Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 12206–12211, October 1996
Biochemistry

Coordinating DNA replication initiation with cell growth: Differential roles for DnaA and SeqA proteins

(*Escherichia coli*/initiation mass)

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Contributed by Nancy Kleckner, July 25, 1996

ABSTRACT We describe here the development of a new approach to the analysis of *Escherichia coli* replication control. Cells were grown at low growth rates, in which case the bacterial cell cycle approximates that of eukaryotic cells with G_1, S, and G_2 phases: cell division is followed sequentially by a gap period without DNA replication, replication of the single chromosome, another gap period, and finally the next cell division. Flow cytometry of such slowly growing cells reveals the timing of replication initiation as a function of cell mass. The data show that initiation is normally coupled to cell physiology extremely tightly: the distribution of individual cell masses at the time of initiation in wild-type cells is very narrow, with a coefficient of variation of less than 9%. Furthermore, a comparison between wild-type and *seqA* mutant cells shows that initiation occurs at a 10–20% lower mass in the *seqA* mutant, providing direct evidence that SeqA is a *bona fide* negative regulator of replication initiation. In *dnaA*(Ts) mutants the opposite is found: the mass at initiation is dramatically increased and the variability in cell mass at initiation is much higher than that for wild-type cells. In contrast to wild-type and dnaA(Ts) cells, *seqA* mutant cells frequently go through two initiation events per cell division cycle, and all the origins present in each cell are not initiated in synchrony. The implications for the complex interplay amongst growth, cell division, and DNA replication are discussed.

Normally, cells growing under steady-state conditions duplicate their chromosomal complement once and only once between each cell division. In any given steady-state culture of *Escherichia coli* cells, initiation of DNA replication at the chromosomal origin, *oriC*, occurs at a specific time in the cell cycle and at a specific cell mass. Two questions regarding the relationship between cell growth and initiation are of particular importance.

First, how is the timing of initiation coupled to cell growth? An early hypothesis suggested that the initiation process responds to cell mass *per se* (1). According to this model, initiation always occurs at a fixed ratio of mass to origins—i.e., the initiation mass is a constant which is independent of the growth conditions. More recent evidence suggests, however, that the situation is more complicated, since the experimentally determined initiation mass varies significantly with growth rate (2, 3).

Second, how variable is the mass at the time of initiation, when the cells grow under steady-state conditions? The extent of variation indicates the tightness of coupling between initiation and cell physiology, of which cell mass is an indicator: the less the variation, the tighter the coupling.

Appropriately controlled replication initiation requires the assembly of the replication machinery at *oriC*. A central component of this assembly is the initiator protein DnaA. DnaA recognizes *oriC* specifically and, together with accessory proteins, promotes the formation of an open complex which permits loading of the DnaB helicase and priming of DNA replication (4). A second component, SeqA, has been identified genetically as a negative modulator of the initiation process (5). Biochemical data suggest that SeqA mediates its effects by binding to *oriC* prior to initiation (6).

The most detailed molecular models of initiation control in *E. coli* have focused on the DnaA protein. The amount of DnaA protein per origin (7), the activity of DnaA protein per cell (8), or the concentration of DnaA (9) have been proposed to trigger initiation of DNA replication. Alternatively, however, genetic interactions between *dnaA* and *seqA* mutations have led to the proposal that DnaA and SeqA act in opposition as a homeostatic pair, together making possible the sensitive response of initiation to physiological signals (5).

Once an origin has undergone initiation, a second process precludes the immediate occurrence of another initiation event at that origin. After initiation, *oriC* is placed in a sequestered state which effectively precludes reinitiation for a considerable period of time, about one-third of the cell cycle (10–12). *oriC* contains a number of GATC sites which are subject to methylation by Dam, the DNA adenine methyltransferase. Initiation normally occurs on fully methylated *oriC*, and replication of that region converts the GATC sites to the hemimethylated form. Sequestration requires the interaction of SeqA with these hemimethylated GATCs and serves to keep *oriC* hemimethylated and inaccessible for further initiation(s).

Analysis of the coupling between replication initiation and cell physiology in *E. coli* is complicated by the fact that under standard laboratory conditions—i.e.. rapid growth rates—several rounds of chromosome replication are going on concurrently. In the present work we have developed an alternative approach to this problem by studying cells that are growing so slowly that their cell cycle contains separate periods for prereplication, DNA replication, and postreplication (13). These periods are temporally analogous to the G_1, S, and G_2 phases of the eukaryotic cell cycle. With such a simple cell cycle it is possible to examine separately the control of the first initiation event as well as the occurrence of possible extra initiation events and to determine the relationship of each process to cell mass. By using this approach, we demonstrate that SeqA and DnaA proteins have opposing roles in the control of replication initiation.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. The *seqA* and *dnaA*(Ts) mutants are all derivatives of the *E. coli* K-12 strain 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^- (14). The *seqA*Δ*10* mutant contains an in-frame deletion which allows transcription/

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Abbreviation: CV, coefficient of variation.

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translation of the downstream *pgm* gene (6). The different isogenic *dnaA*(Ts) mutants were CM740*dnaA*5, CM742*dnaA*46, CM748*dnaA*203, CM2555*dnaA*508, CM2556*dnaA*167, and CM2735*dnaA*601 (15). Growth was in AB minimal medium (16) supplemented with thiamine, sodium acetate (0.4%), and the required amino acids, Met (20 μg/ml), His (22 μg/ml), and Trp (20 μg/ml). Mass growth was monitored by measuring optical density at 450 nm.

Flow Cytometry. The cells were fixed in ethanol, stained with ethidium bromide and mithramycin, and analyzed in an Argus flow cytometer (Skatron, Lier, Norway) as described (17). Determinations of the frequency of cells in the different cell cycle phases were performed with a standard program for analysis of the mammalian cell cycle (Skatron). Cell sorting was performed in a FACStar flow cytometer (Becton Dickinson).

The Coefficient of Variation (CV) of Cell Mass at Initiation. We have taken mass to be represented by scattered light, as measured by flow cytometry. The cells were grown in the same medium and have the same shape, and under such conditions scattered light is a good measure of cell mass (3).

The distribution of cell mass was determined for each DNA content (i.e., each fluorescence channel) in each histogram. The width of the distribution was expressed as the CV, which is the standard deviation of a normal distribution divided by its mean. Thus, a CV of 10% means that the standard deviation of the cell mass distribution is 10% of the average cell mass of the distribution. In each histogram, the CV of the mass distribution was almost constant, and also lowest, in the region between one and two chromosomes. Cells containing exactly one or two chromosomes were excluded from consideration, since such cells are not replicating their DNA but will have varying masses due to cell growth. The CV thus obtained was taken as an upper estimate of the CV at the time of initiation in that particular culture.

Mass at Initiation. The mean mass at several different DNA contents between one and two chromosomes was plotted against DNA content, and a straight line was drawn through the points. This line was extrapolated to the one-chromosome position and the mass value at this point taken to be the mass at initiation (see Fig. 1). The estimate of the initiation mass was accurate to within a few percent because DNA replication occupied only a small fraction of the cell cycle and the slope of the average-mass versus DNA line was therefore not very steep.

RESULTS

A culture of *E. coli* cells growing slowly under steady-state conditions can be established conveniently in the laboratory by employing a minimal medium supplied only with the essential amino acids and vitamins and a poor carbon source. Here we have used flow cytometry to measure the DNA content and the scattered light (closely related to cell mass; see discussion in ref. 3) of individual, slowly growing cells. The distribution of cells in a two-parameter (DNA versus scattered light) histogram reveals the replication/division pattern of the culture (Fig. 1): cells are born with one chromosome at a low mass, whereafter they spend some time of cell growth before DNA replication is initiated at a certain cell mass. After initiation, the DNA content increases from one to two genome equivalents, with a concomitant increase in cell mass. Cells with two chromosomes grow in size for some time until division occurs, thus completing the cell division cycle. From quantitative analysis of such two-parameter flow cytometry histograms we can derive three important parameters. (*i*) The average mass at the time of initiation (M_i in Fig. 1) can be compared for any two or more different situations (e.g., growth conditions or strain genotypes). (*ii*) The degree of coupling of initiation to cell growth is reflected in the variability of cell mass at initiation. This parameter is expressed here as the CV, which gives the relative width of the cell mass distribution of initiating cells. (*iii*) The occurrence of an extra initiation event in a cell results in a cell with more than two genome equivalents of DNA, and the frequency of such cells is revealed in a DNA histogram.

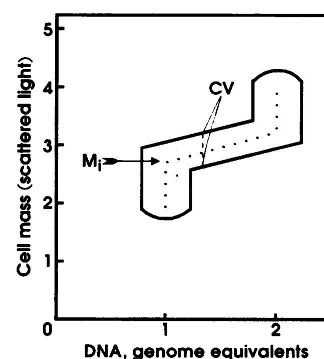

FIG. 1. A schematic representation of a DNA versus cell mass (scattered light) histogram of slowly-growing *E. coli* cells. The closed curve contains all the cells in the measured population, and the dotted line goes through the average values (DNA or scattered light) of the distribution. The CV is derived from the width of the mass distribution and is measured at a given DNA content. The initiation mass (M_i) is determined as the average mass where one-chromosome cell starts to increase their DNA content.

Initiation Control in Wild-Type Cells. The wild-type strain CM735 was grown in acetate minimal medium at 37°C with a doubling time of 190 min and subjected to flow cytometry. Samples were taken at different times and compared to assure that the cultures were growing at steady state. The DNA histogram demonstrates that the cells contained either one or two complete chromosomes or were in the process of replicating their single chromosome (Fig. 2*A*). Very few cells (2–3%) containing more than two chromosomes were observed, demonstrating that two initiations at the same origin in less than one doubling time was extremely rare in the wild-type cell, as inferred from previous analyses (18, 19).

The CV of the cell mass distribution at initiation, measured in a number of independent experiments, is between 9 and 13% (Fig. 2*D* and Table 1). Similar values are found for other commonly used K-12 strains—e.g., AB1157 and C600 (data not shown). Since the lowest CV measured is a maximum estimate of the real, biological variation, we conclude that the true CV is less than 9%.

The flow cytometry data also permit a schematic representation of the replication/division patterns for slowly growing wild-type cells. Most of the cells contain one chromosome (Fig. 2*A*), which means that they spend most of their time at the prereplication stage. Similarly, fewer cells are replicating their DNA (the C period) and even fewer contain two chromosomes. The frequencies of cells in the different periods, corrected for the age distribution (newborn cells are twice as many as dividing cells), can be translated to durations of the respective periods (13). Assuming a monotonous increase of mass with time, the approximate durations of the different periods, represented as mass increase, are as shown schematically in Fig. 3.

The *seqA* Mutation Alters Initiation Control. Isogenic *seqA* null mutant cells were grown in parallel with wild-type cells under the same conditions as described above and analyzed by flow cytometry. The first initiation after division of a *seqA* mutant cell, as shown in a series of repeat experiments, occurs at a lower than normal mass (Fig. 2 *C* and *G*; Table 1): the average mass at initiation is, in repeated parallel experiments, consistently 10–20% lower for the *seqA* strain than for the wild type. This effect cannot result from a failure to sequester *oriC* and suppress extra initiations (see below) and thus implies a change in the regulation of initiations.

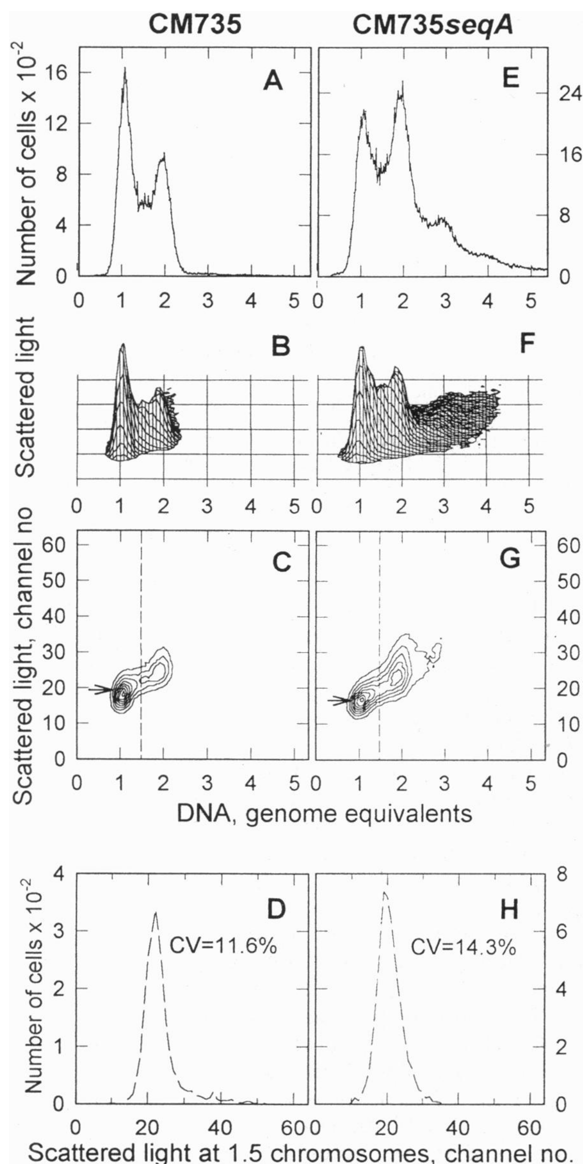

FIG. 2. Flow cytometry histograms of wild-type (CM735) and isogenic *seqA* mutant cells, grown under steady-state conditions at 37°C with a doubling time of 190 min. The parameters measured were DNA fluorescence (*A* and *E*), DNA fluorescence versus scattered light (*B*, *C*, *F*, and *G*), and the light scatter distribution of cells replicating their DNA (*D* and *H*). The three-dimensional plots (*B* and *F*) arise from the DNA histograms (*A* and *E*) when the DNA content and, in addition, the scattered light of each individual bacterium is plotted along perpendicular axes and the number of cells with a given DNA content/scattered light combination is shown along a vertical axis. *C* and *G* result when these three-dimensional plots are viewed along the "number-of-cells" axis. The isocontour lines (*C* and *G*) are drawn through points with the same number of cells. Thus, the collection of almost concentric rings at the one-chromosome position in *C* represent a "mountain" of cells that can clearly be seen in the three-dimensional presentation above (*B*). The arrows in *C* and *G* are placed at the mass (scattered light) values of cells at replication initiation. The light scatter distributions in the lower panels were obtained at 1.5 genome equivalents (stippled line in *C* and *G*). The unit on the light scatter axes is arbitrary (channel no.) and dependent upon instrument settings, but the CV of the light scatter distributions (*C* and *G*) is independent of instrument settings.

Importantly, under the conditions used for this experiment, wild-type and *seqA* mutant cells exhibit indistinguishable mass doubling times. Thus, direct comparisons between mutant and wild-type cells with respect to initiation can be made without having to consider additional complications. Furthermore, we can conclude that the alteration in the regulation of replication initiation observed in a *seqA* mutant has no effect on cell growth rate.

Table 1. Strain growth and DNA replication characteristics

Strain, genotype	Doubling time	Mass at initiation, rel.	CV,* %	Asynchrony index†
CM735 wild type	190 min	1.0	11.6	—
CM735 *seqA*Δ*10*	190 min	0.85	14.3	—
CM735 wild type	4.5 h	1.0	12.6	0.10
CM740 *dnaA5*	4.5 h	2.3	20.0	1.24
CM742 *dnaA*46	4.5 h	2.3	20.2	1.25
CM748 *dnaA*203	5.0 h	3.1	32.6	0.26
CM2555 *dnaA*508	4.5 h	2.0	20.8	0.27
CM2566 *dnaA*167	5.5 h	1.3	17.5	0.38
CM2735 *dnaA*601	5.0 h	2.0	20.9	1.29

*Coefficient of variation of cell mass at initiation.
†From ref. 20.

The distribution of the mass of initiating *seqA* mutant cells exhibits a CV of 13–15% (e.g., Fig. 2*H* and Table 1). This CV is close to that of the wild type, but still significantly higher: repeated experiments show that the CV of initiation mass is always 2–4% higher for the mutant than for the wild-type strain grown and analyzed in parallel (data not shown).

In the seqA Mutant, Decreased Mass at Initiation Is Not Due To an Increased DnaA Content. Oversupply of DnaA protein in wild-type cells results in a reduction of the mass at initiation (21). Therefore, the reason for the low mass at replication initiation in one-chromosome *seqA* mutant cells could conceivably be an increased concentration of DnaA. We have measured the level of DnaA protein in wild-type and *seqA* mutant cells, grown as above, and have found no significant difference (<20%; data not shown). We conclude that an overabundance of DnaA protein is likely not responsible for the perturbed initiation control reported here. Earlier, an increased level of DnaA protein has been reported for rapidly growing *seqA* mutant cells (22). We have also found considerably more DnaA protein in the *seqA* mutant than in wild-type cells when grown at high growth rates (data not shown), confirming the published results.

The seqA Mutant Sometimes Undergoes Extra Initiations. The DNA histogram of the *seqA* strain (Fig. 2*E*) demonstrates

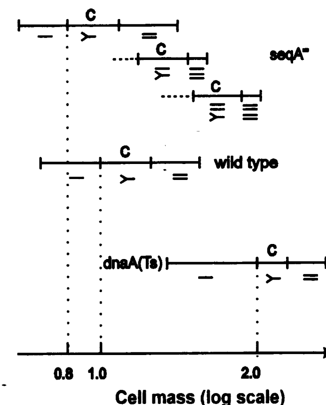

FIG. 3. Schematic representation of the DNA replication/cell division cycles as a function of cell mass (scale, log2) in *seqA* mutant cells (*Top*), wild-type cells (*Middle*), and *dnaA*(Ts) mutants (*Bottom*). The period of DNA replication (the C period) is indicated by Y-shaped chromosomes below the lines. Relative cell mass at the time of initiation is indicated by dotted lines. Since we cannot determine exactly when the extra initiations occur in the *seqA* mutant (see *Discussion*), we also cannot make the appropriate interconnections between the first, second, and third lines. Therefore, the lengths indicated in the three top lines must be understood as estimates.

that a significant fraction of the cells (about 30%) contain more than two genome equivalents, and there are distinct peaks corresponding to cells with three and four fully replicated chromosomes. Therefore, in the *seqA* mutant, at a significant frequency, initiation occurs twice at one or more origins without an intervening cell division.

We have considered and excluded an alternative explanation for the occurrence of three- and four-chromosome cells. If some of the *seqA* mutant cells do not separate completely when dividing, the cells that had apparently undergone an extra round of initiation in the same cell division cycle could instead be two individual, but not separated, cells initiating replication normally and being detected as one cell by flow cytometry. If this were true, most of the three-chromosome cells should contain septa. Fixed and stained cells were run in a FACS (Becton Dickinson) flow cytometer and the three-chromosome cells were sorted out for investigation by combined phase contrast and fluorescence microscopy. The frequency of septated cells was found to be <15%, much too low to support the above alternative explanation.

The appearance of a distinct three-chromosome peak implies that, in some two-chromosome cells, only one of the two *oriC* copies has been initiated. Such asynchronous initiation events are very rare in wild-type cells (23) but are rather frequent in the *seqA* mutant (ref. 5; Fig. 2*E*).

Taken together, the above observations imply that the replication/division cycle of the *seqA* mutant is more multifaceted than that of the wild type. More specifically, *seqA* mutant cells may go through several alternative cell cycles (Fig. 3). The majority of *seqA* mutant cells initiate and complete DNA replication, segregate the two chromosomes, and divide (top line). An alternative pathway for the two-chromosome cells is to replicate one or both of the chromosomes again, before dividing. Cells with one or two extra initiation events move down to the replication/division cycles depicted in line two or three (Fig. 3). These three- and four-chromosome cells divide to give one- and two-chromosome cells. Newborn cells with one chromosome that are daughters of three-chromosome cells will probably have a mass above the average for a newborn one-chromosome cells, allowing them, upon division, to enter the scheme (Fig. 3) at the top line somewhere into the prereplication period. There is a possibility that these cells contribute to the somewhat increased variability of initiation masses of the *seqA* strain. A newborn two-chromosome cell would enter into the top line after the C period and proceed from there to line two or three. A two-chromosome cell that has finished DNA replication in the top line may also initiate both chromosomal origins, thereby skipping the three-chromosome stage to become a four-chromosome cell (not shown in Fig. 3). Intermediate pathways, including cell division during the C periods of lines two and three, may also occur.

The average inter-division time must be equal to the mass doubling time in a steady state culture. Therefore, the long inter-division time of some *seqA* mutant cells (e.g., those developing from newborn with one chromosome to dividing with four chromosomes) must be compensated by a shorter inter-division time in other cells (e.g., those born with two chromosomes and replicating one of them before division).

The scheme presented (Fig. 3) is a simplified version consistent with the data, but there are certainly other replication/division patterns that are possible and that do occur but would complicate the picture further. Because of the complexity we are not able to give all the alternative replication/division cycles or to give exact numbers for the durations of the different periods of the *seqA* mutant cells.

Initiation Control in *dnaA*(Ts) Mutant Cells. Strain CM735 and a set of isogenic *dnaA*(Ts) mutants were grown as described above, except at the permissive temperature of 30°C, and with mass doubling times around 5 h for all strains (Table 1). The DNA histograms did not reveal any major differences between wild-type and mutant cultures (Fig. 4 *A* and *D*; data for other mutants not shown). There are relatively fewer *dnaA46* mutant cells than wild type cells containing between one and two genome equivalents, indicating that the DNA replication period is shorter in the mutant (see *Discussion*). Very few *dnaA*(Ts) mutant cells undergo extra initiations, as shown by a low frequency of three- and four-chromosome cells.

The two-parameter DNA/light scatter histograms, in contrast, reveal two important differences between mutant and wild-type cells. First, four of the six mutants have a mass at initiation that, compared with the wild type, is about twice as high, and that of the *dnaA203* mutant is threefold higher (Fig. 4 and Table 1). The mass at initiation of the *dnaA167* mutant is 30% higher than that of the wild type. Thus, *dnaA* mutant cells initiate replication at their single origin at a significantly larger cell mass than do wild-type cells. At any given DNA content, the *dnaA46* mutant cells are larger than the wild type cells (Fig. 4 *B* and *E*). Indeed, the cell mass distributions of wild-type and mutant cells only partly overlap.

Second, the CV of the mass distributions of initiating *dnaA*(Ts) cells are significantly larger than that of the wild-type parent (Fig. 4 *C* and *F*; Table 1). At any given DNA content and, correspondingly, in the cell population as a whole, the variation of cell mass is much higher in the mutant than in the wild type. Thus, in *dnaA* mutant cells initiation is not well coupled to cell mass.

Relationships Among Different *dnaA*(Ts) Phenotypes. Among the different *dnaA*(Ts) mutants investigated, there is

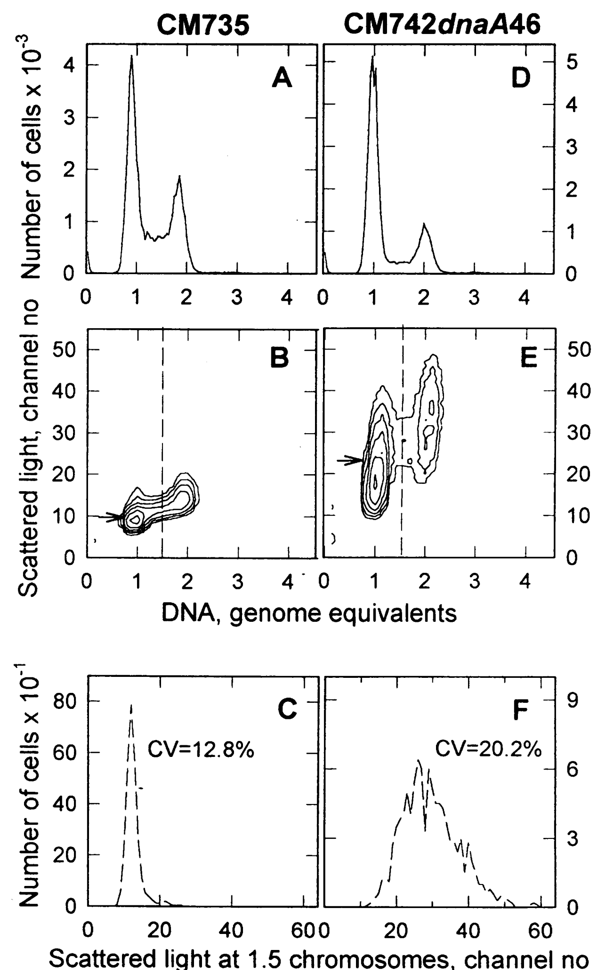

FIG. 4. Flow cytometry histograms of wild-type and the isogenic *dnaA46* mutant cells grown under steady-state conditions at 30°C with a doubling time of about 5 h. For details, see Fig. 2. The light scatter unit is again arbitrary and not the same as that in Fig. 2.

a direct correlation between increased cell mass at initiation and decreased coupling to cell mass, as expressed by the widths of the initiation mass distributions (Table 1). The CVs show the same hierarchy as do the values for average cell mass at initiation: the *dnaA167* mutant is closest to wild type and *dnaA203* is most aberrant in both respects; the remaining four mutants are intermediate.

Rate of Fork Movement in the *dnaA46* Mutant. The difference in the fraction of cells performing DNA replication at any one time in the wild-type and the *dnaA46* mutant cultures was investigated further in four independent sets of wild-type and *dnaA46* mutant cultures. The fractions of cells replicating their DNA (see *Materials and Methods*) were 33.6 ± 5.1% for the wild-type and 20.4 ± 5.4% for mutant cells, which reflect a difference in the duration of their C periods (Fig. 3, bottom two lines). Since the rate of mass doubling, and hence the average interdivision time, is the same for mutant and wild-type cells, this result implies that the replication forks move, on average, faster in *dnaA* mutant cells than in the wild type.

Earlier, an increased level of DnaA protein has been shown to have the opposite effect—i.e., a reduced rate of replication fork progression (21, 24, 25). Perhaps both situations are explained by changes in the level of replication factors present at the time of initiation. DnaA overproduction causes initiation to occur at a lowered cell mass, at which stage cells may not have aquired the full set of factors needed for replication (e.g., nucleotide pools, replication proteins), causing the replication fork to progress more slowly. Conversely, a low DnaA activity gives rise to initiations at an increased cell mass (Fig. 4 and Table 1), which is a situation where they probably have accumulated excess amounts of other factors required for replication, and the replication forks can progress without interruption.

DISCUSSION

In the present work, by applying flow cytometry to slowly growing bacterial cells, we have been able to study in detail the relationships between initiation of DNA replication, cell division, and general cell growth. Slow growth conditions are not only analytically advantageous but comprise a natural state of *E. coli* growth. Similarly poor growth conditions are periodically experienced by *E. coli* cells in one of its most important habitats, the mammalian intestinal tract.

Replication Initiation Is Tightly Coupled to Cell Mass. During steady-state growth, the distributions of single-cell parameters (e.g., individual cell mass, cellular DNA content) are by definition constant. Therefore, it is self-evident that the mean mass at initiation is constant in a given culture, with a certain biological variation. The data presented here demonstrate that, for wild-type cells, the cell mass at initiation is nearly identical amongst all the cells in a steady-state population, with a CV of less than 9%. This low CV reflects an exceedingly tight coupling between initiation of DNA replication and cell mass (and hence cell growth). Tight coupling implies that the cell responds very sensitively to a small incremental change in cellular physiology at the moment of initiation. The CV reported here is significantly smaller than the CVs of 15–22% reported in earlier work (26, 27).

The tight coupling of replication initiation to cell physiology is conceptually distinct from the proposed constancy of cell mass per origin at the time of initiation, the initiation mass. Whether the initiation mass is varying with growth rate is a separate issue and, indeed, it has been found to vary considerably (2, 3), implying that mass *per se* is not the parameter which triggers replication initiation.

SeqA and DnaA Proteins Are, Respectively, Negative and Positive Regulators of Initiation. Analyses of *seqA* and *dnaA* mutant cells, in parallel with wild type, reveal that SeqA and DnaA are both required for normal replication initiation, but in opposite senses and in different ways. In a *seqA* mutant, initiation occurs too early in the division cycle, at a 10–20% lower average cell mass than in wild type, whereas in a *dnaA* mutant, the mass at initiation is nearly twice as high as normal. The *seqA* mutation has relatively little effect on the coupling of initiation to cell physiology, increasing the CV of mass at initiation only slightly. The *dnaA* mutation, in contrast, essentially eliminates any coupling: the CV for the *dnaA* mutants is twice that of wild-type cells or more.

The present observations confirm the role of SeqA as a negative modulator of replication initiation. However, they leave open the crucial question of what is limiting initiations in wild-type cells. It is interesting to consider the possibility that all factors required for initiation are present at the origin at an early time, but that SeqA negatively affects the initiation process at this stage. Our data raise the possibility that SeqA should be considered primarily a timing factor for the initiation process. This leaves open the question of what is limiting initiations in the absence of SeqA.

The phenotype conferred by the *dnaA* mutations is fully compatible with the known positive role of DnaA as the major origin-binding initiator protein. Irrespective of what is limiting initiations in wild-type and *seqA* mutant cells, delayed initiation in the *dnaA* mutants is most simply understood by the hypothesis that a certain level of DnaA activity is required per origin or per genome equivalent for initiation to occur, that the specific activity of the mutated DnaA proteins is lower, and thus that mutant cells must grow to a larger size than normal to attain this critical level of DnaA protein activity. Even though DnaA activity may be limiting in the mutants, the situation in wild-type cells is not necessarily the same—i.e., DnaA activity may be sufficient in the wild type long before initiation occurs and initiation may be regulated by another event (above).

The Nature of Extra Initiations. The *seqA* and *dnaA* mutations have different effects with regard to extra initiation events as well. There is no indication of extra initiations in the dnaA mutant case, consistent with the fact that sequestration is normal in these mutants under permissive growth conditions (5). In the *seqA* mutant, in contrast, extra initiations are prominent. Moreover, in a significant fraction of these extra initiations, initiation occurs at only one of two already-replicated origins, an event never observed in wild-type cells under any conditions.

The occurrence of extra initiations is expected in the *seqA* mutant case, as sequestration is defective. But the extra initiations observed in these experiments are unexpectedly complicated in some way as yet not understood. The simplest interpretation is that the absence of SeqA allows extra initiations to occur on newly initiated, and therefore hemimethylated, origins. Such "hitch-hiked" initiations presumably occur shortly after another initiation event of the same origin, at a time when initiation activity is high. Cells where this has happened should have two sets of replication forks following closely after one another. For the following reason we do not think that this is the case.

Consider a cell that initiates replication at an origin which has been initiated shortly before. With twice as many replication forks it should increase its DNA content relative to mass twice as fast, compared with a cell with only one set of replication forks. Cells with extra initiations should therefore contribute to a broadening of the mass distribution as the DNA content increases from one to two chromosomes. Contrary to this prediction, we observe no change in the CV in this DNA interval (data not shown). In support of the validity of these results, we do find an increase in the CV in the interval from two to three genome equivalents per cell. In this interval, we presumably find two-chromosome cells replicating either one or both chromosomes (see above)—i.e., cells with different rates of DNA replication.

Thus, two alternative explanations remain. First, two initiations may actually occur at the same origin within a short time

interval, but the last set of forks is for some reason not allowed to proceed until at a later time. Second, the extra initiations are not occurring until the first round of replication is finished. In the latter case, initiations occur on fully methylated origins, since the period of hemimethylation is very short in the *seqA* mutant (5). At present we cannot discriminate between these two alternatives or exclude other, intermediate alternatives.

Coordination Between DNA Replication and Cell Division. The present data provide further insight into the interplay amongst three different aspects of the bacterial "cell cycle": cell mass increase, cell division, and DNA replication. Neither of two mutations which significantly perturb replication initiation, and in opposite senses, has any effect on the rate of increase of cell mass. Thus, nutrient conditions govern the rate of cell mass increase independent of the replication cycle. Furthermore, since steady-state growth could be achieved with both *seqA* and *dnaA* mutants, and at the same mass doubling times as the wild type, we conclude that neither of the initiation mutations significantly perturbs the (average) time between successive cell divisions.

However, steady state growth condition is achieved in two somewhat different ways in the *seqA* and *dnaA* mutant cells. In the *seqA* mutant, if extra initiations are excluded from consideration, initiation occurs at a lower than normal cell mass, replication forks move at approximately the normal rate, and a longer than normal period of time elapses between the completion of replication and the onset of the next cell division. Thus, in this case, the timing of cell division is determined by factors external to the replication cycle—e.g., the time required to accumulate relevant components.

A different aspect of the DNA replication/cell division relationship is displayed by newborn *dnaA* mutant cells: they have a much higher mass than dividing wild-type cells and yet they are not ready to divide. These cells do not fulfill another requirement for cell division: the chromosomes must be replicated and segregated before division can occur (28). Inhibition of cell division in the absence of segregated chromosomes appears to be independent of the SOS response (29), in keeping with the absence of SOS induction in *dnaA*(Ts) mutant cells (30, 31).

In contrast, DNA replication does not seem to be dependent upon cell division. For example, *seqA* mutant cells can initiate an origin twice in the same cell division cycle. Also, *E. coli* cells which increase their growth rate may go through two rounds of DNA replication without an intervening cell division. These findings support the notion that DNA replication and cell division are separately regulated but interconnected biochemical processes, in accordance with earlier data (32–34).

Synchrony of Replication Initiation. Analysis of rapidly growing cells has also defined a feature of replication initiation known as "synchrony": in a cell containing more than a single copy of the origin, all copies are initiated within a narrow time window. A procedure in which replication initiation and cell division are rapidly blocked and ongoing rounds of replication permitted to continue (23, 35) catches very few cells that contain a number of origins other than 2^n (n = 1, 2, 3. . .). Synchrony could result either from a coordinate response of all origins to the same internal cellular cues and/or from direct communication amongst the several copies. The former mechanism would presumptively be related to the finding reported here, that different cells containing a single origin all respond similarly to physiological cues. But among *dnaA* mutants, replication synchrony, as defined at faster growth rates, is not affected coordinately with mass at initiation and its variability (CV). This finding may implicate additional features in the synchrony phenomenon.

SeqA is clearly required to maintain synchrony of replication initiation (5). Extra initiation events, as observed here for slowly growing *seqA* cells, presumably explain the reason for the asynchrony phenotype observed in rapidly growing *seqA* mutants.

Excellent technical assistance from Marit Osland and Anne Wahl and help with the flow cytometry from Mette Walberg and Harald B. Steen are gratefully acknowledged. This work was supported by the Norwegian Cancer Society (E.B., T.S., and K.S.), the Norwegian Research Council (K.S.), and the National Institutes of Health (Grant R01 GM25326 to N.K.).

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