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SeqA Foci Dynamics in Slow Growing Cells

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The study of the cell cycle has a long and rich history. By combining results from separate cell cycle and DNA replication investigations through modelling efforts, classical studies have managed to greatly advance our understanding of the rules governing both the cell cycle and DNA replication processes. However, separate investigations of the cell cycle and DNA replication may miss correlations between the two processes. In this work we simultaneously follow the cell cycle and the DNA replication processes *in vivo* and in real time by fluorescently labeling the DNA sequestering SeqA protein and performing timelapse microscopy. A regular 0-1-2-1-0 pattern in the number of SeqA foci over time for slowly growing cells is observed, corresponding to an absence of replication at the beginning and end of the cell cycle and bidirectional movement of two replication forks during replication. Interestingly, our study reveals a strong anticorrelation between cell interdivision time and its elongation rate. Moreover, the period between the birth and start of DNA replication (B period) and between the end of replication and division (D period) are found to be highly variable, while the period during DNA replication (C period) is relatively constant, moderately correlated with cell elongation rate. Our results provide support for the critical mass model for DNA replication initiation.

3.1 Introduction

The study of the cell cycle dates back to as early as 1932, when movies of budding *Saccharomyces cereviciae* cells were recorded by Bayne-Jones and Adolph, detailing cell size and shape over time [8]. Their work was followed by later investigations of bacterial cell growth using manual timelapse microscopy, while at approximately the same time, various techniques involving radioactive labeling were performed separately to investigate the DNA replication process during the cell cycle. Results from the cell cycle and the DNA replication processes were then connected to each other by modelling to better understand the rules governing each process. One classic example is the concept of critical mass as suggested by Donachie [33]. By combining the observation of Schaehter, Maaloe, Kjeldgaard [104] on the sizes of *Salmonella* that increase exponentially with growth rate and the DNA replication pattern in *E.coli B/r* by Cooper and Helmstetter [27], Donachie showed that DNA replication is initiated at certain masses, an integer multiple of a particular critical mass.

By investigating the cell cycle and DNA replication separately, possible correlations between the two processes may be missed. In this work we present novel data obtained by simultaneously and directly following the cell cycle and the DNA replication processes *in vivo* and in real time. We propose that following SeqA foci dynamics over time leads to a unique opportunity to directly follow the DNA replication process, as well as to simultaneously obtain cell cycle parameters for the same cell, and to correlate these. This implies a direct and simultaneous measurement of the B period (the period between birth and the start of DNA replication), the C period (the DNA replication period), and the D period (the period after DNA replication until cell division) of the cell. Moreover, in this way we can also express the two processes mentioned above in the currency of both time and cell size, each of which is important for different cell processes.

Current advances in microbiology and imaging techniques make it possible to directly follow various intracellular processes. In *E.coli*, when fully methylated parental DNA undergoes replication, the resulting daughter strand is hemimethylated and is sequestered by DNA sequestering protein SeqA [82]. *In vivo*, this results in visible foci when SeqA is fluorescently labeled. Here, we label SeqA by fusing it with the fluorescent protein mCherry and quantify the number and position of the resulting foci *in vivo* as an indicator of the DNA replication process. We simultaneously investigate cell growth by phase contrast microscopy.

The study reveals a regular 0-1-2-1-0 pattern in the number of SeqA foci over time for slowly growing cells, which indicates an absence of replication at the start and

end of the cell cycle, and bidirectional movement of two replication forks during replication. Furthermore, quite surprisingly, we find a strong anticorrelation between cell interdivision time and its elongation rate, a relationship we believe has never explicitly been shown before. We observe that the B period (in between birth and start of DNA replication) and D period (in between end of replication and division) are highly variable periods, while the C period (during DNA replication) is relatively constant and moderately anticorrelated with cell elongation rate. Our results support the previously suggested critical mass model for DNA replication initiation.

3.2 Results

3.2.1 SeqA foci in rapid and slow-growing cells

We first set out to determine how growth rate affects the number of SeqA foci. Isogenic cells are grown exponentially either in defined rich medium, resulting in a division time Td of about 24 minutes, or in MOPS minimal medium supplemented with succinate, which results in a Td of about 120 minutes. The cells were then spread onto an agarose slab and imaged with phase contrast and fluorescence microscopy. Distinct SeqA foci are observed in both cases (fig. 3.1). On average 3.5 foci per cell are observed for the fast growing cells, and 1.3 foci for slow growing cells. These observations are consistent with the Cooper-Helmstetter model of DNA replication involving multiple nested replications for fast growing cells [27].

3.2.2 Regular pattern of SeqA foci dynamics in slow growing cells

To follow SeqA dynamics in real time, we perform timelapse microscopy as the cells continued to grow and divide. Microcolonies of about 30 cells are formed over a period of about 8-10 hours on MOPS-succinate agar. In total about 150 single-cell trajectories are monitored and analyzed. The majority of the cells (54%) show the same pattern in the number of SeqA foci. The data of the other cells appear irregular and diverse, which may have been due to SeqA foci that cluster or are out of focus, or caused by insufficient mCherry-SeqA expression.

A typical sequence of images is shown in figure 3.2A. The white arrow indicates the cell of which the SeqA pattern is being followed. At birth, no foci are observed. At some point within the cell cycle, one SeqA focus appears. After some time, this focus elongates, and then separates into 2 nearby foci. The distance between the two foci increases over time and then decreases until a single focus is again observed, which subsequently disappeared nearing cell division. Five out of eight

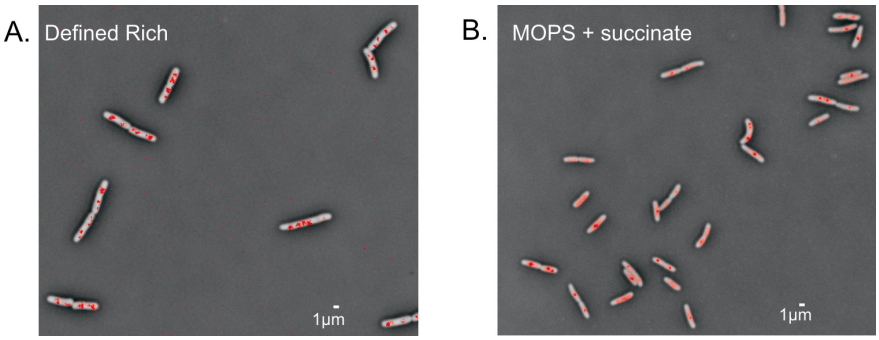

Figure 3.1 SeqA foci *in vivo*.
Due to a time delay of the methylation process following the replication process, nascent DNA is hemimethylated. DNA sequestering protein SeqA binds to hemimethylated DNA and when fluorescently labelled, SeqA aggregate appears as discrete foci. Images show cells deposited on agar: phase contrast micrographs are in black and white, overlaid with the background corrected mCherry-SeqA fluorescence signal.
(A) Cell previously grown in defined rich medium.
(B) Cell previously grown in MOPS minimum medium supplemented with succinate. More SeqA foci are observed in faster growing cells.

cells in this particular microcolony displayed this 0-1-2-1-0 pattern. Several SeqA foci trajectories covering one cycle are shown in figure 3.2C.

The observed SeqA pattern is consistent with current models of DNA replication and SeqA function in *E.coli*. In *E.coli* DNA replication starts at *oriC*, and therefore a single SeqA foci at the beginning of the cell cycle represents the early phase of DNA replication, when the hemimethylated part of the DNA is near *oriC* (fig. 3.2B). Consequently, the two foci represent the two bidirectional forks moving away from *oriC* during chromosome replication. At the end of the cell cycle the two forks end at the terminus, resulting in only one visible SeqA focus. This model agrees very well with the results of two recent studies, the first being a ChIP assay showing that the SeqA protein binds sequentially to hemimethylated nascent DNA segments following replication fork movement in synchronized cultures of *E.coli* [125]. The second study using immunofluorescence microscopy shows colocalization of labelled SeqA and BrdU (5-bromo-2'-deoxyuridine) foci in slow growing cells [1]. Our results suggest that the ratio of SeqA foci to number of replication fork in slow growing cells is 1:1.

Interestingly, we only rarely (<1%) observe a 0-1-2-1-2-0 pattern, which would point to a separation of the two hemimethylated termini after the chromosome

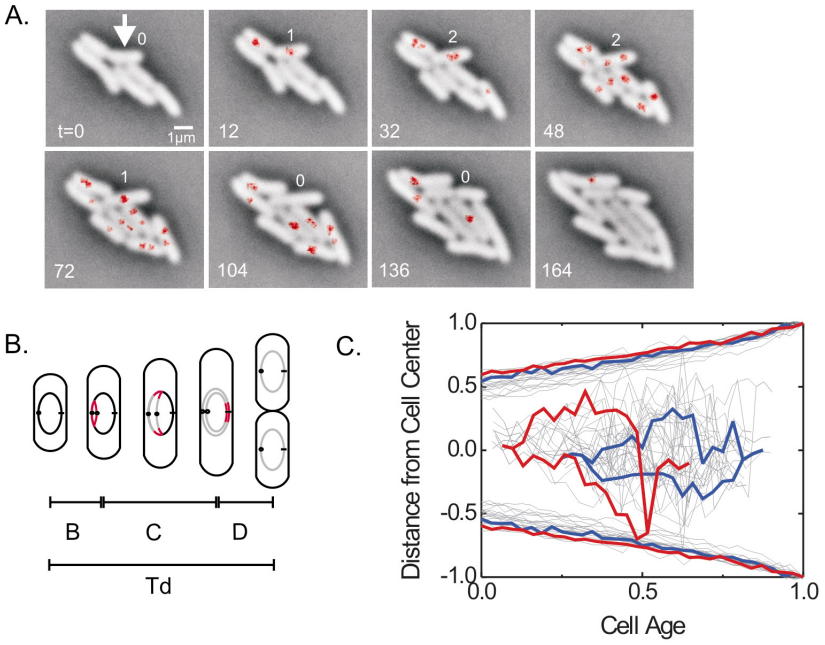

Figure 3.2 SeqA foci dynamics in slow growing cells.
(A) SeqA foci in a cell (indicated by the white arrow) within a growing microcolony are followed over time. Distinct SeqA foci pattern according to the number 0-1-2-1-0 is observed.
(B) Schematic representation of bidirectional chromosomal DNA replication *in vivo*. The unreplicated chromosome is colored black and the replicated part, grey. Red areas indicated SeqA foci. Td is the cell interdivision time. It consists of the B period (the period between birth and the start of DNA replication), the C period (the DNA replication period), and the D period (the period after DNA replication until cell division).
(C) Typical trajectories of SeqA foci during one cell cycle normalized to the cell long axis. The maximum cell length at division is normalized to 1 and a newly born cell has a value around 0.5. Cell age is shown from birth (cell age =0) to division (cell age= 1).

replication. By using a fluorescence *in situ* hybridization (FISH) technique, Bates and Kleckner [7] probe *ter* loci of synchronized cells and observe that separated *ter* foci are only visible long after replication has ended, and they stayed next to each other until the cell divided. Because in our experiment we probe the hemimethylated DNA, and the time Dam methylase takes to fully methylate the DNA is much smaller than the time it takes for the two *ter* to separate as observed by Bates and Kleckner, we observe one focus before all foci disappear.

We limit our investigation to slowly growing cells due to the complexity of data interpretation for faster growing cells. In faster growing cells where the new replication round would already have started before cell birth, the difficulty in recognizing foci that belong to the old replication cycle and the new replication cycle can lead to misinterpretations of SeqA foci pattern. Furthermore, for fast growing cells, different patterns are expected for only a small change of interdivision time, therefore pattern subclasses are to be expected. A small percentage (about 10%) of the slowly growing cells also show this phenomenon and are excluded from further analysis, thus the variances for parameters produced by SeqA foci observation below reflect a minimum variance value.

3.2.3 Quantification of SeqA foci dynamics and cell cycle parameters

We have analyzed and quantified SeqA foci dynamics and cell cycle parameters for about 80 cycles. First we characterize cell growth by measuring cell interdivision time (Td) and elongation rate (μ) from the phase contrast images. The elongation rate is obtained from the slope of a linear regression of logarithmic cell length over time and is the measure of the time it took for each cell to double in size. We find a mean elongation rate of 0.47 (size doubling per hour) with coefficient of variation (CV) of about 22% for both interdivision times and elongation rate among these cells. Large variation in interdivision times was observed previously [95]. We then simply plot cell elongation rate versus cell interdivision time to investigate if there is any relationship between the two parameters. Interestingly, a strong anticorrelation between cell division time and its corresponding elongation rate is observed as shown in figure 3.3A (correlation coefficient, ρ =0.7). This anti-correlation suggests that cells with slower cellular growth (processes) display longer division times.

To our knowledge, no study has explicitly correlated the interdivision time and elongation rate. Early microscopy work measuring elongation rate did not quantify the extent of variation among individual cells, but qualitatively concluded that cell elongation rate is very uniform within a steady state culture [105]. In subsequent cell cycle modelling studies, a single elongation rate has been frequently assumed for all cells, indirectly implying that cells with longer Td are larger. Therefore, we next investigate whether or not a correlation exists between cell size at birth and the measured interdivision time of that cell (fig. 3.3A, lower panel). Cells are divided into three different sub-populations according to their size at birth. Interestingly, for a certain elongation rate, the smallest cells are predominantly positioned towards the longer interdivision time, while the largest cells are positioned towards the shorter interdivision time.

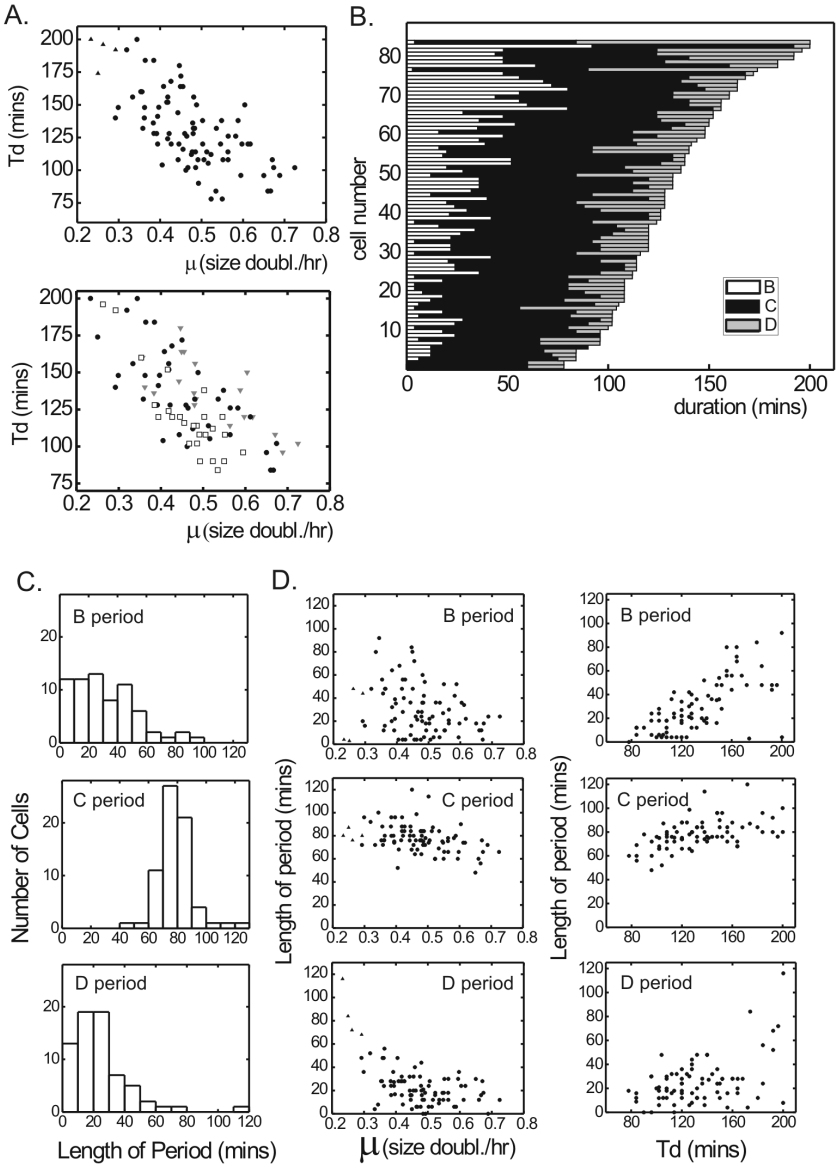

Figure 3.3 Cell cycle and DNA replication parameters of slow growing cells.
(A) A strong anticorrelation is observed between the cell elongation rate (μ) and interdivision time (correlation coefficient, ρ =0.7). Cells are then divided into three sub-populations according to their sizes at birth (lower panel). The smallest (grey triangles), intermediate (closed circles) and largest (open squares). For a certain elongation rate value, larger cells tend to have shorter interdivision time.

(B) The quantified B, C and D periods are sorted according to the cells interdivision time.
(C) Histograms of the duration of B, C and D periods (in minutes).
(D) Correlation of each period to the cell interdivision time.

Next, we investigate how the DNA replication process associates with the cell cycle. First, we quantify time periods preceding the appearance of the first focus (B period), the total time foci are observed (C period), and the time after the disappearance of the second single focus (fig. 2B). A previous investigation [23] estimates that the time chromosomal DNA stays in the hemimethylated form (except *oriC*) is about 1.5 minutes. Moreover, it has been shown that SeqA binds one to two orders of magnitude more strongly at hemimethylated DNA compared to fully methylated DNA [109]. Thus, we expect that SeqA foci do not persist for long after the nascent hemimethylated DNA become methylated. The effect of longer persisting foci will be the overestimation of the C period and the underestimation of the D period, but it should not strongly affect correlations.

Figure 3.3B shows the B, C and D periods for 80 cells, sorted according to their cell division time. The histogram of each period is shown in figure 3.3C, which shows that the B period is most widely distributed (coefficient of variation, CV=70 %), followed by the D period (CV=60 %, excluding the slowest growing cells denoted by closed triangles), and then the C period with the least spread (CV=16%). The narrower distribution of the C period compared to the spread in elongation rate (CV=22%) suggests that the DNA replication process is relatively insensitive to overall growth efficiency.

The B period shows a strong positive correlation with the interdivision time (correlation coefficient, ρ =0.7), while the C period shows only a moderate positive correlation (ρ =0.5) (fig. 3.3D). The D period shows almost no correlation with division time (ρ =0.2, excluding a few rare slow growing cells) and spreads around an average value of about 21 minutes. When plotted against elongation rate, all three periods are observed to have slight anti correlation with elongation rate (ρ = -0.3, -0.4 and -0.4 for B,C and D period respectively). The results for the C period suggest that cells with slower metabolic processes also replicate their DNA at a slower pace. The results for the B period may be explained by the accumulation of a certain molecular entity in parallel with the accumulation of cell mass or size. Certain cells with more efficient metabolic processes may accumulate more mass and therefore elongate faster.

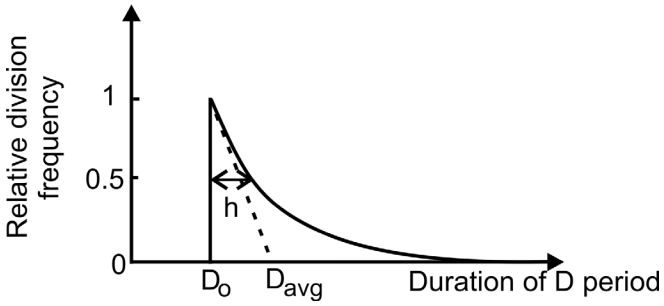

Figure 3.4 Idealistic distribution of cell division frequency as a function of D period duration. D_o is the minimum D period for each cell before division could take place. D_{avg} is the mean division time, and h is the half life of the exponential decay of the curve [18].

Previous attempts to quantify variability in the B and D periods are scarce. By means of modelling and data fitting, an estimated variation coefficient for the B period in a synchronized culture was 60%, close to what we observe [64]. Previous methods investigating the variability of the D period consider the D period to have two components [18,108]. The first component is the minimum D period, D_o, which is a constant value. The other is the stochastic component of D, which distributes the D period beyond D_o as an exponential decay (fig. 3.4). The variability in the D period is often expressed as a half-life time, h, the time it took for half of the population to divide. The proposed D period distribution has zero occurrence before D_o, then a sharp peak starting at D_o followed by an exponentially decreasing curve characterized by h. The average D period, D_{avg}, is the sum D_o+(h/ln 2).

By means of direct observation, we measure an average D period that is very similar to the commonly reported value of about 20 minutes. However, the distribution appears rather different from that of the proposed model (fig. 3.4). We observe a significant number of cells with a very short D period. Multiple interpretations can be considered for this result. It can result from a certain sub-population of cells with longer persisting SeqA foci, and therefore giving the apparent shorter D period. Alternatively, it may indicate that the D period is more loosely regulated than previously thought. The classical interpretation of the D period is the time for a cell to prepare for cell division and that it has a rather constant value. However, if it is assumed that its high variability did not arise from persisting SeqA foci, then our data suggests that the completion of DNA replication has little influence on starting the process of cell division.

Early work with timelapse microscopy has revealed that familial relationships influence cell cycle parameters such as cell size and the correlation between the B period daughter and D period mother cell [88]. Unfortunately, only a few cells with mother daughter relationships showing clear SeqA pattern are observed, and thus prevent a reasonable interpretation from data plots.

Next, we investigate cell length at different times in the cycle as shown in figure 3.5A. We observe much less variation in cell sizes throughout the whole cell cycle as compared to the observed variation in interdivision time. Coefficient of variation is smallest for the length at division (CV=11.5 %), followed by a slightly higher coefficient of variation at the start of replication (12%) and a similar coefficient of variation for both the length at birth and at the end of replication (13.5% and 13.7%). This variation in cell size, however, is not only caused by the unequal division of sister cells. In general we observe relatively equal sized daughter cells immediately after division (CV 7% from the exact half size of mother cell).

Next, we investigate whether cell length at the beginning of the B, C, and D periods correlates with the duration of that period. Interestingly, for the B and D period a moderate anticorrelation exists (ρ = -0.35 and -0.45 respectively), but not for C period (fig. 3.5B). Plots of cell size at the start of DNA replication (the end of B period) or at division (the end of cell cycle) versus cell interdivision time or elongation rate are shown in figure 3.5C. Intriguingly, a rather large variation in elongation rate (almost a factor of 3 between the slowest and the fastest) result in about only a micron spread of cell length at the time of DNA replication initiation. In contrast to earlier findings with *Salmonella typhimurium* grown in various media [104], we observe no strong correlation between cell size and either elongation rate or interdivision time in our experiment (the correlation coefficient value never exceeds 0.25). These results suggest that at least in our experimental conditions, different elongation rates have little effect on cell size.

3.3 Discussion and outlook

Since the first observation of SeqA as discrete foci *in vivo* by immunofluorescence staining [51], a number of unresolved issues have remained regarding the correlation between SeqA foci and the number of replication forks as well as the interpretation of SeqA dynamics. In most studies, the distribution of the number of SeqA foci per cell is determined from images of many cells taken at one time point. Using this method, it has been reported that the ratio between the number of SeqA foci and replication forks is about 1:1 [19,51], based on the observation of up to 14 foci for rapidly growing cells, which is close to the predicted number of replication

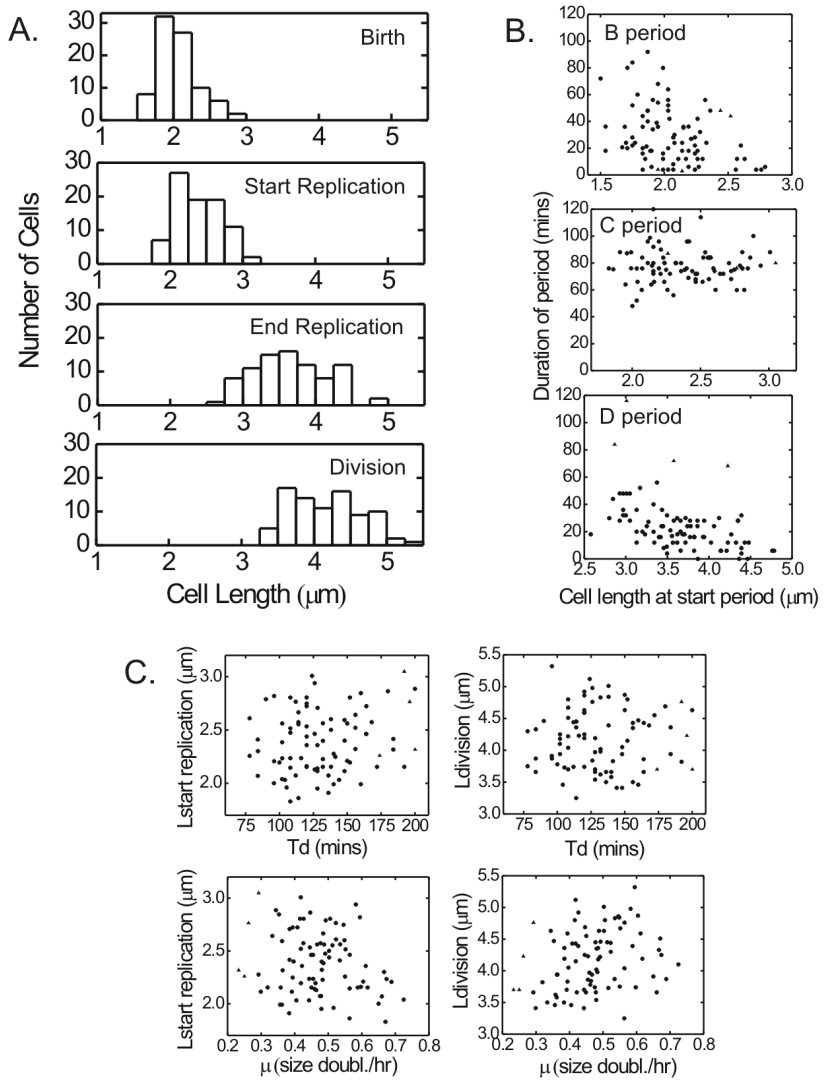

Figure 3.5 Correlations between various cell parameters.
(A) Histograms of cell lengths at various times within the cell cycle.
(B) Correlation between the duration of the B, C, and D periods and cell length at the beginning of the corresponding period.
(C) Correlation between cell lengths at different times and the cell division times or elongation rates.

forks in the Cooper-Helmstetter model. However, a ratio of 1:2 has been suggested as well [4,32,85,91]. For cells growing at a moderate growth rate, the observation of a single SeqA focus in smaller (younger), and two foci in longer (older) cells has been observed [32]. The appearance of the second focus may indicate the start of a new replication round, thus implying that each focus represents two co-localized replication forks. However, these observations are also consistent with a 1:1 ratio, as the two foci may also represent two separate replication forks instead of a new round of replication. These issues can be resolved directly by tracing the SeqA foci in real time.

By performing timelapse imaging experiments in slow growing cells, we find a pattern of 0-1-2-1-0 for the number of SeqA foci. The results show that the second focus does not represent the initiation of a new replication round but rather a single replication fork, as evidenced by the close initial proximity of the two foci. The results are consistent with a model where two replication forks are co-localized at initiation, but then separate in a bidirectional manner. If a second round of replication were to occur, it would not be expected to initiate in close proximity to the replication machinery of the first round, so we can eliminate the possibility of a second replication round. This method has led to a unique opportunity to not only directly follow the chromosomal DNA replication and the cell-cycle process, but also to extract multiple parameters for the same cell cycle. These data can easily provide information about correlations between DNA replication and the cell cycle.

We study cell-to-cell heterogeneity in *E.coli* division and the chromosome replication cycle, for isogenic populations in a constant environment. The resulting spontaneously occurring variability both contrasts and complements traditional studies of the cell cycle for different steady state growth rates in different media. We observe a strong correlation between the cell interdivision time and its elongation rate, showing that elongation rate is an important contribution to the noise in interdivision times. The data also shows that the B period is the main contributor to this trend: slower cells have longer B periods, while faster cells have shorter B periods. Such a trend is expected if a certain critical mass (or certain molecular entities accumulate in parallel with the mass) would need to accumulate before DNA replication initiation can proceed.

Since it was first proposed, the concept of a critical mass at the time of DNA replication initiation has been challenged many times (for reviews: [14,29,49]). Several studies using various growth media have found a growth dependent initiation mass [26,120]. However, their results contradict one another and large variations in growth rate (as much as 8 times) result in a maximum factor of two difference in initiation mass. Alternatively, the time elapsed after a certain event

can be viewed as the determining factor for DNA replication initiation. Recently, a study with synchronized cells obtained by a membrane elution method proposed that cell division itself may license the chromosome(s) for the next round of replication initiation. In other words, the time after cell division highly influences the time of the next round of DNA replication initiation [7]. Our observation of a highly variable B period contradicts this proposal. Moreover, we find a mild anti-correlation between cell length and the duration of the B period, indicating the tendency of smaller cells to have longer B periods. A large spread in growth rates also results in a relatively small spread in cell length at the start of the C period. Taken together, the results provide support for models predicting that the start of DNA replication is triggered by the accumulation of a critical mass [33]. However, some variability in cell size already exists at the start of replication, indicating the limitations on the precision of this trigger.

Although great advances have been made in understanding the details of the DNA replication initiation process, the way it is linked to cell growth is still unclear. Currently, DnaA protein is believed to be the molecular entity that accumulates in parallel with cell mass. However, it is very likely that it is not the DnaA concentration itself that limits replication initiation, since it has been shown that DnaA is constitutively expressed during the cell cycle [102] and a high overexpression of DnaA only slightly affects the timing of replication initiation [2]. The ratio between the number of *oriC* and DnaA is also not the determinant of replication initiation since synchronous initiation is still observed at normal cell size for cells with extra *oriC* copies. The current hypothesis is that the ratio between the active form of DnaA (DnaA-ATP) and the inactive form (DnaA-ADP) is the critical entity, however the dynamics of the active and inactive forms of DnaA between successive replication rounds is not yet known [107]. It is of much interest to investigate how variation in the ratio of DnaA-ATP and DnaA-ADP *in vivo* would affect the conditions for DNA replication initiation in our system, as this could be the first step toward understanding how cell mass relates to DNA replication initiation at the molecular level.