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Timing of initiation of chromosome replication in individual *Escherichia coli* cells

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The synchrony of initiation of chromosome replication at multiple origins within individual *Escherichia coli* cells was studied by a novel method. Initiation of replication was inhibited with rifampicin or chloramphenicol and after completion of ongoing rounds of replication the numbers of fully replicated chromosomes in individual cells were measured by flow cytometry. In rapidly growing cultures, with parallel replication of several chromosomes, cells will end up with $2n$ ($n = 1, 2, 3$) chromosomes if initiation occurs simultaneously at all origins. A culture with asynchronous initiation may in addition contain cells with irregular numbers ($\neq 2n$) of chromosomes. The frequency of cells with irregular numbers of chromosomes is a measure of the degree of asynchrony of initiation. After inhibition of initiation and run-out of replication in rapidly growing B/r A and K-12 cultures, a small fraction of the cells (2–7%) contained 3, 5, 6 or 7 chromosomes. From these measurements it was calculated that initiation at four origins in a single cell occurred within a small fraction, 0.1, of the doubling time (τ). A *dnaA*(Ts) mutant strain grown at permissive temperature exhibited a very large fraction of cells with irregular numbers of chromosomes after drug treatment demonstrating virtually random timing of initiation. A similar pattern of chromosome number per cell was found after treatment of a *recA* strain.

Key words: dnaA/flow cytometry/initiation at *oriC*/*recA*

Introduction

Initiation of chromosomal replication in *Escherichia coli* is a complex process involving >10 different proteins (Kaguni and Kornberg, 1984), some of which are initiation specific (Hirota *et al.*, 1968; Carl, 1970; Wechsler and Gross, 1971; Zyskind and Smith, 1977; Hansen and von Meyenburg, 1979). *De novo* protein synthesis is required at each initiation (Maaløe and Hanawalt, 1961; Lark *et al.*, 1963) as well as transcription by RNA polymerase (Lark, 1972; Messer, 1972; von Meyenburg *et al.*, 1979; Ogawa *et al.*, 1985). The initiation event occurs at a specific site, *oriC* (Nagata and Meselsohn, 1968; Bird *et al.*, 1972), and appears to be regulated in such a way that it occurs when a certain initiation mass is reached (Donachie, 1968). When growth is rapid, with parallel replication of several chromosomes, initiation has earlier been assumed to occur simultaneously at all origins within a cell (Cooper and Helmstetter, 1968; Bremer and Churchward, 1977). Recently, mathematical analyses of DNA distributions of exponentially growing cultures measured by flow cytometry have indeed indicated that initiations within a single cell do occur essentially simultaneously rather than sequentially (Skarstad *et al.*, 1985).

Here we describe a novel method of measuring the degree of synchrony of initiation at the different origins within single cells in rapidly growing cultures. Initiation was inhibited by chloramphenicol (CAP) which inhibits proteins synthesis (Vazquez, 1979) or by rifampicin (RIF) which inhibits initiation of RNA synthesis (Wehrli and Staehelin, 1971), while DNA synthesis was allowed to continue until all cells had completed replication and ended up with fully replicated chromosomes. The number of chromosomes per cell was then measured by flow cytometry. If initiation occurred simultaneously at all origins in a rapidly growing cell, the culture would be expected to contain two, four or eight fully replicated chromosomes per cell. Thus, if the cells with chromosome numbers different from $2n$ ($n = 1, 2, 3$) were found their frequency would reflect the degree of asynchrony of initiation (numbers of chromosomes different from $2n$ will be termed 'irregular numbers of chromosomes'). We have used this method to measure the degree of synchrony of initiation in wild-type cells of B/r and K-12 and in *dnaA*(Ts) and *recA* mutants.

Results

DNA and mass distributions obtained by flow cytometry

Flow cytometry was performed with a microscope-based flow cytometer where stained cells pass one by one through the focus of a modified fluorescence microscope (Steen and Lindmo, 1979). Each cell gives rise to a pulse of fluorescent light, the size of which is proportional to the amount of DNA-bound dye and thus proportional to the cellular DNA content. The fluorescence light pulses are transformed into equivalent electrical signals, sized and stored as counts added to the memory channels in a multichannel anlayzer. The light scattered by each cell is proportional to the mass and protein content of the cell (Boye *et al.*, 1983) and is detected simultaneously and stored in the same multichannel analyzer. Thus, a three-dimensional frequency histogram accumulates for DNA content and light scatter (mass) of individual cells in the population (Figure 2).

From such flow cytometric analyses of expontentially growing cultures we have found earlier that initiation of DNA replication in *E. coli* B/r A was essentially synchronous; we concluded that the initiations occurred within a time interval of <15% of the doubling time (Skarstad *et al.*, 1985). Below we describe a novel method for estimation of the degree of synchrony of initiation in rapidly growing cells with multiple replication origins. First, initiation of DNA replication was inhibited by RIF or CAP and ongoing rounds of replication allowed to run to completion. Then the DNA and light scatter distributions of the drug-treated cells were measured by flow cytometry. Virtually all the cells ended up with fully replicated chromosomes giving histograms with discrete peaks at DNA contents representing integral numbers of chromosomes (Figures 2B and 3). The number of cells in each peak was counted and the fraction of cells with irregular numbers of chromosomes was taken as a measure of the degree of asynchrony of the initiation events in single cells. The calculation procedure used when determining the degree of asynchrony of initiation is described below.

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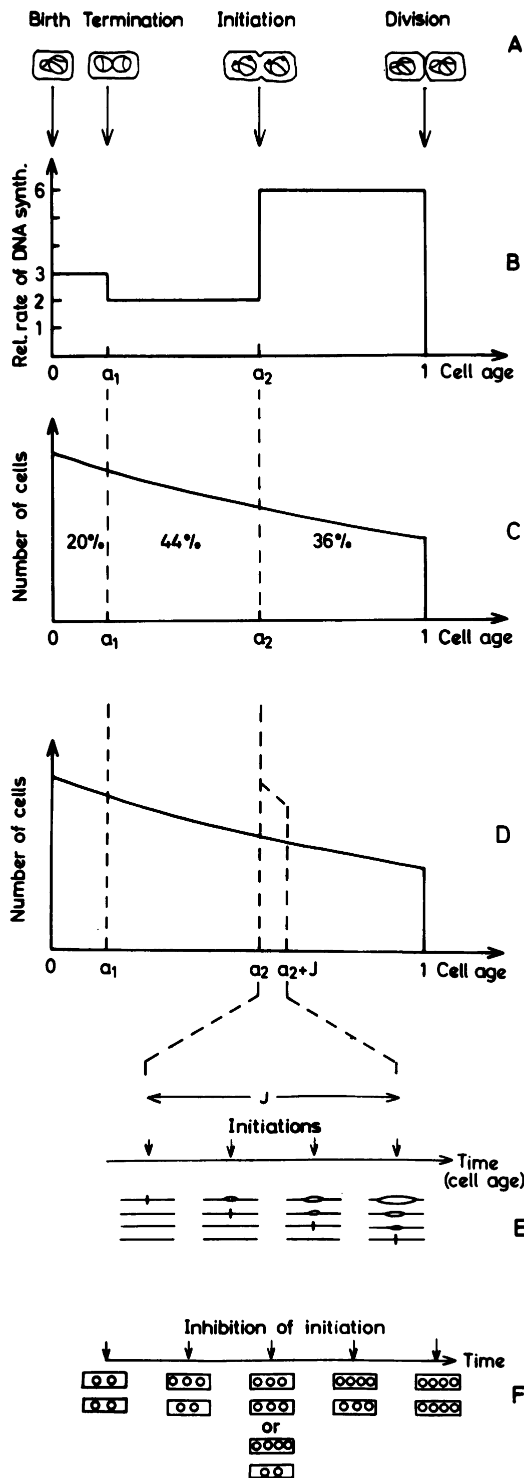

Fig. 1. (A) Schematic representation of chromosome configurations in cells with τ=27 min, C=43 min and D=23 min at birth (age=0), termination (a_1) and initiation (a_2) of replication, and at cell division (a=1). (B) Relative rate of DNA synthesis per cell as a function of cell age. (C) Age distribution of an exponential culture with no cell-to-cell variation in cell cycle parameters and synchronous initiation at all four origins in a cell. The percentages refer to the fraction of cells present in the three parts of the cell cycle. (D) The same age distribution as in (C) with a period, J, within which four initiations at the four origins occur. (E) Schematic representation of J with asynchronous, consecutive initiation (arrows) at four origins. In the lower part, the origins are depicted as lines before initiation and bubbles when initiation has occurred. (F) Inhibition of initiation (arrows) at times between the four initiation events and (lower part) chromosome configurations in cells after run-out of replication and cell division.

Determination of initiation synchrony; theoretical considerations

In an exponentially growing culture of *E. coli* B/r A with a doubling time (τ) of 27 min, the DNA replication time (C) and post-replication period (D) have been estimated to be 43 min and 23 min, respectively (Skarstad *et al.*, 1985). The expected chromosome configurations at birth, termination and initiation of DNA replication and at cell division (Cooper and Helmstetter, 1968) are depicted schematically in Figure 1A. Newborn cells (age a_0 = 0) contain one replicating structure with six replication forks and a rate of DNA synthesis of three chromosomes per C period (Figure 1B). At age $a_1 = (\tau - D)/\tau = 0.15$ one pair of forks reaches the terminus, resulting in two replicating chromosomes with two forks each and a total rate of DNA synthesis of two chromosomes per C period. Initiation occurs at age $a_2 = [3\tau-(C+D)]/\tau=0.56$ at four origins and the relative rate of DNA synthesis rises to six chromosomes per C period.

The age distribution of an ideal exponential cuture of such cells is shown in Figure 1C. Twenty percent of the cells are of age 0 to a_1, containing a single replicating structure with six forks, 44% are of age a_1 to a_2, containing two replicating structures with two forks each, while 36% are of age a_2 to 1, containing two replication structures with six forks each.

When we treat such a culture with a drug which has been shown to inhibit initiations (RIF or CAP) and allow ongoing rounds of replication to run to completion, 64% (20% + 44%) of the cells will contain four chromosomes and 36% will end up with eight chromosomes, provided no cells divide. If all cells with two separate replicating structures at the time of inhibition (80% of the culture) eventually divide, the culture should consist of 49% two-chromosome cells and 51% four-chromosome cells after completion of replication.

If the four initiation events occur at different times at the four origins within a single cell, i.e. over a time interval a_2 to a_2 + J (Figure 1D), RIF or CAP addition during the period of initiation (J) will inhibit replication from some but not all origins in the cell. The situation where asynchronous initiations occur consecutively at four origins within a cell over a period J is shown schematically in Figure 1E. Inhibitions of initiation occurring at times between the first and the last initiation event causes the cell, after completion of replication, to end up with five, six or seven fully replicated chromosomes or, if division occurred, with two, three or four chromosomes (Figure 1F). Asynchrony of initiation is, therefore, expected to lead to cells with numbers of chromosomes $\neq 2n$. The greater the time interval, J, during which the four initiation events occur, the greater the percentage of cells with irregular numbers of chromosomes. From this percentage we can estimate J as a fraction of the doubling time (see below). Thus, J represents the degree of asynchrony.

Treatment with RIF or CAP

In a B/r A culture growing exponentially with τ = 27 min the DNA distribution was continuous (Figure 2A) and there was near proportionality between DNA and light scatter, i.e. protein content. Also, the ridge in the histogram was very narrow, which means that cells with given DNA contents have protein contents only within narrow limits. Exponential cultures were treated with RIF for 90 min or with CAP for 110 min, at which time DNA synthesis had come to a halt, as judged from [^{3}H]thymidine incorporation (data not shown). Flow cytometry histograms of these cells showed peaks at DNA contents representing integral numbers of chromosomes (Figures 2B and 3).

The major fraction of both RIF- and CAP-treated cells contained two, four or eight fully replicated chromosomes, and only a few cells had irregular numbers of chromosomes (Figures 2B

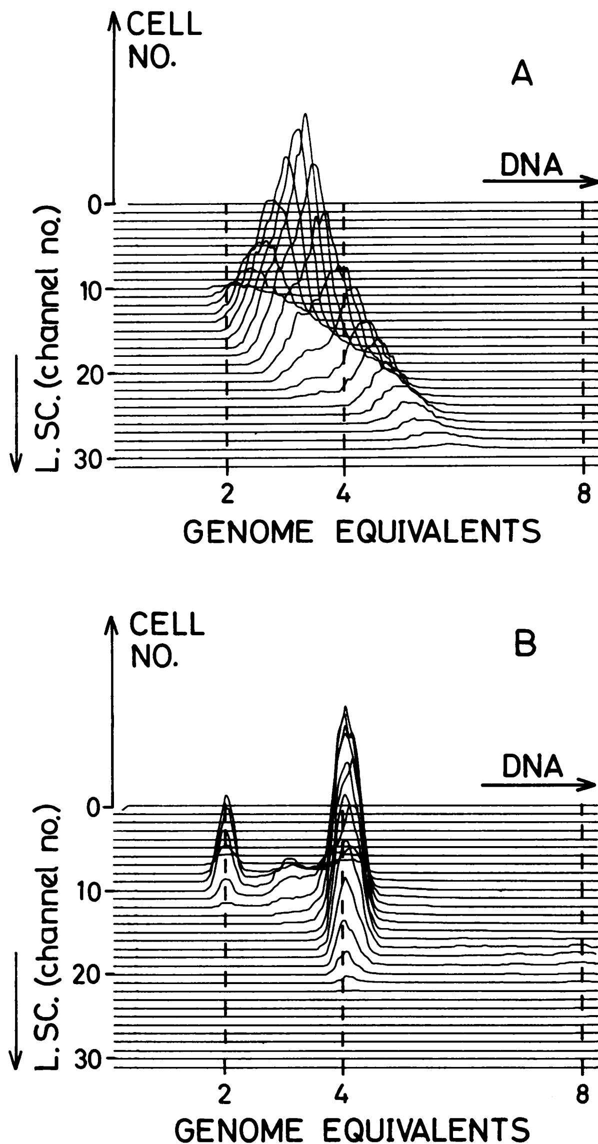

Fig. 2. DNA/light scatter histograms (128 by 32 channels) of an exponential B/r A culture with τ=27 min (**A**) treated with 150 μg/ml of RIF for 90 min (**B**). The two main peaks in (**B**) represent cells with two and four chromosomes.

and 3 and Table I). The presence of cells with irregular numbers of chromosomes indicated that initiation was not perfectly synchronous.

Residual cell division in the presence of RIF or CAP

If cell division were totally inhibited 36% of the cells would end up with eight chromosomes after drug treatment (see Figure 1). The low percentages of eight-chromosome cells (Figures 2B and 3) show that the majority of cells which were in the latter part of the D-period and had initiated chromosome replication (cells of age a_2 to 1) were able to divide. This conclusion is supported by the reduction in average protein content after RIF treatment, as evidenced by the reduction in light scatter values (Figure 2). If all cells in the D period (between cell age a_1 and 1) at the time of drug action divided, 49% of the cells would be expected to contain two chromosomes. The actual percentage of two-chromosome cells was, however, considerably lower (5 and 8% for RIF and CAP treatment, respectively). From these figures we can calculate that about half of the cells in the D period did not divide after inhibition of protein synthesis.

Since insignificant synthesis or degradation of protein occurs after drug action (Willetts, 1967) the protein content of a drug-

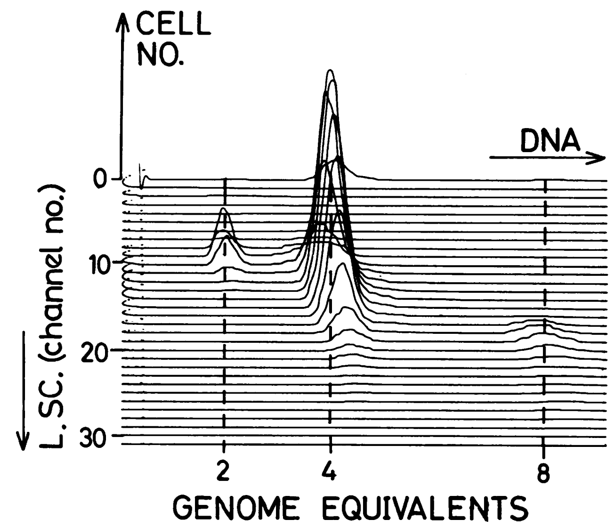

Fig. 3. DNA/light scatter histogram (128 by 32 channels) of an exponential B/r A culture with τ=27 min treated with 200 μg/ml of CAP for 110 min. The amplification settings were slightly different from those of Figure 2.

Table I. DNA content of *E. coli* cells treated with RIF or CAP

Strain	Doubling time (min)	Drug	Time after drug addition (min)	Number of cells measured	Percentage of cells with different numbers of chromosomes								J	Figure
					1 chr	2 chr	3 chr	4 chr	5 chr	6 chr	7 chr	8 chr		
B/r A	27	RIF	90	58 830	0	8	5	76	0	1	1	2	0.11	2B
B/r A	27	CAP	110	87 846	0	5	1	81	1	0	0	8	0.03	3
K-12 AB1157	35	RIF	90	95 344	0	5	1	81	2	1	1	8	0.07	ns
K-12 AB1157	36	CAP	180	210 869	0	2	1	70	–	6[a,b]	–	12	0.10	ns
K-12 AB1157 *recA*56	53	RIF	135	97 077	nd	20	28	33	4	2	2	nd	–	5B
K-12 CM735	54[c]	RIF	135	228 813	0	28	3	57	2	2	0	5	0.10	4A
K-12 CM742 *dnaA*46	57[c]	RIF	135	163 991	4	12	16	24	14	12	9[b]	6[b]	–	4B

ns = not shown; nd = not determined; J = degree of asynchrony (expressed as fraction of τ).
[a]Percentage of cells with five, six and seven chromosomes.
[b]Peaks were not well separated.
[c]Grown at 29°C.

treated cell reflects this cell's (or its mother cell's) protein content at the time of drug action. The eight-chromosome cells had relatively low protein contents (channels number 16–22), which indicates that they had initiated with a small cell size and were not able to divide. Cells constituting the two-chromosome peak had light scatter values from seven to 12 and were smaller than most exponentially growing cells. The mothers of these cells (with light scatter values from 14 to 24) had not initiated at the time of drug action (since the daughters end up with only two chromosomes each) but were sufficiently far into the D period to be able to divide. It may thus be noted that among cells within the same size range some were able to divide but had not initiated (mothers of two-chromosome cells), while others had initiated but were not able to divide (eight-chromosome cells). This reflects the cell-to-cell variation in initiation and termination ages within the exponential culture.

After RIF treatment, all three-chromosome cells and part of the four-chromosome cells (Figure 2B) were smaller than the smallest cells in the exponential culture (Figure 2A) and were probably daughters of cells that had initiated shortly before the time of drug action. The large four-chromosome cells either had old and large mothers at the time of drug action or were cells that had neither initiated before, nor divided after, drug action.

The CAP-treated culture had a protein distribution similar to that after RIF treatment. A slightly larger fraction of the cells accumulated with eight chromosomes and a smaller fraction with two chromosomes, indicating that fewer cells were allowed to divide after CAP treatment than after RIF treatment. This is probably because CAP treatment immediately blocks protein synthesis required for cell division and for initiation. After RIF treatment, protein synthesis (and thus cell division) ceases more slowly as a result of 'run-out' of mRNA synthesis and gradual mRNA degradation (Pato and von Meyenburg, 1971) while initiation of replication is blocked almost immediately through the effect of RIF on transcription (von Meyenburg *et al.*, 1979).

Determination of initiation synchrony; calculation of J

Estimation of the degree of asynchrony, J, was performed for an ideal culture with a doubling time of 27 min and the C and D values mentioned above. Experimental cultures have cell-to-cell variations in these growth parameters but this will only influence the calculated values of J to a small extent. It was assumed that the duration of J was the same for all cells in a culture. Furthermore, it was assumed that if division occurred cells destined to end up with seven chromsomes would divide into three- and four-chromosome cells, 50% of six-chromosome cells would divide into two- and four-chromosome cells and 50% into three-chromosome cells, while all five-chromosome cells would divide into two- and three-chromosome cells (Figure 1F).

The estimation of J was performed by: (i) calculating the initiation age, a_2, from the values of C, D and τ, (ii) measuring the percentage of cells with three, five, six or seven chromosomes after inhibition of replication, (iii) calculating the percentage of five-, six- or seven-chromosome cells there would have been if no residual division had taken place after inhibition of initiation and (iv) using the age distribution (Figure 1D) to estimate the age interval, J (from a_2 to a_2 + J), corresponding to this percentage of cells.

The following example for the *E. coli* B/r A culture treated with RIF (Figure 2B and Table I) illustrates how J was estimated. From the percentages in Table I it was found that 40% of the culture (half of the cells in the D period at the time of drug action) had divided, increasing the cell number by a factor of 1.4. The 5% three-chromosome cells are daughters of cells that initiated at the time of RIF action. Thus 10% of the culture had mother cells that were in the process of initiation at the time of drug action. The six- and seven-chromosome cells were also in the process of initiation at the time of RIF action. Altogether, these six- and seven-chromosome cells and the mothers of the three-chromosome cells constituted 9.8% of the culture before division. By considering the age distribution it can be found that 9.8% of the culture corresponds to an age interval (J) of 0.11τ or 3 min. In CAP-treated cells J was 0.03τ (1 min) (Table I). Thus, the CAP-resistant stage of initiation was reached within a somewhat smaller time period than the RIF-resistant stage.

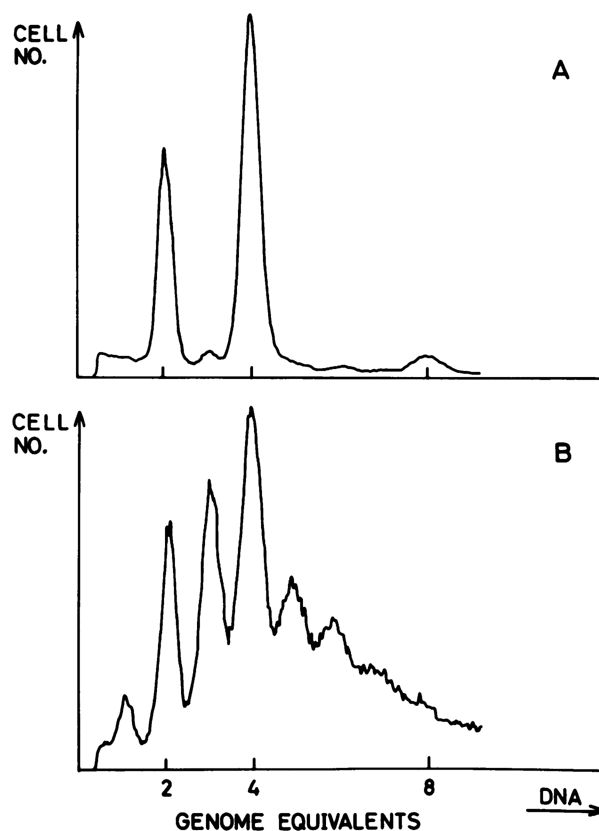

Fig. 4. Single parameter DNA histograms (128 channels) of an exponential CM735 (*dnaA*$^+$) culture with τ=54 min (**A**) and an exponential CM742 (*dnaA46*) culture with τ=57 min (**B**), both grown at 29°C and treated with 150 μg/ml of RIF for 135 min.

The synchrony of initiation in *E. coli* strain K-12 was also investigated. When grown in the same medium as B/r A, strain K-12 AB1157 had a somewhat longer doubling time (τ = 35 min) but essentially the same amount of cells with irregular numbers of chromosomes after RIF treatment (Table I). The increase in cell number after drug treatment was estimated to be 35%. The degree of asynchrony, J, was found to be 0.07τ assuming the same C/τ and D/τ ratio as in the B/r A culture. Another K-12 strain (CM 735) grown at 29°C (τ = 54 min) showed a similar degree of synchrony of initiation (J = 0.10τ) after RIF treatment (Figure 4A, Table I). Thus, the precision with which the RIF-resistant state is reached within single cells seems to be the same in wild-type B/r and K-12. After CAP treatment of AB1157 J was found to be 0.10τ, so in this strain the attainment of the RIF- and CAP-resistant states occurred with the same degree of precision.

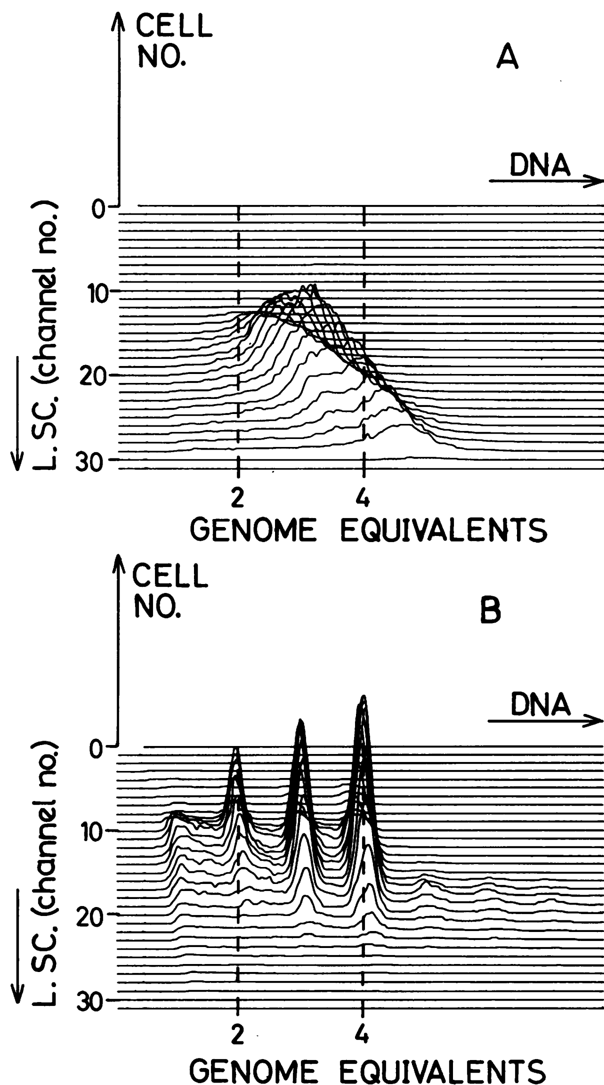

Fig. 5. DNA/light scatter histograms (128 by 32 channels) of an exponential AB1157 *recA56* culture with τ=53 min **(A)** incubated with 150 μg/ml of RIF for 135 min **(B)**. The three main peaks in **(B)** represent cells with two, three and four chromosomes. The fluorescence signal discriminator level was such that cells with < 1 genome equivalent per cell were not recorded. Histograms have the same amplification settings as those of Figure 2.

Synchrony of initiation in a dnaA46 mutant

Initiation at *oriC* requires a functional DnaA protein (von Meyenburg *et al.*, 1979; Schaus *et al.*, 1981; Fuller *et al.*, 1981). Temperature-sensitive mutations in the *dnaA* gene allow cells to initiate at low but not at high temperatures (Hirota *et al.*, 1968; Wechsler and Gross, 1971). We have investigated whether a temperature-sensitive mutation in the *dnaA* gene would affect the timing of initiation at the permissive temperature. A *dnaA46* culture growing exponentially at 29°C was treated with RIF. After completion of ongoing rounds of replication the population consisted of cells containing one, two, three, four, five, six, seven or eight fully replicated chromosomes (Figure 4B and Table I). The large amount of three-, five-, six-, and seven-chromosome cells indicates a high degree of asynchrony in initiation. (The possibility of aberrant division but non-perturbed initiation may be excluded because division normally occurs before forks have reached the termini, see Figure 1.) RIF treatment of an isogenic *dnaA*$^+$ strain yielded mostly two- and four-chromosome cells and a J value of 0.10τ (Figure 4A and Table I).

These results show that the *dnaA46* mutation perturbs the synchrony of initiation even at the permissive temperature. J values were not estimated because this required assumptions about initiation age and interinitiation interval which were difficult to make with such a large asynchrony.

A similar degree of asynchrony was found after CAP treatment of a *dnaA46* mutant (data not shown). Peaks were wider than after RIF treatment and average DNA content per cell was greater, presumably due to less residual cell division (see above).

Synchrony of initiation in a recA56 mutant

The *recA* gene product is involved in initiation of stable DNA replication (Kogoma *et al.*, 1985) which is an alternative replication pathway occurring in *rnh*$^-$ cells. Initiation of this replication has been shown to be asynchronous, as measured with flow cytometry (Kogoma *et al.*, 1985), and random, as measured with density shift experiments (Koppes and von Meyenburg, personal communication). Here we have investigated whether lack of RecA protein affects normal initiation of DNA replication.

The histogram of an exponentially growing culture of AB1157 *recA56* (Figure 5A) shows that the distribution of DNA and protein contents was somewhat wider than what is typical in a wild-type strain (Figure 1A). Furthermore the average protein content and the range of protein contents for each DNA value was greater than in a wild-type, indicating that the coupling between DNA replication and cell division was perturbed in the *recA* mutant. After incubation with RIF for 135 min most of the cells contained integral numbers of chromosomes (Figure 5B and Table I). Some cells had DNA contents between one and two chromosomes, possibly indicating some degree of DNA degradation. Cells with < 1 chromosomes are non-dividing (Capaldo and Barbour, 1975) and were disregarded (not counted). The substantial amount of cells with three chromosomes may indicate a large asynchrony of initiation in the *recA56* cells compared with the *recA*$^+$ counterpart (Table I).

A similar frequency of cells with irregular numbers of chromosomes was found after CAP treatment of the *recA56* culture (data not shown). As in the case of the *dnaA* mutant, peaks were wider and the average DNA content per cell greater after CAP than after RIF treatment.

Discussion

Evaluation of the method

We have presented a novel method which makes it possible to monitor the timing of initiation of DNA replication in individual cells. The method opens up a new source of information concerning the process of initiation of chromosomal replication. Previously, DNA distributions of exponentially growing cultures measured by flow cytometry have been analyzed and used to determine the durations of the C and D periods and the cell-to-cell variability in these parameters (Skarstad *et al.*, 1985). From these analyses we concluded that initiation was essentially synchronous rather than sequential. It was, however, not possible to differentiate between a slight asynchrony of initiation within a single cell and cell-to-cell variation in DNA content at initiation within the population. An upper estimate for the degree of asynchrony was found to be $\sim 0.15\tau$ (4 min) in an exponential B/r A culture with τ = 27 min, assuming no cell-to-cell variation in initiation age. The present results, giving J periods of 0.03 and 0.11τ in which the CAP- and RIF-resistant states were reached, agree well with the earlier upper estimate of J.

The precision in timing of initiation has previously also been measured by determining the constancy of the interreplication

Table II. Bacterial strains used

Strain	Type	Relevant genotype	Source/Reference
B/r A	B	Wild-type	C.E.Helmstetter via H.E.Kubitschek
AB1157	K-12	Wild-type	P.Howard-Flanders (Bachmann, 1972)
AB1157 *recA*	K-12	*recA56 srl300*::Tn10	N.Nanninga
CM735	K-12	Wild-type	(Hansen and von Meyenburg, 1979)
CM742	K-12	*dnaA46*	(Hansen and von Meyenburg, 1979)

time. This time period was determined by density shift experiments (Eberle, 1968; Nagata and Meselsohn, 1968; Koppes and von Meyenburg, personal communication) or by bromouracil photolysis induction, i.e. incorporation of bromouracil and measurement of the time from incorporation to increased resistance to u.v. radiation (Newman and Kubitschek, 1978). It was found that the interreplication time had a relatively small coefficient of variation. In comparison, the coefficient of variation of the interdivision interval (generation time) was considerably larger (Kubitschek, 1962; Schaechter *et al.*, 1962; Koppes *et al.*, 1978; Bremer and Chuang, 1981) indicating that the timing of initiation of replication was more tightly regulated than cell division. Our results indicating a precise timing of initiation within individual cells also suggest that the initiation event is very strictly regulated.

Timing of initiation in wild-type cells

We show here that initiation of replication was essentially synchronous at the four origins of replication within single cells. The degree of synchrony was constant for different strains (B/r A and K-12) and at different temperatures (29°C and 37°C).

The attainment of the RIF-resistant state in B/r A cells was somewhat less synchronous than attainment of the CAP-resistant state. RIF inhibits initiation of replication by preventing transcription at *oriC* while CAP inhibits the *de novo* protein synthesis which is obligatory for initiation of chromosomal replication. The results indicate that the stage of initiation requiring *de novo* protein synthesis is the more precisely timed step in the initiation process for strain B/r A. In K-12 cells attainment of the CAP-resistant state occurred with approximately the same precision as attainment of the RIF-resistant state, so in these cells the stage of initiation requiring *de novo* protein synthesis did not seem to be more precisely timed than the stage requiring transcription.

Timing of initiation in a dnaA(Ts) mutant

A high degree of asynchrony of initiation was observed in a *dnaA46* mutant grown at the permissive temperature. This shows that the *dnaA* mutation affects replication initiation even at the permissive temperature. Initiations at the four origins within a cell were not coordinated but occurred at different times at the different origins. Initiation in a *dnaA46* mutant may therefore be totally random, occurring at any available *oriC* site when enough mutant DnaA protein has accumulated. However, totally random initiation is rather unlikely as cells seem to contain mechanisms that prevent initiation at newly formed origins (lack of methylation by *dam* methylase, lack of proper supercoiling; Leonard *et al.*, 1982; Hughes *et al.*, 1984; Messer *et al.*, 1985; Smith *et al.*, 1985). It is more reasonable to assume that even though the timing is perturbed, initiations occur preferentially at older origins. In support of this, density shift experiments with *dnaA5* and *dnaA46* mutants at the permissive temperature (Tippe-Schindler *et al.*, 1979) showed that 90% of radioactively labelled DNA was found in the hybrid density fraction after two generations, indicating that by this time practically all chromosomes had replicated once.

Several experiments indicate that the DnaA protein has a regulatory function in initiation of replication (for a review, see von Meyenburg and Hansen, 1986). The present data show that mutant DnaA protein causes asynchronous initiation at the permissive temperature. A regulatory function of the DnaA protein may thus be that of coordinating initiations at the different origins so that the initiation event occurs simultaneously at all origins in a cell. This coordinating function is defective at permissive temperatures in some *dnaA*(Ts) mutants but almost intact in others (unpublished data). A study of the timing of chromosome replication in different *dnaA* mutants will be presented elsewhere.

Timing of initiation in a recA mutant

The RecA protein is involved in several enzymatic activities: homologous recombination, post-replication repair and cleavage of different repressors including its own (Little and Mount, 1982). It is also required for initiation of stable DNA replication from origins other than *oriC* (Kogoma *et al.*, 1985). Here we show that the timing of initiation of normal replication seems to be perturbed in *recA*$^-$ cells. There is a certain amount of DNA degradation in *recA*$^-$ strains (Capaldo and Barbour, 1975) and therefore one has to consider the possibility that the three-chromosome cells observed are due to selective degradation of one chromosome in cells initially destined to end up with four chromosomes. The extent of such selective degradation of single chromosomes is currently being investigated. Preliminary results indicate that initiations occur asynchronously in *recA*$^-$ strains, suggesting that *recA* directly or indirectly affects initiation of replication at *oriC*.

The *recA*$^-$ genetic background is frequently used in experiments where integration of plasmids or minichromosomes into the chromosome is undesirable. The present results indicate that *recA*$^-$ cells may not be suitable hosts in experiments where an undisturbed replication pattern is necessary, e.g. studies of initiation of chromosomal replication.

Materials and methods

Bacteria and growth conditions

The strains used are shown in Table II. B/r A and AB1157 wild-type and *recA* cultures were grown at 37°C in K glucose medium (Boye and Krisch, 1980). CM 735 and CM 742 were grown at 29°C in Luria Broth. Culture doubling times were determined by optical density (OD) measurements at 450 and 600 nm for growth in K glucose and LB medium, respectively.

RIF and CAP treatment

Cultures were grown exponentially for a minimum of 10 generations and then treated with 150 μg of RIF per ml or 200 μg of CAP per ml at OD = 0.2 – 0.4.

Fixation and staining

Culture samples were washed and resuspended in ice-cold 10 mM Tris, pH 7.4, fixed by 10-fold dilution in ice-cold 77% ethanol and stored at 4°C. Fixed cells were washed in ice-cold 10 mM Tris, pH 7.4, containing 10 mM $MgCl_2$ and stained with 100 μg/ml of the DNA-specific fluorescent dye Mithramycin (Pfizer Inc., New York, NY) and 20 μg/ml of ethidium bromide (Calbiochem, La Jolla,

CA) in the same buffer. DNA-bound Mithramycin was directly excited by the incident light and transferred its energy to DNA-bound ethidium bromide which emits light with a high quantum yield. Ethidium bromide was not directly excited by the incident light and fluorescence from RNA-bound ethidium bromide was therefore negligible.

Flow cytometry

Flow cytometry was performed as described elsewhere (Steen and Lindmo, 1979; Steen, 1983; Skarstad *et al.*, 1983). The instrument used is particularly sensitive and based on a modified fluorescence microscope. Fluorescent light pulses from each cell are transformed by a photomultiplier detector into equivalent electrical pulses which are sized by a multichannel pulse height analyzer and stored as counts added to the memory channels. The light scattered by each cell is detected simultaneously by a second photomultiplier tube, transformed into electrical pulses and sized by the same multichannel analyzer which thus accumulates a three-dimensional DNA/light scatter histogram of the culture. The light scattering measured under these conditions is proportional to the mass and protein content of the cells (Boye *et al.*, 1983). The excitation wavelength was primarily at 405 and 436 nm, while fluorescence was detected above 470 nm. Cells were measured at a rate of ~1000 per s.

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References

Bachman,B.J. (1972) *Bacteriol. Rev.*, **36**, 525–557.
Bird,R., Louarn,J., Martuscelli,J. and Caro,L. (1972) *J. Mol. Biol.*, **70**, 549–566.
Boye,E. and Krisch,R.E. (1980) *Int. J. Radiat. Biol.*, **37**, 119–133.
Boye,E., Steen,H.B. and Skarstad,K. (1983) *J. Gen. Microbiol.*, **129**, 973–980.
Bremer,H. and Chuang,L.(1981) *J. Theor. Biol.*, **88**, 47–81.
Bremer,H. and Churchward,G. (1977) *J. Theor. Biol.*, **69**, 645–654.
Capaldo,F.N. and Barbour,S.D. (1975) *J. Mol. Biol.*, **91**, 53–66.
Carl,P.L. (1970) *Mol. Gen. Genet.*, **109**, 107–122.
Cooper,S. and Helmstetter,C.E. (1968) *J. Mol. Biol.*, **31**, 519–540.
Donachie,W.D. (1968) *Nature*, **219**, 1077–1079.
Eberle,H. (1968) *J. Bacteriol.*, **31**, 149–152.
Fuller,R.S., Kaguni,J.M. and Kornberg,A. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7370–7374.
Hansen,F.G. and von Meyenburg,K. (1979) *Mol. Gen. Genet.*, **175**, 135–144.
Hirota,Y., Ryter,A. and Jacob,F. (1968) *Cold Spring Harbor Symp. Quant. Biol.*, **33**, 677–693.
Hughes,P., Squali-Houssaini,F.-Z., Forterre,P. and Kohiyama,M. (1984) *J. Mol. Biol.*, **176**, 155–159.
Kaguni,J.M. and Kornberg,A. (1984) *Cell*, **38**, 183–190.
Kogoma,T., Skarstad,K., Boye,E., Von Meyenburg,K. and Steen,H.B. (1985) *J. Bacteriol.*, **163**, 436–444.
Koppes,L.J.H., Woldringh,C.L. and Nanninga,N. (1978) *J. Bacteriol.*, **134**, 423–433.
Kubitschek,H.E.(1962) *Exp. Cell. Res.*, **26**, 439–450.
Lark,K.G. (1972) *J. Mol. Biol.*, **64**, 47–60.
Lark,K.G., Repko,T. and Hoffman,E.J. (1963) *Biochim. Biophys. Acta*, **76**, 9–24.
Leonard,A.C., Hucul,J.A. and Helmstetter,C.E. (1982) *J. Bacteriol.*, **149**, 499–507.
Little,J.W. and Mount,D.W. (1982) *Cell*, **29**, 11–22.
Maaløe,O. and Hanawalt,P.C. (1961) *J. Mol. Biol.*, **3**, 144–155.
Messer,W. (1972) *J. Bacteriol.*, **112**, 7–12.
Messer,W., Bellekes,U. and Lother,H. (1985) *EMBO J.*, **4**, 1327–1332.
Nagata,T. and Meselsohn,M. (1968) *Cold Spring Harbor Symp. Quant. Biol.*, **33**, 553–557.
Newman,C.N. and Kubitschek,H.E. (1978) *J. Mol. Biol.*, **121**, 461–471.
Ogawa,T., Baker,T., van der Ende,A. and Kornberg,A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3562–3566.
Pato,M.L. and von Meyenburg,K. (1971) *Cold Spring Harbor Symp. Quant. Biol.*, **35**, 497.
Schaus,N., O'Day,K., Peters,W. and Wright,A. (1981) *J. Bacteriol.*, **145**, 904–913.
Schaechter,M., Williamson,J.P., Hood,J.R.,Jr. and Koch,A.L. (1962) *J. Gen. Microbiol.*, **29**, 421–434.
Skarstad,K., Steen,H.B. and Boye,E. (1983) *J. Bacteriol.*, **154**, 656–662.
Skarstad,K., Steen,H.B. and Boye,E. (1985) *J. Bacteriol.*, **163**, 661–668.
Smith,D.W., Garland,A.D., Herman,G., Enns,R.E., Baker,T. and Zyskind,J.W. (1985) *EMBO J.*, **4**, 1319–1326.
Steen,H.B. (1983) *Histochem. J.*, **15**, 147–160.
Steen,H.B. and Lindmo,T. (1979) *Science*, **204**, 403–404.
Tippe-Schindler,R., Zahn,G. and Messer,W. (1979) *Mol. Gen. Genet.*, **168**, 185–195.
Vazquez,D. (1979) In Kleinzeller,A.,Springer,G.F. and Wittman,H.G.W. (eds), *Molecular Biology, Biochemistry and Biophysics: Inhibitors of Protein Biosynthesis.* Springer Verlag, Berlin, pp. 108–112.
von Meyenburg,K. and Hansen,F.G. (1986) In Ingraham,J., Low,K.B., Magasanik,B., Neidhardt,F.C., Schaechter,M. and Umbarger,H.E. (eds) *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology.* American Society of Microbiology, Washington, DC, in press.
von Meyenburg,K., Hansen,F.G., Riise,E., Bergmans,H.E.N., Meijer,M. and Messer,W. (1979) *Cold Spring Harbor Symp. Quant. Biol.*, **43**, 121–128.
Wechsler,J.A. and Gross,J.D. (1971) *Mol. Gen. Genet.*, **113**, 273–284.
Wehrli,W. and Staehelin,M. (1971) *Bacteriol. Rev.*, **35**, 290–309.
Willetts,N.S. (1967) *J. Biochem.*, **103**, 453–461.
Zyskind,J.W. and Smith,D.W. (1977) *J. Bacteriol.*, **129**, 1476–1486.

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