cephalexin (see text) for an additional 120 min, and then analyzed by flow cytometry. In the DNA distributions shown, the absolute number of cells with a particular number of chromosome equivalents is not essential; it is the relative heights of the different peaks that should be compared. DNA-less (nonfluorescent) cells were not recorded.

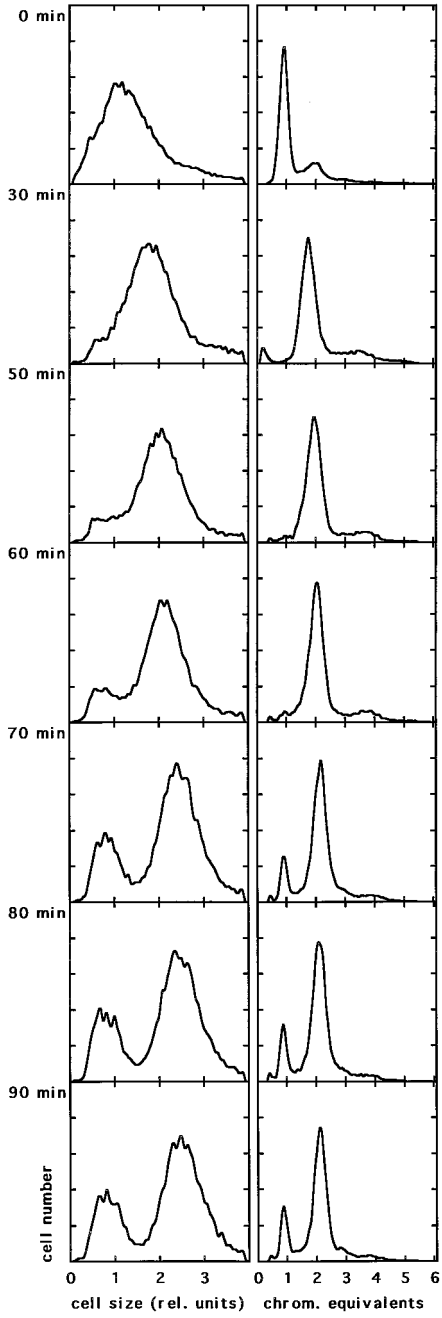

FIG. 9. Induction of a single synchronous round of chromosome replication and cell division. Strain EC::71CW containing plasmid pOU420 was grown exponentially overnight in M9 medium at 39°C and shifted to 36°C for 120 min. A single synchronous round of chromosome replication was initiated by shifting the culture (at time zero) to 39°C for 8 min, after which it was returned to 36°C to inhibit further initiations. Samples were removed at different time points and analyzed by flow cytometry. In the cell size and DNA distributions, it is the relative height of the different peaks that should be compared (see legend to Fig. 8). DNA-less (nonfluorescent) cells were not recorded.

stretches. Examples of different classes of septation events are given in Fig. 10A, e.g., central (frames 6 to 8) and asymmetric (frame 4) septation between segregated nucleoids, as well as formation of DNA-less cells varying in size (frames 1, 2, 3, and 5). The proportion of cells with septa located between segregated nucleoids or near a nucleoid was high (not shown), although occasionally septation took place at some distance from the nearest nucleoid (frame 5).

The relative placements of the septa along the length axis of the cells are shown in Fig. 10B to D, in which the length of the longer of the daughter cells (cell 2) is plotted against that of the shorter (cell 1). In an unshifted population growing at 39°C, the septation events preferentially (>90%) took place in the center of the cells, yielding a 1:1 ratio between the daughter lengths (Fig. 10B), and few chromosomeless cells were formed. In a population grown at 36°C for 120 min and then shifted back to 39°C (Fig. 10C), the septation events after the upshift fell into two main classes, corresponding to a ratio of 1:1 (about 25% of the septations) or 1:3 (50%) between the daughter cell lengths. Few septations gave rise to a length ratio of 1:2. Strikingly, the shorter cell (cell 1 axis) was usually of a length (1.5 to 3.5 μm) close to that of unshifted cells growing at 39°C (Fig. 10B; see also Fig. 7J and L). Thus, septation events that resulted in one daughter cell of approximately normal length were significantly more frequent than other septations. In addition, these normal-sized cells usually contained DNA (Fig. 10A, frames 4, 6, and 7). A significant fraction, about 20% of the septation events, gave rise to chromosomeless cells (Fig. 10A, frames 1, 2, 3, and 5; open circles in Fig. 10C). The newborn chromosomeless cells were often shorter than the shortest chromosome-containing cells, and a large length variation was observed. Formation of chromo-

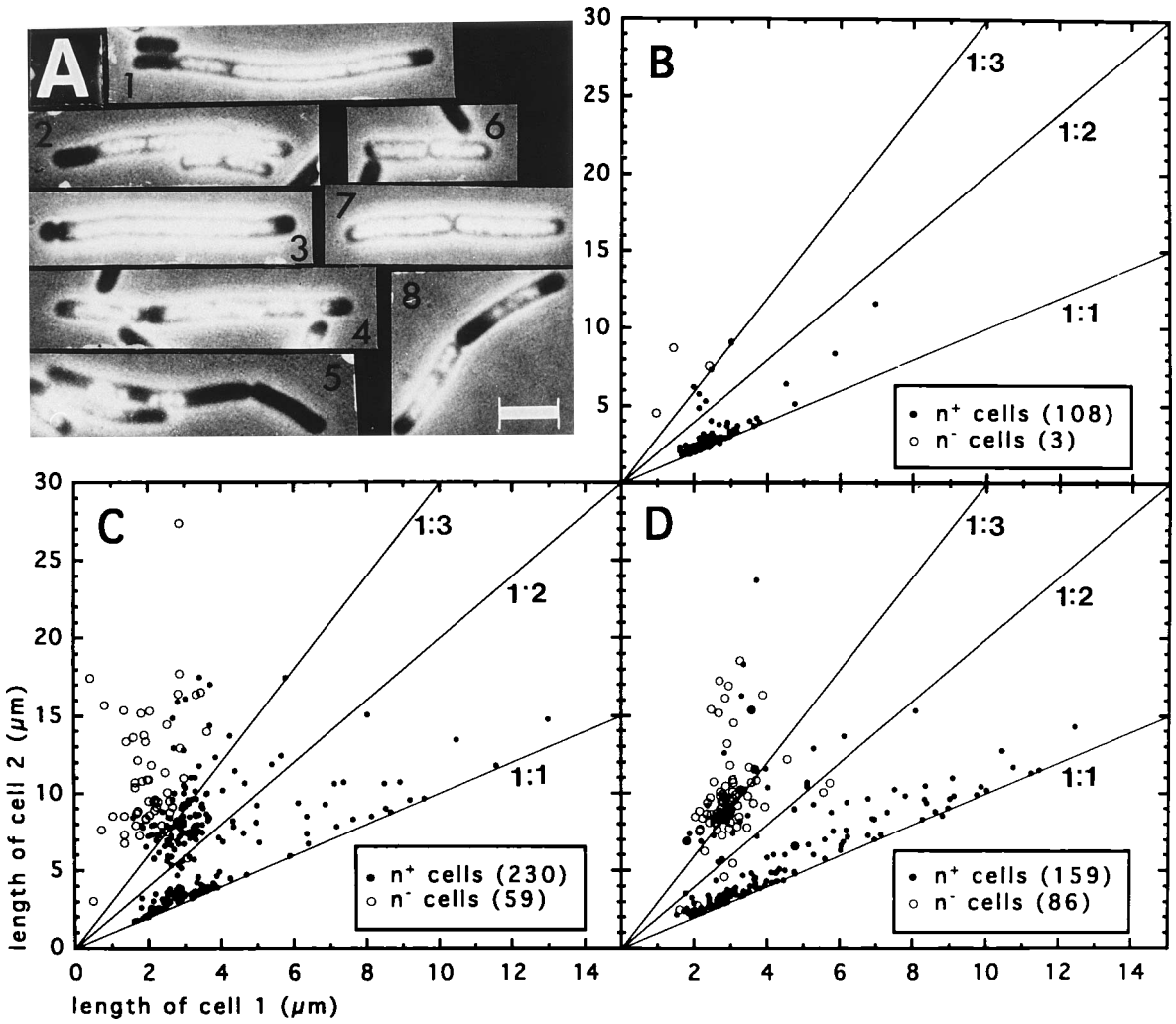

FIG. 10. Septum localization patterns after temperature upshifts from 36 to 39°C. Strain EC::71CW containing plasmid pOU420 was exponentially grown overnight in M9 medium at 39°C, shifted to 36°C, and then shifted back to 39°C. At different time points after the upshift, samples were stained such that nucleoid condensation was avoided, and the septum localization patterns were studied by image analysis (see Materials and Methods). (A) Phase-fluorescence micrographs of cells from the same experiment as in panel C. Note that the frames were chosen to illustrate different types of septation events; they do not reflect their relative abundance. The bar equals 2.4 μm. (B to D) The lengths of the two daughter cells produced in each septation event plotted against one another, with the shorter cell always chosen for the *x* axis (cell 1). The straight lines represent different length ratios between the two daughter cells. (B) Culture grown at 39°C without shifts; (C) same culture shifted to 36°C for 120 min and then shifted to 39°C and sampled after 54, 69, and 84 min; (D) as panel C, but the culture was returned to 36°C after 8 min at 39°C and sampled after 51 min. Note that in this frame, the apparent cluster of filled circles along the 1:3 line is in fact composed mostly of a large number of closely spaced open circles. Symbols: ●, both daughter cells contained nucleoids; ○, one nucleoid-free cell formed. In all frames, the DNA-less cell was invariably the shorter of the two daughter cells.

someless cells was not observed in centrally dividing cells (1:1 ratio).

The analysis was repeated for a population in which only a single synchronous round of chromosome replication was allowed to take place (see above). The main differences compared with the experiment in which multiple initiations were allowed were as follows (Fig. 10D). A majority, about 80%, of the septations that resulted in two DNA-containing daughter cells took place centrally in the cells in the single-round experiment, resulting in a 1:1 ratio. The corresponding figure for the multiple-initiation experiment was approximately 40%. Thus, central septations between separated nucleoids were favored after a single initiation of replication, or alternatively, asymmetric septations between separated nucleoids were favored after multiple initiations. The proportion of chromosomeless cells within the group of divisions that yielded a 1:3 ratio increased, from about 25% in the experiment in which multiple rounds took place (Fig. 10C) to approximately 75% in the single-round experiment (Fig. 10D). (This difference is obscured by the closely spaced dots in the 1:3 group in Fig. 10D; see figure legend.) Thus, in the single-round experiment, a ratio of 1:3 usually resulted in one chromosomeless cell, whereas in the multiple-initiation experiment, both daughter cells more often contained DNA. Interestingly, the cell size distribution for the chromosomeless cells was shifted toward larger values in the single-round experiment: no chromosomeless cells smaller than the smallest DNA-containing cells were detected.

In conclusion, resumption of chromosome replication resulted in a return to rapid increase in optical density and CFU as well as in a large increase in the frequency of septated cells. This finding demonstrates that normal growth and cell division

are directly correlated to ongoing replication and nucleoid processing. The septation patterns indicated that there were preferred locations for cell division along the length axis of the cells, and one of the newborn cells in each pair was often of normal length and DNA content. The septation patterns differed depending on whether multiple rounds or a single round of chromosome replication was induced.

DISCUSSION

We have constructed *intR1* strains in which initiation of chromosome replication in growing *E. coli* cells can be specifically turned off and on by changing the growth temperature by only 3°C. The large effect of this small temperature shift is due to the combined effect of changing the rates of synthesis of the positively acting initiator protein for replication in these strains, RepA, and the inhibitor of initiation of replication, the CopA RNA. In contrast to other methods of turning replication off and on (see the introduction), other aspects of cellular physiology are not interfered with, and the 3°C temperature changes are likely to have minor effects on growth and division relative to the dramatic specific effects of the inhibition of replication.

Blockage of initiation of chromosome replication resulted in runout of replication. After completion of ongoing rounds of replication and subsequent cell division, further divisions were inhibited. This was shown neither to be due to induction of the SOS regulon nor to be mediated by the SOS-inducible division inhibitor SfiA (SulA). Cellular growth continued in the absence of cell division, resulting in the formation of elongated cells and filaments, and a majority of the cells ended up with a single centrally located nucleoid, usually consisting of a single chromosome. The growth rate gradually declined and approached a linear instead of exponential increase after some time.

When DNA replication was restarted by returning the growth temperature to 39°C, resumption of cell division was observed after more than 1 h, and the cultures returned to rapid increase in optical density and CFU. The divisions appeared to occur at preferred locations along the cells, and often one of the daughter cells was of normal size and DNA content. With a transient temperature upshift, it was possible to induce a single synchronous round of chromosome replication followed by a synchronous round of cell divisions after 60 to 70 min, which resulted in some differences in the septation pattern (discussed below) from that when multiple rounds were allowed.

In discussing the results and the regulation of cell division, it is important to distinguish between different possibilities for how division sites may be formed and activated during the cell cycle. First, specific division sites may not exist; divisions may simply be confined to regions in the cell that are large enough to allow the septum machinery to act and that are devoid of chromosome DNA or other structures that might prevent division (39, 50). Alternatively, specific division sites may be generated during cellular growth, e.g., when chromosome replication is initiated or terminated, or when nucleoid partition occurs. It is also possible that the division cycle is independent of the replication cycle (42), such that generation of division sites is not linked to any particular chromosome replication or nucleoid-processing event (23, 26). Regardless of mechanism of site formation, a second level of control may also exist: it is possible that a division site becomes available, or activated, only after detachment of an associated nucleoid (30).

The nature of the division inhibition that took place in the experiments reported here is unknown, except that the SOS regulon was not involved (9). Jaffé et al. (28) reported that in strains mutated in the *sfiA*, *sfiB*, or *sfiC* genes, all of which mediate SOS-inducible division inhibition, cell division restarted about 90 min after inhibition of chromosome replication to yield chromosomeless cells, regardless of method chosen for blocking replication. We observe a considerably tighter block to division than that reported in the study above and in other studies (50), although a residual septation frequency of about 2% contributed to increase the proportion of chromosomeless cells over time. The division block is compatible with nucleoid detachment being necessary for division site formation or utilization (see above), such that an unreplicated and unprocessed nucleoid may act as a block that prevents the action of the cell division machinery either directly (discussed in references 17 and 50) or through the production of hypothetical diffusible activators and inhibitors (39). An interesting possibility is that the site where subsequent division will take place is defined by association of the replication apparatus with an intracellular structure, e.g., in the cell envelope (15). Thereby a complex may be formed with which cell division proteins may subsequently associate, such that replisomes turn into "divisomes" by assembly of the cell division machinery around a membrane-bound replication complex. The observation that for the category of divisions that yielded two DNA-containing cells, the ratio of central to noncentral divisions was increased in the experiment in which only a single round of replication was induced may thus be explained by assuming that in this experiment, most cells contained only a single centrally located replication complex. In contrast, in the experiment in which multiple rounds of replication were allowed, the number of replication sites, and thereby also the number of possible locations for division sites, may have been increased. Division might subsequently be initiated by nucleoid detachment after completion of replication, providing an example of how regulation might occur through cues generated by changes in intracellular structure (6). However, a population of elongated cells with more than one nucleoid was also detected in the microscopic studies, indicating that in these cells, division had not taken place despite replication termination and nucleoid partition. Finally, the fact that cell division stopped after the replication block argues against the suggestion that nucleoid-free stretches within the cell are sufficient to allow cell division to be initiated (50); additional requirements must be fulfilled before division can take place.

Resumption of replication allowed rapid mass increase to resume. It is therefore clear that the rate of mass increase in a growing cell is dependent on the rate of increase in the DNA content. Gene products whose production rates normally increase with each round of replication in a wild-type cell may eventually become limiting in the absence of chromosome replication, or cellular growth may be slowed down by diffusion and transport limitations as the cells become elongated.

Restart of chromosome replication also resulted in resumption of cell division, after a time delay roughly corresponding to the sum of the lengths of the C and D periods in *E. coli* K-12 (3), confirming that division is strongly coupled to ongoing replication and nucleoid processing. Also, this observation is in accordance with the suggestion by Donachie and Begg (16) that the main requirements for division are a period of protein synthesis after termination of replication and a minimum distance between the partitioned nucleoids.

The presence of extended nucleoid-free stretches within the cells allowed an analysis of whether preferred division sites exist, e.g., between two newly segregated nucleoids or at a particular distance from the cell center or a cell pole. After restart of replication, the majority of the septation events took

place between two nucleoids, or close to a nucleoid (39), but no absolute requirement was evident since septations were also observed at a distance from the nearest nucleoid. The septation patterns indicated that the choice of division sites in the elongated cells was not random: the septations usually resulted in a length ratio between the daughter cells of 1:1 or 1:3, whereas septations that yielded a 1:2 length ratio were rare. By adding together the length of the two daughter cells for the septation events in Fig. 10C, it is evident that cells of all sizes were capable of division. Thus, the absence of the 1:2 class appeared to reflect a preference for central septations up until a certain cellular length was reached, at which asymmetric septations that yielded a 1:3 ratio began to occur.

Since a majority of the cells contained a single centrally located nucleoid before replication was restarted, the DNA must often have been transported from the center to a position near a pole after replication, even in the longer cells, to yield the 1:3 class. It has been suggested that two newly replicated nucleoids are transported a fixed distance in opposite directions during partition in *E. coli* (5). Alternatively, there may be preformed sites to which the nucleoids are transported along the interior of the cell (13, 25), which may be easier to reconcile with the partition pattern observed here. Another mechanism which would be compatible with the results is that the nucleoids are simply transported as far apart as possible. Filaments spanning the cell from one end to the other have been observed after overproduction of the *cafA* gene product (45), and if similar structures exist in wild-type cells, the partition system might use these to position each nucleoid close to a cell pole, thereby perhaps also preventing unwarranted polar divisions from occurring. This would provide a simple and elegant way for the cell to ensure that both daughter cells receive a nucleoid, without a requirement for an intracellular measuring device. However, if both nucleoids are partitioned in this manner, there would presumably be two possible positions for division in the elongated cells, one at each polarly located nucleoid. Since the cells divided only once, the number of divisions may be correlated to the number of replication complexes or partition events.

Formation of chromosomeless daughter cells was observed after the restart of replication. It is not known whether production of chromosomeless cells is governed by the same rules as divisions that yield two chromosome-containing daughter cells or whether it represents an aberrant division pathway that is not informative about the normal mechanisms. Here, these two possibilities are therefore treated separately. The appearance of the chromosomeless cells might be taken to indicate that cell division can take place wherever DNA-free areas appear in an *E. coli* cell, provided that replication and nucleoid processing continue. However, it was only in the experiment in which multiple rounds of chromosome replication occurred that chromosomeless cells smaller than chromosome-containing cells were observed, whereas in the single-round experiment, the size distribution was similar to that of the chromosome-containing cells. This finding indicates that some aspect of replication or nucleoid structure and position influences the localization of the site for cell division, e.g., such that when the size of the nucleoids increases, the divisions are forced to occur closer to the poles in a higher proportion of the population. Nucleoid influence on septum positioning and septum geometry has been suggested before (39), and we have shown that deficient nucleoid processing results in aberrant septation patterns in *E. coli*, including formation of branched cells (2). It was striking that chromosomeless cells were rarely observed to be formed from cells that divided centrally, indicating that central divisions took place only after distribution of chromosome DNA to either side of the cell center, as would be the normal case in a wild-type cell.

The main findings reported in this communication may be summarized as follows. We find a strong negative coupling, apparently stronger than reported by others in similar studies, between ongoing replication, nucleoid processing, and cell division, such that little or no residual division took place after inhibition of replication. A correspondingly strong positive coupling was not observed, since only a subpopulation of the cells that went through a single round of chromosome replication went on to divide. Thus, initiation of chromosome replication is not sufficient for cell division to occur; other requirements, e.g., successful decatenation and partition of the replicated nucleoids, must also be fulfilled. Although it is not clear what specifically determines where division should take place in the cell, the experiments reported here show that the nucleoids do influence septum positioning. This conclusion follows from the observation that the size distribution of the chromosomeless cells differed depending on whether single or multiple rounds of replication were allowed. The mechanism is not clear but might involve generation of additional division sites when new rounds of replication are initiated or simply be an effect of nucleoid size as multiple rounds of replication contribute to filling up a larger part of the cells with DNA.

Finally, by combining the constructed system with mutations in genes known to be involved in different stages of chromosome replication and cell division, it may be possible to investigate their functions by studying whether the mutations result in the cell division block being relieved or the septum localization pattern being affected. Also, after a downshift to 36°C, chromosome replication became synchronized in the entire population of cells of different sizes, and with a transient temperature shift, a single round of chromosome replication and cell division could be induced with little disturbance of the cellular physiology. Therefore, the system has a considerable potential for analysis of transcription, translation, and phosphorylation patterns, as well as enzymatic or other activities, during different stages in replication, nucleoid processing, and cell division. Thus, the system should be useful for further analyses of the regulation and coordination of cell cycle events.

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REFERENCES

1. **Åkerlund, T., R. Bernander, and K. Nordström.** 1992. Cell division in *Escherichia coli minB* mutants. Mol. Microbiol. **6:**2073–2083.
2. **Åkerlund, T., K. Nordström, and R. Bernander.** 1993. Branched *Escherichia coli* cells. Mol. Microbiol. **10:**849–858.
3. **Allman, R., T. Schjerven, and E. Boye.** 1991. Cell cycle parameters of *Escherichia coli* K-12. J. Bacteriol. **173:**7970–7974.
4. **Bachmann, B. J.** 1987. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12, p. 1190–1219. *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.
5. **Begg, K. J., and W. D. Donachie.** 1991. Experiments on chromosome separation and positioning in *Escherichia coli*. New Biol. **3:**475–486.
6. **Bernander, R.** 1994. Universal cell cycle regulation? Trends Cell Biol. **4:**76–79.

7. **Bernander, R., A. Merryweather, and K. Nordström.** 1989. Overinitiation of replication of the *Escherichia coli* chromosome from an integrated runaway-replication derivative of plasmid R1. J. Bacteriol. **171:**674–683.
8. **Bi, E., and J. Lutkenhaus.** 1993. Cell division inhibitors SulA and MinCD prevent formation of the FtsZ ring. J. Bacteriol. **175:**1118–1125.
9. **Burton, P., and I. B. Holland.** 1983. Two pathways of division inhibition in UV-irradiated *E. coli*. Mol. Gen. Genet. **190:**309–314.
10. **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.
11. **Dam, M., and K. Gerdes.** 1994. Partitioning of plasmid R1: ten direct repeats flanking the *parA* promoter constitute a centromere-like partition site *parC*, that expresses incompatibility. J. Mol. Biol. **236:**1289–1298.
12. **D'Ari, R., and O. Huisman.** 1983. Novel mechanism of cell division inhibition associated with the SOS response in *Escherichia coli*. J. Bacteriol. **156:**243–250.
13. **deBoer, P. A. J., W. R. Cook, and L. I. Rothfield.** 1990. Bacterial cell division. Annu. Rev. Genet. **24:**249–274.
14. **Diaz, R., K. Nordström, and W. L. Staudenbauer.** 1981. Plasmid R1 replication dependent on protein synthesis in cell-free extracts of *E. coli*. Nature (London) **289:**326–328.
15. **Donachie, W. D.** 1969. Control of cell division in *Escherichia coli*: experiments with thymine starvation. J. Bacteriol. **100:**260–268.
16. **Donachie, W. D., and K. J. Begg.** 1989. Cell length, nucleoid separation, and cell division of rod-shaped and spherical cells of *Escherichia coli*. J. Bacteriol. **171:**4633–4639.
17. **Donachie, W. D., K. J. Begg, and N. F. Sullivan.** 1984. Morphogenes of *Escherichia coli*, p. 27–62. *In* R. Losick and L. Shapiro (ed.), Microbial development. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Donachie, W. D., D. G. Hobbs, and M. Masters.** 1968. Chromosome replication and cell division in *Escherichia coli* 15*T* after growth in the absence of DNA synthesis. Nature (London) **219:**1079–1080.

18a. **Gerdes, K.** Personal communication.

19. **Grinsted, J., J. R. Saunders, L. C. Ingram, R. B. Sykes, and M. H. Richmond.** 1972. Properties of an R factor which originated in *Pseudomonas aeruginosa* 1822. J. Bacteriol. **110:**529–537.
20. **Gustafsson, P., K. Nordström, and J. W. Perram.** 1978. Selection and timing of replication of plasmids R1*drd-19* and F′*lac* in *Escherichia coli*. Plasmid **1:**187–203.
21. **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.
22. **Heitman, J., and P. Model.** 1991. SOS induction as an in vivo assay of enzyme-DNA interactions. Gene **103:**1–9.
23. **Helmstetter, C., S. Cooper, O. Pierucci, and E. Revelas.** 1968. On the bacterial life sequence. Cold Spring Harbor Symp. Quant. Biol. **33:**809–822.
24. **Helmstetter, C. E., and O. Pierucci.** 1976. DNA synthesis during the division cycle of three substrains of *Escherichia coli* B/r. J. Mol. Biol. **102:**477–486.
25. **Hiraga, S., T. Ogura, H. Niki, C. Ichinose, and H. Mori.** 1990. Positioning of replicated chromosomes in *Escherichia coli*. J. Bacteriol. **172:**31–39.
26. **Hirota, Y., F. Jacob, A. Ryter, G. Buttin, and T. Nakai.** 1968. On the process of cellular division in *Escherichia coli*. I. Asymmetrical cell division and production of deoxyribonucleic acid-less bacteria. J. Mol. Biol. **35:**175–192.
27. **Huisman, O., and R. D'Ari.** 1981. An inducible DNA replication-cell division coupling mechanism in *E. coli*. Nature (London) **290:**797–799.
28. **Jaffé, A., R. D'Ari, and V. Norris.** 1986. SOS-independent coupling between DNA replication and cell division in *Escherichia coli*. J. Bacteriol. **165:**66–71.
29. **Jensen, K. F.** 1993. The *Escherichia coli* K-12 "wild types" W3110 and MG1655 have an *rph* frameshift mutation that leads to pyrimidine starvation due to low *pyrE* expression levels. J. Bacteriol. **175:**3401–3407.
30. **Jones, N. C., and W. D. Donachie.** 1974. Protein synthesis and the release of the replicated chromosome from the cell membrane. Nature (London) **251:** 252–254.
31. **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.
32. **Kornberg, A., and T. A. Baker.** 1992. DNA replication, second edition. W. H. Freeman and Company, New York.
33. **Lark, K. G., T. Repko, and E. J. Hoffman.** 1963. The effect of amino acid deprivation on subsequent deoxyribonucleic acid replication. Biochim. Biophys. Acta **76:**9–24.
34. **Larsen, J. E. L., K. Gerdes, J. Light, and S. Molin.** 1984. Low-copy-number plasmid-cloning vectors amplifiable by derepression of an inserted foreign promoter. Gene **28:**45–54.
35. **Light, J., and S. Molin.** 1981. Replication control functions of plasmid R1 act as inhibitors of expression of a gene required for replication. Mol. Gen. Genet. **184:**56–61.
36. **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.
37. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. **Monk, M., and J. Gross.** 1971. Induction of prophage λ in a mutant of *E. coli* K12 defective in initiation of DNA replication at high temperature. Mol. Gen. Genet. **110:**299–306.
39. **Mulder, E., and C. L. Woldringh.** 1989. Actively replicating nucleoids influence positioning of division sites in *Escherichia coli* filaments forming cells lacking DNA. J. Bacteriol. **171:**4303–4314.
40. **Neidhardt, F. C., and R. A. VanBogelen.** 1987. Heat shock response, p. 1334–1345. *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.
41. **Nordström, K., R. Bernander, and S. Dasgupta.** 1991. Analysis of the bacterial cell cycle using strains in which chromosome replication is controlled by plasmid R1. Res. Microbiol. **142:**181–188.
42. **Nordström, K., R. Bernander, and S. Dasgupta.** 1991. The *Escherichia coli* cell cycle: one cycle or multiple independent processes that are co-ordinated? Mol. Microbiol. **5:**769–774.
43. **Nordström, K., and E. G. H. Wagner.** 1994. Kinetic aspects of control of plasmid replication by antisense RNA. Trends. Biochem. Sci. **19:**294–300.
44. **Ogawa, T., and T. Okazaki.** 1994. Cell cycle-dependent transcription from the *gid* and *mioC* promoters of *Escherichia coli*. J. Bacteriol. **176:**1609–1615.
45. **Okada, Y., M. Wachi, A. Hirata, K. Suzuki, K. Nagai, and M. Matsuhashi.** 1994. Cytoplasmic axial filaments in *Escherichia coli* cells: possible function in the mechanism of chromosome segregation and cell division. J. Bacteriol. **176:**917–922.
46. **Pierucci, O., and M. Rickert.** 1994. Duplication of *Escherichia coli* during inhibition of net phospholipid synthesis. J. Bacteriol. **162:**374–382.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **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.
49. **Skarstad, K., R. Bernander, S. Wold, H. B. Steen, and E. Boye.** Cell cycle analysis of microorganisms. *In* M. Al-Rubeai and A. N. Emery (ed.), Flow cytometry applications in cell culture, in press. Marcel Dekker Inc., New York.

49a. **Söderbom, F.** Personal communication.

50. **Tang, M.-S., and C. E. Helmstetter.** 1980. Coordination between chromosome replication and cell division in *Escherichia coli*. J. Bacteriol. **141:**1148–1156.
51. **Theisen, P. W., J. E. Grimwade, A. C. Leonard, J. A. Bogan, and C. E. Helmstetter.** 1993. Correlation of gene transcription with the time of initiation of chromosome replication in *Escherichia coli*. Mol. Microbiol. **10:**575–584.
52. **Walker, G. C.** 1987. The SOS response of *Escherichia coli*, p. 1346–1357. *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.