The EMBO Journal vol.13 no.8 pp.1856–1862, 1994

The initiation cascade for chromosome replication in wild-type and Dam methyltransferase deficient *Escherichia coli* cells

Anders Løbner-Olesen[1,3,4], Flemming G.Hansen[2], Knud V.Rasmussen[2], Brad Martin[1] and Peter L.Kuempel[1]

[1]University of Colorado at Boulder, Molecular Cellular and Developmental Biology, Porter Biosciences Building, Campus Box 347, Boulder, CO 80309-0347, USA and [2]Department of Microbiology, The Technical University of Denmark, Building 221, DK-2800 Lyngby, Copenhagen, Denmark
[3]Present address: University of Copenhagen, Department of Molecular Cell Biology, Øster Frimagsgade 2A, DK-1353 Copenhagen K, Denmark
[4]Corresponding author

Communicated by E.Boye

'Newborn' *Escherichia coli* B/r cells, obtained by membrane elution, were used to study the cell cycles of wild-type and Dam methyltransferase mutants. In wild-type cells, initiation of chromosome replication was synchronous and tightly controlled. In *dam* mutants, initiation was altered, but not random. We propose that this is due to the absence of an initiation cascade caused by liberated DnaA molecules, and that this cascade normally synchronizes initiation. The *dam*$^-$ cells contained mainly two, three or four replication origins, and this affected nucleoid partitioning as well as cell division. In cultures growing with a 50 min doubling time, a variety of cell cycles were present and half the origins were used every 25 min. Some cells had a 25 min interdivision time, whereas others had an interdivision time longer than the generation time. Partitioning of nucleoids containing unequal numbers of replication origins could also be readily observed by fluorescence microscopy in the *dam* mutant. Based upon these observations we propose that the *dam* mutant is also an initiation cascade mutant.

Key words: chromosome replication/Dam methylation/DnaA protein/*Escherichia coli*/initiation cascade

Introduction

Initiation of chromosome replication in *Escherichia coli* occurs when a certain mass (the initiation mass) has accumulated per origin (Donachie, 1968). The factor controlling the frequency of initiation from *oriC* seems to be the availability of active DnaA protein, since a good correlation between the *dnaA* gene expression and the time of initiation in the cell cycle has been established (Løbner-Olesen *et al.*, 1989; Atlung and Hansen, 1993). Under growth conditions where initiation of chromosome replication occurs at multiple origins in the cell, this process is remarkably precise and all origins are initiated virtually simultaneously (Skarstad *et al.*, 1986). Therefore, wild-type cells always contain 2^n origins (where n = 0, 1, 2, 3...).

A number of mutant strains are known in which a large fraction of the cells contain numbers of origins different from 2^n. This is referred to as the asynchrony phenotype, and is generally interpreted as being the result of an initiation rather than a segregation defect (Boye and Løbner-Olesen, 1991). Mutations leading to the asynchrony phenotype occur in the *dnaA*, *dam*, *recA* and *fis* genes as well as in the genes encoding the IHF protein complex (Skarstad *et al.*, 1988; Boye *et al.*, 1988, 1992; Skarstad and Boye, 1993). It was recently demonstrated that the asynchrony phenotype of *recA* mutants is due to complete degradation of whole chromosomes (Skarstad and Boye, 1993).

The role of Dam methyltransferase in maintaining synchronous initiations is also being clarified. After initiation of DNA replication has occurred, the newly replicated origins remain hemimethylated and are sequestered from the Dam methyltransferase for approximately one-third of a doubling time (Campbell and Kleckner, 1990). This sequestration of hemimethylated origins, most likely into the cell membrane (Ogden *et al.*, 1988), may render the origins inaccessible to the replication machinery. This provides an explanation why hemimethylated chromosomal origins are not initiated *in vivo* (Russel and Zinder, 1987), but are good substrates in a soluble *in vitro* replication assay (Landoulsi *et al.*, 1989; Boye, 1991). The transient hemimethylation and sequestration of origins was proposed to prevent reinitiation of origins in the period when initiation potential within the cell is high, and thus to provide a mechanism ensuring that each origin is only initiated once per cell cycle (Boye and Løbner-Olesen, 1990).

In *dam* mutants, some origins are used more than once per cell cycle (Bakker and Smith, 1989), and others are not used. It has been proposed that initiations occur randomly in time, but with the appropriate average frequency (Bakker and Smith, 1989; Boye and Løbner-Olesen, 1990).

To get further insight into the initiation of chromosome replication in normal cells and in cells deficient in Dam methyltransferase, we have used radioactive labelling techniques and flow cytometry to follow the time course of DNA synthesis, amount of DNA and the number of origins in cells which were selected by the membrane elution technique. The results demonstrate that although *dam* mutants do not exhibit the synchronous initiation typical of *dam*$^+$ cells, the pattern of replication is not entirely random. These results are discussed with respect to the initiator titration model (Hansen *et al.*, 1991), and to how initiation is influenced by a 'cascade' of DnaA protein released after initiation.

Results

Properties of exponentially growing and 'newborn' cells

The generation times for the *dam*$^+$ and *dam*$^-$ strains, growing exponentially in M9-glucose medium at 37°C, were

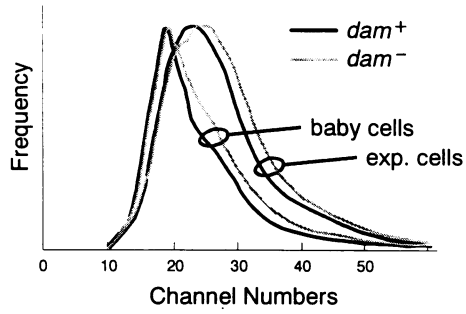

Fig. 1. The sizes of exponentially growing and 'newborn' cells. Cell size distributions of wild-type and *dam* mutant cultures. 'Newborn' cells were obtained by the membrane elution technique.

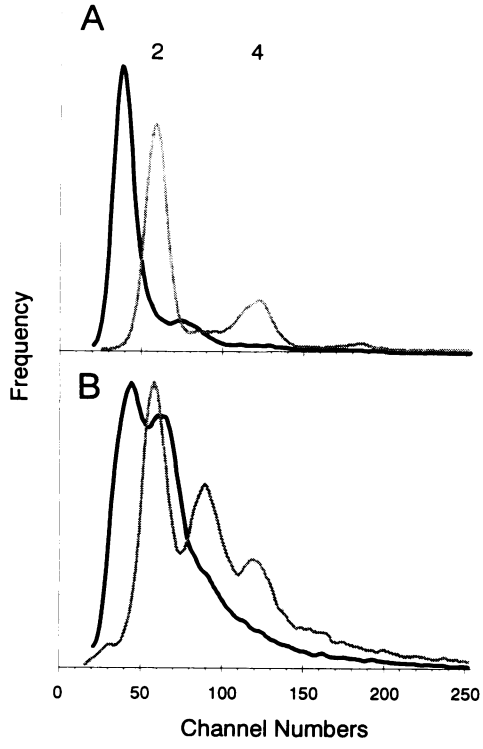

Fig. 2. DNA and origin content of 'newborn' cells. 'Newborn' cells of the wild-type (**A**) and *dam* mutant (**B**) strains were obtained by the membrane elution technique. Total DNA content (solid line) and cellular origin content (hatched line) were determined as described in Materials and methods.

42 and 50 min, respectively. Analysis of the cell sizes with a Coulter counter demonstrated that the two strains had similar, but not identical, size distributions (Figure 1).

Elution of growing cells from a membrane filter (Helmstetter *et al.*, 1992) was used to obtain 'newborn' cells. The *dam*$^+$ cells eluted from the filter were considerably smaller than the average cells in the population before selection (Figure 1), as expected for 'newborn' cells. Elution of the *dam* mutant cells led to a comparable selection of small cells (Figure 1). A comparison of selected *dam*$^+$ and *dam*$^-$ cells showed that the size distributions were similar, except that the *dam*$^-$ distribution was more skewed towards larger cells (Figure 1). The larger size of the selected 'newborn' *dam*$^-$ cells does not reflect a random wash-off from the membrane, since the cell numbers in the effluent were constant for hours of elution (data not shown). If cells stick less well to the membrane, a rapid decrease in cell concentration in the effluent is observed (Helmstetter *et al.*, 1992).

Table I. DNA concentration in wild-type and *dam* mutant cells

Strain	DNA concentration[a]	
	Exponential	'Newborn'
dam$^+$	1.00	0.86
dam$^-$	0.93	0.95

[a]The DNA concentration was estimated as the fluorescence per light scatter given relative to the exponential wild-type cells (Boye and Løbner-Olesen, 1990).

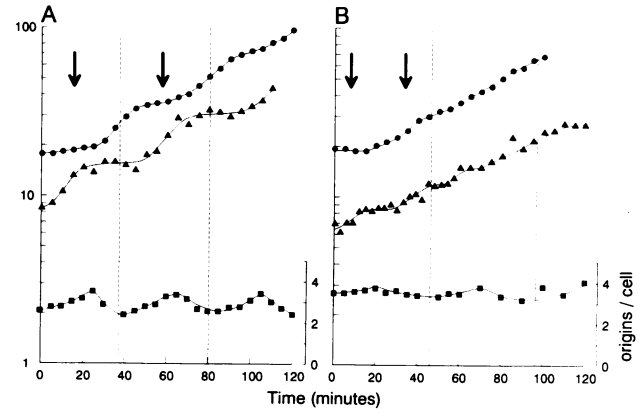

Fig. 3. Cell cycle parameters of synchronized cultures. 'Newborn' cells of wild-type (**A**) and *dam* mutant (**B**) cultures were collected and allowed to grow as batch cultures. Cell cycle parameters were monitored as described in Materials and methods. (●) Cell number; (▲) DNA synthesis rate; (■) average number of origins per cell. The times of initiation of DNA replication are indicated by arrows. Broken lines indicate the expected times of division based upon the growth rates of exponential cultures.

When the eluted 'newborn' cells were analysed for DNA and origin content, the data indicated that a major difference existed between the *dam*$^+$ and the *dam* mutant strains. The eluted wild-type cells were homogeneous in terms of DNA content (Figure 2A, solid line), with the average DNA content being between one and two genome equivalents. When the cells were treated with rifampicin to inhibit initiation of DNA replication and with cephalexin to inhibit cell division prior to flow cytometric analysis, cells were found to contain either two or four fully replicated chromosomes (Figure 2A, hatched line). This demonstrates that the treated cells contained either two or four origins, and that initiation occurred synchronously on two origins in this strain.

'Newborn' *dam*$^-$ mutant cells were heterogeneous in terms of DNA content (Figure 2B, solid line). Some cells contained between one and two genome equivalents of DNA while others contained more than two genome equivalents of DNA. The *dam* mutant cells contained mainly two, three or four replication origins (Figure 2B, hatched line), indicating asynchrony of initiation in this strain. Since the fraction of cells containing fewer than two genome equivalents of DNA was similar to the fraction having two replication origins, these cells most likely were the same.

The remaining 'newborn' cells that contained more than two genome equivalents of DNA presumably correspond to the cells that contained more than two origins of replication. Thus, the population of 'newborn' *dam*$^-$ cells appeared to be dominated by a few distinct subpopulations.

Flow cytometric data were also used to estimate the average cellular DNA concentration. This was done for exponentially growing and 'newborn' cells of the wild-type and *dam* mutant strains (Table I). In agreement with previous observations (Boye and Løbner-Olesen, 1990), no major difference was observed between exponentially growing cells of the two strains, indicating that the coupling of DNA replication to mass increase is still intact in *dam*$^-$ cells. The DNA concentrations in the selected 'newborn' cells of both strains were similar to that of exponential cells.

Cell division and chromosome replication patterns

We used batch cultures of 'newborn' cells collected for 4 min to study cell cycle parameters. This allowed us to take samples for determination of cell number and the rate of DNA replication, as well as for flow cytometry at different times throughout the division cycles of the selected cells.

Cell division in wild-type cells (Figure 3A), as determined by the middle of each increase in cell number, occurred ~38 min and 80 min after the time of elution (Figure 3A, stippled lines). Since collection of 'newborn', eluted cells occurred for 4 min prior to sampling, this is in good agreement with the 42 min doubling time that was observed for the exponential culture. Initiation of DNA replication, as determined by the middle of the increase in the rate of DNA synthesis, occurred at 15 min and 57 min. When corrected for the 4 min sampling period, this corresponds to initiations at 19 and 61 min.

Helmstetter *et al.* (1992) determined that for strain B/r F26 the time taken to replicate the chromosome (C period) was 42 min, and that the period from termination of chromosome replication to cell division (D period) was 18 min. Our data suggest that the duration of the C+D period, calculated as the time from initiation of DNA replication to the pertinent cell division, was 65 min.

Flow cytometry was used to calculate the number of replication origins in the cells (Løbner-Olesen *et al.*, 1989). The number of origins per cell (Figure 3A, lower curve) increased simultaneously with the increase in the rate of replication, as would be expected for the initiation of replication cycles. The number of origins per cell then started decreasing as cell division occurred in the cells. The cells contained between 2.5 and 3.5 origins; a variation between two and four origins would be expected for perfectly synchronous initiation.

The cell division pattern of cultures of the *dam* mutant was different from that observed with the *dam*$^+$ strain. The expected times of division for a batch culture growing with a 50 min doubling time are 46 and 96 min (Figure 3B, stippled lines). No doubling in cell number was observed at these times. We consistently observed that the cell number was constant for the first 20–25 min of growth, after which it increased steadily for the rest of the experiment. This lag in cell division, as well as the small size of the eluted cells (Figure 1), strongly suggested that the cells collected were truly 'newborn' and not a random wash-off from the membrane.

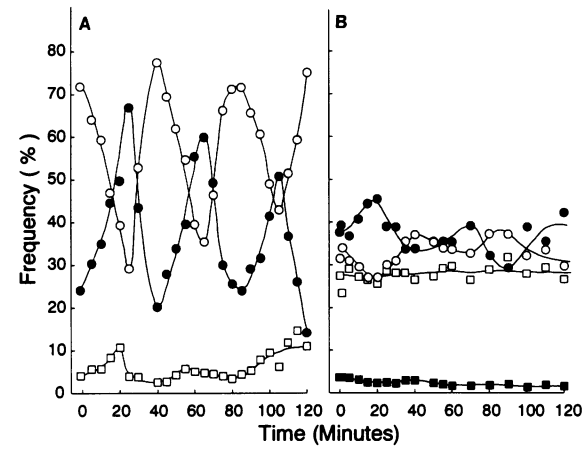

Fig. 4. Variation in number of origins per cell. 'Newborn' wild-type (**A**) and *dam* mutant (**B**) cells were collected and grown as batch cultures. At the times indicated samples were taken for determination of the frequency of cells containing one (■), two (○), three (□) or four origins (●). For the *dam* mutant strain the frequency of cells with five or more origins never exceeded 15% and these are included in the fraction of cells containing four origins.

Cultures of the *dam* mutant also exhibited a reproducible pattern in the rate of DNA synthesis which differed from that of the *dam*$^+$ strain. A repeated pattern was, however, only observed for the first two doubling times, indicating that synchrony is rapidly lost in this strain. The rate of DNA synthesis increased by ~30% during the first 15 min following birth of the cells, and another increase of ~50% occurred at 30–45 min. The total rate of DNA synthesis consequently doubled during the first 50 min, or one doubling time. This suggests that during the first mass doubling time, initiations took place at two discrete periods around 10 min and 35 min after elution.

During the first two mass doubling times following elution, the *dam* mutant showed minor fluctuations in the average number of origins per cell. A small peak was repeatedly observed around 25–30 min, just prior to the onset of cell division. A second small peak was observed ~50 min later, at ~70–75 min.

Variation in number of origins per cell

In the wild-type strain a cyclic variation between two and four origins per cell was observed (Figure 4A), in agreement with the predictions of Cooper and Helmstetter (1968). Initiation converts cells containing two origins to cells containing four origins. Therefore a decrease in the frequency of two-origin cells and concomitant increase in four-origin cells reflect an initiation event. Division has the opposite effect, it increases the number of cells with two origins and decreases the number with four origins. Cells containing three origins of replication were not abundant and never exceeded 15% of the population, an indication of initiation synchrony within single cells. The frequency of three-origin cells showed peaks at around 18–20 and 55–60 min—shortly before the four-origin peak appeared. These peaks most likely represent cells in which one of the two origins has initiated replication at the time of drug addition, and may therefore reflect the time sequence within a short-lived cascade of initiations.

Initiation of replication, detected as the transition from

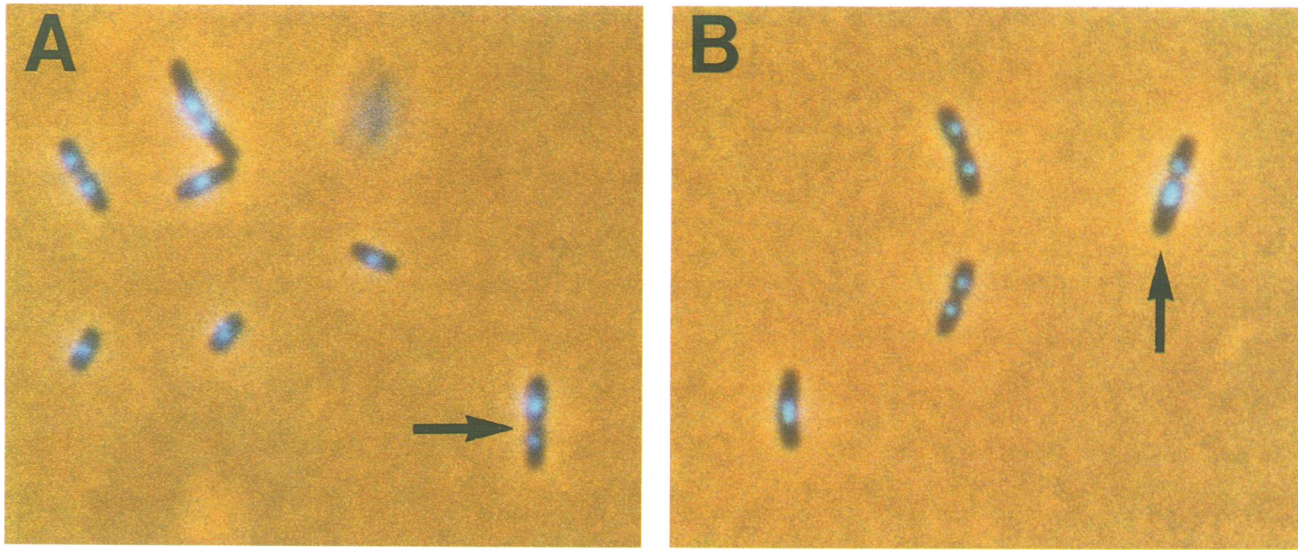

Fig. 5. Nucleoid partitioning. Dividing pairs of *dam*$^-$ cells were allowed to form on microscope slides dipped in LB agar containing 300 μg/ml of chloramphenicol. Nucleoids were fixed and stained with DAPI before visualization in a fluorescence microscope. For details see Materials and methods. (**A**) A cell pair with equal size daughters but unequal size nucleoids is indicated. (**B**) A pair of cells with unequally sized daughters and unequally sized nucleoids is indicated.

two- to four-origin cells, took place over periods of 25 min (from 0 to 25 min and from 40 to 65 min). Cell division, detected as the transition from four- to two-origin cells, took place more rapidly (from 25 to 40 min and again from 65 to 85 min). This indicates that initiation in the wild-type cell cycle is not very well timed relative to cell division.

The data obtained for the *dam* mutant indicate that cells eluted from the membrane were heterogeneous with respect to origin content; 80% of the cells contained two, three or four origins (Figures 2B and 4B). The remaining 20% of the cells contained either one (3–5% of the cells) or more than four origins (~15% of the cells). Since the cells had apparently just divided and had sizes comparable to those of the smallest cells in the culture before selection, such heterogeneity in the number of origins was unexpected.

Due to the heterogeneity of the eluted cells, there is no simple explanation for the various changes that occur in the *dam* culture. However, initiation and cell division can be used to explain the basic cell cycle patterns of the *dam* mutant strain. The data shown in Figure 4B demonstrate that, during the first 20 min after elution when no cell division took place (Figure 3B), the fraction of cells containing four origins increased, the fraction of two-origin cells decreased, and the fraction of three-origin cells remained constant. This suggests that initiation took place in this period, in good agreement with the increase in DNA synthesis rate (indicated in Figure 3B). A simple explanation for this pattern is that initiation occurred in cells with two origins, which decreased that class and increased the fraction of three-origin cells. However, this increase was balanced by initiations in cells with three origins, which decreased that class and increased the number of four-origin cells. Cell divisions then occurred from 20 to 50 min. This decreased the number of four-origin cells and increased the number of two-origin cells. The fraction of cells containing three origins again remained constant. Note that except for the first 25 min, periods of cell division and initiation overlapped and are more difficult to interpret. The subsequent variations in classes thus reflect the relative rates of initiation and division.

Partitioning

The heterogeneity observed in the number of origins present in 'newborn' *dam*$^-$ cells indicated that this strain frequently partitioned nucleoids that contained different numbers of origins. For example, 28% of the 'newborn' cells contained three origins of replication, yet very few cells with six origins were ever present. An alternative source of these cells would be partitioning of unequal nucleoids from cells with four origins.

To test this possibility we analysed nucleoids in cells stained with DAPI. Exponentially growing cells were incubated for 60 min on microscope slides coated with agar medium containing chloramphenicol, and cell pairs that formed during this incubation were examined. Only cells that had already completed a replication cycle and had progressed into the D period should be able to divide in these conditions (Donachie and Begg, 1989). Since other, on-going replication cycles would be completed in these conditions, the nucleoids would contain completed chromosomes. The amount of DNA would depend on the number of replication origins present on the chromosomes partitioned to the daughter cells.

Pairs with unequal levels of fluorescence in the two daughter cells were readily detected in *dam*$^-$ cultures (Figure 5A and B) and represented ~25% of the dividing pairs. In *dam*$^+$ cultures, few of the dividing pairs exhibited this unequal fluorescence (not shown). As indicated in Figure 5B, dividing *dam*$^-$ cells that contained nucleoids of different DNA contents also sometimes produced daughters of unequal lengths. The longer sisters would contribute to the skewness in the *dam*$^-$ curve(s) shown in Figure 1.

Discussion

Newly divided cells which were eluted from the 'baby cell machine' exhibited surprisingly similar size distributions; the *dam*$^-$ cells being only marginally bigger than the wild-type cells. The majority of the wild-type 'baby' cells contained a replicating chromosome in the so-called 'theta' structure

with two origins. This is what should be expected for *E.coli* cells growing with a 42 min doubling time. At division, the replication forks should be half way through the next replication cycle and the cell would contain two 'theta' structure chromosomes. The *dam* mutant strain was different. A large fraction of the 'newborn' *dam*$^-$ cells also contained a replicating chromosome with two origins, but in addition there were many cells which contained DNA corresponding to more than two genome equivalents. These latter cells have three or four origins, and a significant fraction of the cells have more than four origins (Figure 2).

Cell cycle events were followed for at least two doublings. The wild-type strain behaved as expected and showed variations in DNA synthesis rate, cell number and origins/cell ratio as should be expected for a synchronous culture (Figure 3A). When the same parameters were followed in the *dam*$^-$ strain, the fluctuations that occurred were more subtle. The cell number stayed constant for 20−25 min and then increased. The rate of DNA synthesis appeared to exhibit stepwise increases every half doubling time. The origins/cell ratio stayed virtually constant, somewhat higher than for the wild-type strain in agreement with previous observations (Boye and Løbner-Olesen, 1990). The DNA/mass and the origin/mass ratios, the latter being proportional to the initiation mass, were virtually the same in the two strains.

The flow cytometer allowed us to analyse the initiation synchrony in the two strains in a little more detail. The data presented in Figure 4A show cyclic oscillations of wild-type cells having either two or four origins. There were also cyclic oscillations in the number of cells with three origins. The three-origin cells built up to a maximum after 20 min of growth and most of these disappeared 5 min later. At this time the four-origin cells showed a maximum. The increase in three- and four-origin cells thus parallel each other for the first 20 min. The '5 min' difference between the maxima of the three- and four-origin cells is the first experimental indication that initiations of chromosome replication, in cells which have more than one origin to initiate, could be separated significantly in time for the individual origins.

The *dam* mutant strain also showed oscillations in numbers of origins (Figure 4B), but these were moderate relative to the wild-type. Surprisingly, the fraction of cells with three origins stayed constant (~28%) for the whole experiment, whereas increases and decreases in the number of cells with two origins were paralleled by simultaneous decreases and increases in the number of cells with four or more origins. The constancy of the three-origin cells indicates that there was a fraction (10%) of newly divided cells with two origins which initiated and became cells with three origins, and there was a similar fraction of newly divided cells with three origins which initiated and became cells with four or more origins. The variations in cells with different numbers of origins were for the first 20 min accompanied by an ~30% increase in DNA synthesis rate (Figure 3B). These oscillations indicate that some cells of the *dam* mutant strain initiate soon after division.

The initiation cascade

Many of the properties that were observed in the *dam*$^+$ and *dam* mutant cells can be explained by the initiator titration model (Hansen *et al.*, 1991). In this model accumulation of a critical amount of DnaA protein leads to an initiation event

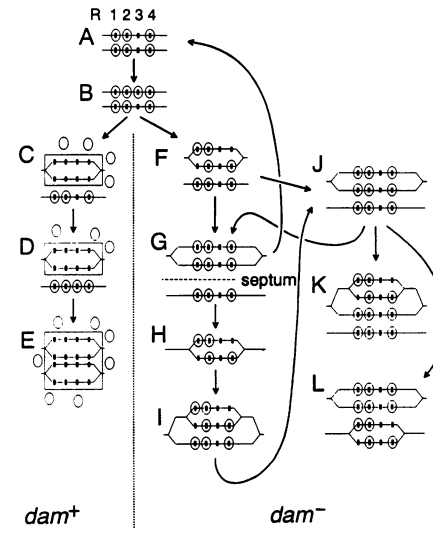

Fig. 6. The cascade mechanism for initiation. A cell containing two fully replicated chromosomes approaches the initiation mass. The three high affinity DnaA binding sites within *oriC* of both chromosomes (R1, R2 and R4) have DnaA protein (ovals) bound to them, whereas the low affinity sites (R3) are empty (**A**). When the initiation mass is reached, site R3 of one of the origins binds DnaA protein and initiation occurs (**B**). It is assumed that the bound DnaA protein is released from the binding sites in this process. This initiation will have different consequences in wild-type (**C**−**E**) or *dam* mutant (**F**−**L**) cells. The boxed area represents hemimethylated origins sequestered from the replication machinery. DnaA protein binding sites outside *oriC* are not indicated in the figure. Initiation has just occurred in panels C, H, I and K/L. Termination has just occurred in panel J. Figure not drawn to scale; see text for details.

(Atlung *et al.*, 1987; Pierucci *et al.*, 1987; Løbner-Olesen *et al.*, 1989; Skarstad *et al.*, 1989; Atlung and Hansen, 1993). Although the details of this model will not be described here, its essential aspects can be illustrated (Figure 6) by the four DnaA binding sites (R1 to R4) in the *oriC* sequence (other sites could also be involved, but the principle remains the same). One of these, R3, has a lower affinity than the other three (Samitt *et al.*, 1989). When DnaA protein accumulation approaches the level critical for initiation, all origins within the cell will have DnaA protein bound to sites R1, R2 and R4 (Figure 6A). Initiation of replication is proposed to occur when site R3 binds DnaA protein as well (Figure 6B). As replication commences at the first origin within a single cell, the bound DnaA protein is assumed to be released.

We propose that this burst-like release of DnaA protein leads to different consequences in *dam*$^+$ and *dam* mutant cells. In the wild-type, the released DnaA protein will bind to other origins present within the same cell and trigger new initiations (Figure 6C, D and E). This situation is similar to the forced initiation observed when DnaA protein synthesis was suddenly induced (Atlung *et al.*, 1987; Pierucci *et al.*, 1987; Løbner-Olesen *et al.*, 1989; Skarstad *et al.*, 1989). Since the newly replicated origins are transiently hemimethylated and membrane-bound (Ogden *et al.*, 1988), they are transiently inaccessible to the DnaA protein (Landoulsi *et al.*, 1990; Figure 6C boxed region), and the ratio of free DnaA protein to accessible origins is momentarily increased. As a consequence initiations will occur as a cascade at 'old' origins only (Figure 6D and E). Therefore all origins within the single cell are initiated within a relatively narrow time

interval. When the sequestered origins are released after approximately one-third of a generation (Ogden *et al.*, 1988; Campbell and Kleckner, 1990), replication will have produced a number of new DnaA binding sites (not indicated in Figure 6), and the DnaA/origin ratio will be low.

A different situation occurs in *dam* mutant cells, following initiation. When the amount of DnaA protein builds up to the initiation level, replication is initiated at one of the origins (Figure 6B). In contrast to the situation in the wild-type cell, the two newly synthesized origins are unmethylated, and will not be sequestered into the cell membrane (Figure 6F; Ogden *et al.*, 1988; Campbell and Kleckner, 1990). They will therefore immediately start titrating the free DnaA protein (Hansen *et al.*, 1987) that is released by the initiation event and very soon bring the DnaA/origin ratio below the threshold level. This will prevent the occurrence of the initiation cascade in the *dam* mutant cells.

It is only a fraction of the origins that are initiated in the *dam*$^-$ cells, thus the decrease in DnaA/origin ratio will not be as marked as in the wild-type cells, leading to a shorter time period for the build-up of initiation potential (accumulation of DnaA protein) for the next initiation. As an example we can look at a *dam*$^-$ cell with two origins at the time of initiation (Figure 6A and B) that initiates only one origin (Figure 6F). As these origins in the *dam*$^-$ cell are not sequestered to the membrane, the resulting three origins would have an equal probability of being initiated the next time. In some cases the next initiation will occur before cell division (Figure 6J) leading to the chromosome configuration shown in Figure 6K or L. In other cases the other initiation will not occur until the next cell cycle, after cell division has occurred (Figure 6G). The nascent daughter cell that receives two origins will have a low DnaA/origin ratio resulting in a delayed time interval before initiation, but it will eventually repeat the pattern shown in Figure 6A, B and F.

The nascent daughter that receives one fully replicated chromosome will follow a more complicated sequence of events. As soon as the cell is separated by the nascent septum, the compartment receiving one chromosome will have a high DnaA/origin ratio. Replication will be initiated (Figure 6G and H), in most cases before the daughters are physically separated, leading to 'newborn' cells with two origins. Figure 2B shows that eluted 'newborn' cells with one origin are infrequent.

The first initiation in cells with one chromosome will lead to a division ~65 min later. However, the potential for the next (second) initiation will be reached earlier (possibly at ~35 min; Figure 3B) and an initiation will occur at a randomly chosen origin, leading to the chromosome configuration illustrated in Figure 6I. The replication cycle due to the first initiation would terminate at 40 min (Figure 6J), and the resulting cell division (at 65 min) would overlap with the build-up to the threshold potential for a third initiation. If this initiation took place before division, the result would be cells with the chromosome configuration shown in Figure 6K or L. Otherwise, the dividing cells would re-enter the *dam*$^-$ cycle at position Figure 6G. A cell obtaining the configuration illustrated in Figure 6K would at the following division become two cells, one with one origin and the other with three. The cell receiving one chromosome would possibly be even more prone to initiation than the one arising from the division of the three-origin cell in Figure 6G. Note that the initiations leading to Figure 6I and K/L are separated by ~25 min (half a doubling time). Cell divisions corresponding to these two initiation events will consequently also be separated by ~25 min. In agreement with this we observed some newly abscised *dam*$^-$ cells that had already divided after 25 min (Figure 3B).

There is no simple way to explain or characterize the multitude of different cell cycles which occur in the *dam* mutant. The model presented in Figure 6 only illustrates some of the simpler *dam*$^-$ cell cycles, but it also demonstrates the complexity of the phenomena observed in the *dam* mutant. To have a more complete description of these cell cycles it would be necessary to keep track of the cell size and the DNA content of individual cells passing through a multitude of different cell cycles. As an example of this complexity consider the two cells arising from division in the cell in Figure 6L. These two cells might be quite similar in size and they both contain two origins. However, their DNA contents would be different: when will the next initiation occur in these two cells?

According to the above model, origins are randomly selected for initiation in the *dam* mutant strain. Some origins will quickly be reinitiated, while others might avoid replication for a long time. The interinitation time is therefore highly variable (Baker and Smith, 1989) and density transfer experiments would not be expected to indicate a repeated pattern of replication. The model would also predict that rapidly growing cells of the *dam*$^-$ strain would show the asynchrony phenotype (Boye and Løbner-Olesen, 1990). At slow growth rates, when cells only contain one origin at the time of initiation, the model would predict that *dam*$^-$ cells would be similar to wild-type cells, which is in agreement with previous observations (Boye *et al.*, 1992).

The basic postulate of the *dam*$^-$ cell cycle model is that the absence of the initiation cascade in *dam*$^-$ cells is caused by the missing sequestration of newly initiated *dam*$^-$ origins to the membrane, and thereby the rapid rebinding of DnaA protein to the DnaA binding sites in the origin. This rapid rebinding causes the initiation cascade to be very short-lived or absent with the net result that cells which have more than one origin at the time of initiation will initiate only one of these origins. Therefore, we propose that the *dam* mutant is also an initiation cascade mutant.

Partitioning

We have been able to obtain direct, microscopic support of a major prediction of the cascade model: distribution of origins to daughter cells at division will often be unequal in *dam* mutants. We have not attempted to quantify the amounts of DNA in the pairs of daughter cells, but visual examination indicates at least a 2-fold difference in the amounts of DNA present in many of the pairs. Since cells with six origins, which would give a 4:2 split, are rare, this suggests that many of these pairs arise from cells containing three or four origins. If a cell is in the D period when chloramphenicol is added, cell division will still occur but initiation will be blocked, even in the daughter that receives the chromosome with only one origin. As mentioned above, eluted 'baby' cells with only one origin were infrequent, presumably because initiation occurred at or shortly before cell division.

In examining fluorescently stained cells, we have observed

very few cells lacking DNA. This is consistent with the observations of Vinella *et al.* (1992). As suggested by them, this also indicates that the sequestration of newly replicated, hemimethylated origins (Ogden *et al.*, 1988) is not necessary for accurate partitioning. Termination of a replication cycle and septum formation between the nucleoids containing the newly completed chromosomes still occurs, even though the chromosomes contain different numbers of replication origins.

The basis of the unequal location of the division septum in some cells is unclear, but it indicates that DNA affects the location of the septum. Whether this is due to nucleoid occlusion (Woldringh *et al.*, 1991) or is somehow influenced by the number or age of the origins remains to be determined. The partitioning of nonequivalent nucleoids exhibited by these mutants provides a new way to characterize some of the controls that act during the bacterial cell cycle. It will be interesting to determine how the other mutations that give the asynchrony phenotype, such as *dnaA* and *fis*, affect initiation and partitioning.

Materials and methods

Bacteria and growth conditions

The strains used were *E.coli* B/r F26 (*thyA*, *his*; Helmstetter *et al.*, 1992) and its *dam16*::Km derivative (ALO1141). The *dam16*::Km mutation (Parker and Marinus, 1988) was introduced into the F26 strain by P1 transduction.

Cells were grown at 37°C in M9 minimal medium (Miller, 1972) supplemented with 0.2% glucose, 20 μg/ml histidine and 10 μg/ml thymine. The optical density of the culture was measured in a Beckman DU-50 spectrophotometer. Cell number was monitored using a Coulter counter model Z_B equipped with a 30 μm orifice.

Membrane elution

A 100 ml volume of exponentially growing cells at an OD_{450} of 0.1 (5×10^7 cells/ml) was filtered onto a nitrocellulose membrane (Millipore type GS 142 mm) coated with poly-D-lysine (Sigma) as described by Helmstetter *et al.* (1992). This procedure was originally developed for applying the membrane elution technique to *E.coli* K-12 strains, but we used it for synchronization of the *E.coli* B/r derived strains used here. Cells were eluted at a flow rate of 2 ml/min.

During the early stages of elution cell numbers in the effluent were $\sim 2-4 \times 10^6$ cells/ml, but this number increased rapidly and stabilized at $\sim 1-2 \times 10^7$ cells/ml after 20–25 min elution. Cells continued to be eluted at concentrations fluctuating around this value for several hours (data not shown).

Cell cycle analysis

When the concentration of cells in the effluent from the membrane had stabilized, 'newborn' cells were collected in a culture flask for 4 min. Since these arose within a time interval of less than 1/10 of the doubling time of the culture, they represent a synchronous culture. Samples were taken for determination of cell number and DNA synthesis rate and for flow cytometry. For determination of the DNA synthesis rate, 150 μl of cells were pulse-labelled at 37°C for 2 min with 1 μCi [^{3}H]thymidine (6.70 Ci/μmol; Dupont NEN). Under these conditions incorporation of ^{3}H into nucleic acids was linear for at least 10 min (not shown). Incorporation was stopped by adding 1 ml of 1 M NaOH containing 1 mg/ml unlabelled thymidine. After 1 h at 37°C to allow hydrolysis of RNA to occur, the samples were mixed with appropriate amounts of [^{35}S]methionine-labelled ALO1141 reference cells, TCA precipitated and counted in a Beckman LS 6800 liquid scintillation counter. Incorporation of ^{3}H into DNA was expressed as the ^{3}H/^{35}S ratio.

For flow cytometric determination of numbers of origins per cell, 200 μl samples were treated with 300 μg/ml rifampicin (Boehringer Mannheim) and 12 μg/ml cephalexin (Sigma) as previously described (Løbner-Olesen *et al.*, 1989). The strains used in this work stopped all cell division 6 min after cephalexin addition (not shown). Cellular DNA content and DNA concentration were determined as described (Løbner-Olesen *et al.*, 1989) in 'newborn' cells killed by sampling directly into 0.01 M ice-cold cyanide.

Flow cytometry

Flow cytometry was performed using an Argus 100 flow cytometer as previously described (Skarstad *et al.*, 1985).

Microscopy

Cells growing exponentially in M9 medium (10 μl at 10^8 cells/ml) were spread on microscope slides that had been dipped (Stewart and D'Ari, 1992) in LB agar (Miller, 1972) containing 300 μg/ml chloramphenicol. The preparations were incubated for 60 min at 37°C in humidified chambers and then fixed and stained with DAPI (4',6'-diamino-2-phenyl-indole) as described by Hiraga *et al.* (1989). Microscopy was done with a Zeiss Axioskop fluorescence microscope with a 100× Neofluar objective.

Acknowledgements

We thank C.E.Helmstetter for providing the *E.coli* B/r F26 strain, and Martin Marinus for the *dam16*::Km allele. We are grateful to Tove Atlung, Erik Boye and Martin Marinus for helpful discussions and critical reading of the manuscript. This work was supported by grants from the Danish Centre for Microbiology (A.L.O., F.G.H. and K.V.R.), by the Danish Natural Science Research Council grant 11-9317 (A.L.O.) and by grant GM 15965 from the National Institutes of Health (P.L.K.).

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Received on November 2, 1993; revised on January 31, 1994