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Transcription of *ftsZ* oscillates during the cell cycle of *Escherichia coli*

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The FtsZ protein is a key element controlling cell division in *Escherichia coli*. A powerful transcription titration assay was used to quantify the *ftsZ* mRNA present in synchronously dividing cells. The *ftsZ* mRNA levels oscillate during the cell cycle reaching a maximum at about the time DNA replication initiates. This cell cycle dependency is specifically due to the two proximal *ftsZ* promoters. A strain was constructed in which expression of *ftsZ* could be modulated by an exogenous inducer. In this strain cell size and cell division frequency were sensitive to the cellular FtsZ contents, demonstrating the rate-limiting role of this protein in cell division. Transcriptional activity of the *ftsZ* promoters was found to be independent of DnaA, indicating that DNA replication and cell division may be independently controlled at the time when new rounds of DNA replication are initiated. This suggests a parallelism between the prokaryotic cell cycle signals and the START point of eukaryotic cell cycles.

Key words: cell cycle/cyclic oscillation/*Escherichia coli*/*ftsZ*/gene expression

Introduction

Cell division and DNA replication are the two main periodic processes of both prokaryotic and eukaryotic cell cycles. In *Escherichia coli*, both processes are tightly coordinated to the growth of the cell (Donachie, 1968; Donachie *et al.*, 1976) so that, under conditions of steady-state growth, they are extremely well regulated temporally. DNA replication is controlled at initiation by the concentration of active DnaA protein (Lobner-Olesen *et al.*, 1989), which acts very early in DNA strand separation at the chromosomal origin, *oriC* (see von Meyenburg and Hansen, 1987, for a review). On the other hand, cell division also seems to be regulated at the earliest known step of septation, where the FtsZ protein plays an essential role (Dai and Lutkenhaus, 1991; Pla *et al.*, 1991). This protein is the target of a number of endogenous cell division inhibitors that participate in the coordination of cell division with DNA replication and in the proper placement of the division septum (see de Boer *et al.*, 1990, for a review). The FtsZ protein has been localized by immunoelectron microscopy to a ring-like structure found at the future septation site (Bi and Lutkenhaus, 1991), and the GTPase activity associated with FtsZ has been proposed to be involved in the assembly of this protein to form the ring (de Boer *et al.*, 1992; Raychaudhuri and Park, 1992, Mukherjee *et al.*, 1993).

In addition to *ftsZ*, the *ftsQAZ* region contains two more essential division genes. Transcription of the two upstream genes, *ftsQ* and *ftsA*, is driven by a gearbox promoter and a growth-rate independent promoter so that, independently of the growth rate and cell size, these genes are transcribed at constant levels per cell and cycle to comply with septal needs (Aldea *et al.* 1990). Gearbox promoters are induced during the entrance to the stationary phase (Aldea *et al.*, 1989) and their activity is inversely dependent on growth rate (Aldea *et al.*, 1990; Vicente *et al.*, 1991). Overall *ftsZ* expression shares both characteristics, but none of the four *ftsZ* promoters displays a clear gearbox behavior (Aldea *et al.*, 1990). Analysis of an *ftsZ* transcriptional fusion to *lacZ*, which only contained the two upstream promoters, reported rather conflicting results with regard to a possible cell-cycle regulated expression (Dewar *et al.*, 1989; Robin *et al.*, 1990).

In this report we present the results obtained by using a powerful transcription titration assay (Becker-André and Hahlbrock, 1989) to measure the *ftsZ* mRNA levels during the cell cycle of *E.coli*. The membrane elution technique (Helmstetter, 1969) is a reliable method of obtaining synchronous populations of *E.coli*. Substantial amounts of work on the cell cycle of this bacterium have been based on the use of this technique. Its main advantage is that cells are not subjected to cyclic nutrient deprivations or temporal osmotic stress, which could disturb the cell. A drawback of the method is that because the cell densities in synchronized cultures are very low, many procedures for monitoring gene expression cannot be applied. A transcription titration assay, using the PCR methodology, has been developed allowing the quantification of mRNAs present at the femtogram level (Becker-André and Hahlbrock, 1989). This assay makes it possible to titrate mRNA levels in samples obtained from cultures synchronized by the membrane elution technique. By combining these two methodologies we detect an oscillatory pattern of transcription of the *ftsZ* gene during the cell cycle of *E.coli*. The use of different forward primers in the transcription titration assay has allowed us to evaluate the contribution of the different *fts* promoters to the overall transcriptional regulation of *ftsZ*. To determine whether the FtsZ protein is rate-limiting for cell division in steady-state conditions and whether the observed cell-cycle dependent transcription of *ftsZ* is essential for cell division regulation, we have uncoupled the *ftsZ* gene in the chromosome from its natural promoters so that its transcription can be controlled by an exogenous inducer.

Results

Levels and half-life of ftsZ mRNA

To test if the transcription titration assay used is quantitative under the conditions used we first measured the absolute *ftsZ* mRNA levels per cell in *E.coli* B/rK growing exponentially in M9+glucose. A fixed amount of total RNA was titrated with different amounts of competitor, *ftsZ* cRNA, and the resulting mixtures were subjected to reverse transcription and amplification steps (see Materials and methods) using TG2 and MA8 as forward and reverse primers, respectively (Figure 1). These two primers define the minimal sequences coding for FtsZ, thus providing a measure of what can be considered as translatable mRNA. Figure 2 shows that the mRNA/cRNA ratios obtained correlated well with the amounts of cRNA added to the assay, indicating that the *ftsZ* mRNA is present at about 3 molecules per cell in B/rK cells growing exponentially with a generation time of 45 min. We then measured the *ftsZ* mRNA decay in conditions where RNA synthesis is inhibited by the addition of rifampicin. Figure 3 shows that the half-life of the *ftsZ* mRNA is 1.5 min. From these data it can be deduced that turning off *ftsZ* transcription would cause an arrest of FtsZ synthesis after 5−10 min in most cells. This timing would, in principle, allow the operation of an expression pattern regulated by cell cycle events.

The transcription of ftsZ oscillates during the cell cycle

Levels of *ftsZ* mRNA during the cell cycle were measured in synchronized cultures of *E.coli* B/rK. Cells that had just divided were obtained by membrane elution from cultures growing exponentially in M9+glucose. Samples from the resultant synchronously dividing population were taken at short time intervals to measure growth, cell number and *ftsZ* mRNA levels. The total amount of *ftsZ* mRNA was determined by using TG2 and MA8 as forward and reverse primers, respectively (Figure 1). Figure 4 shows the results of two independent experiments with slightly different generation times, 42 and 45 min. In both cases the amount

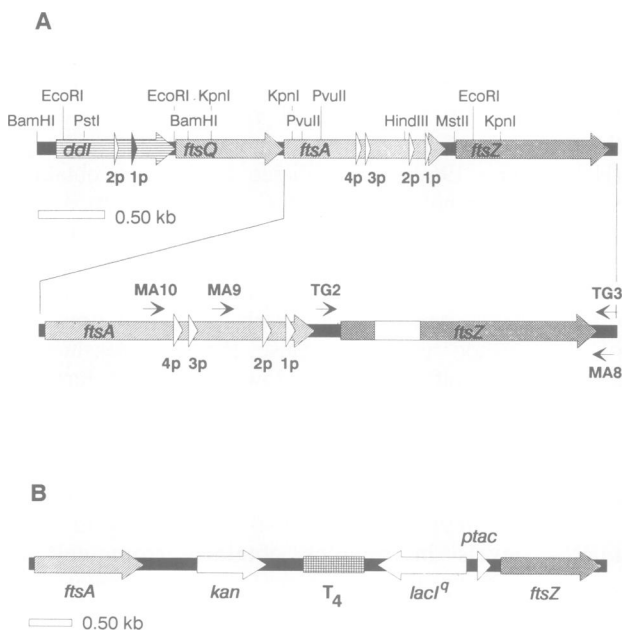

Fig. 1. Structure of the *ftsQAZ* operon in wild-type and VIP205 strains of *E.coli*. (**A**) Map of the *ftsQAZ* operon. Promoters are indicated by white arrowheads and the position of the *ftsQ* gearbox promoter by a black arrowhead. Small arrows symbolize the primers used for the transcription titration assay to measure *ftsZ* mRNA levels. The empty box indicates the *ftsZ* region deleted to obtain the competitor cRNA for the transcription titration assays. (**B**) Structure of the altered *ftsAZ* chromosomal locus constructed in VIP205. The four copies of a strong transcription terminator (T_4) uncouple the *ftsZ* gene from its natural promoters. The *ftsZ* gene is under the control of the *tac* promoter, which is in turn repressed by the high amounts of repressor produced by $lacI^q$ in the absence of IPTG (see Materials and methods for details).

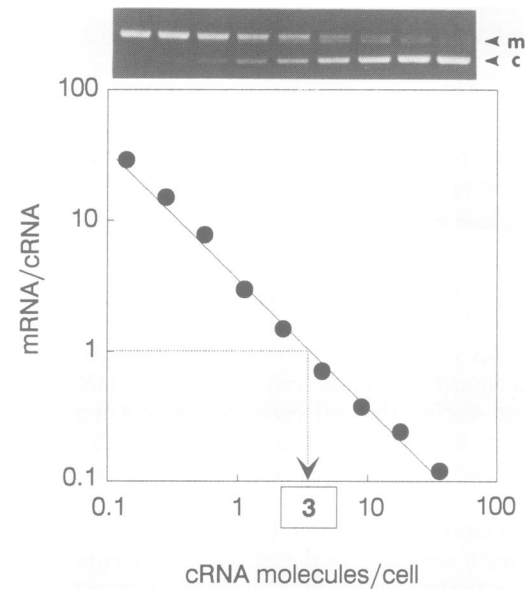

Fig. 2. Absolute *ftsZ* mRNA contents per cell. Total RNA from B/rK cells growing exponentially in M9+glucose was obtained and analyzed by the transcription titration assay as described in Materials and methods. Constant amounts of total RNA (corresponding to 2×10^5 cells) were titrated with different amounts (from 2.8×10^4 to 7.2×10^6 molecules) of competitor cRNA. The reaction products from mRNA (m) and cRNA (c) molecules, which are shown at the top, were quantified by laser densitometry, and the *ftsZ* molar mRNA/cRNA ratios were calculated. The obtained ratios are plotted against the number of competitor cRNA molecules relative to the equivalent number of cells used for each assay.

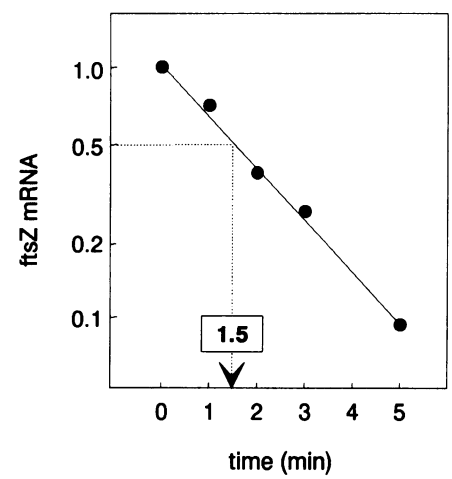

Fig. 3. *ftsZ* mRNA half-life. B/rK cells were grown exponentially in M9+glucose and rifampicin was added (250 μg/ml) at time 0 to inhibit RNA synthesis. Total RNA samples were taken at short time intervals and titrated with a fixed amount of competitor cRNA. The *ftsZ* mRNA levels are plotted as molar mRNA/cRNA ratios relative to that obtained at time 0.

of *ftsZ* transcripts was not constant and did not show a simple (linear or exponential) accumulation pattern. We can only interpret the results as being a wave during the cell cycle peaking ~15 min before cell division. The same oscillatory pattern for *ftsZ* mRNA was observed in each of the two cycles of an experiment where two consecutive division cycles were analyzed (data not shown). The C and D periods for the B/rK strain have been determined previously (Helmstetter and Pierucci, 1976) and last 42 and 14 min, respectively. Thus, for a generation time of 42 min, initiation of DNA replication for any given cycle will take place 56 min before division or, in other words, 14 min before the previous division event, which in turn coincides with the end of the previous C period. Consequently, the observed *ftsZ* transcription peak 15 min before division may be correlated with either initiation or termination of DNA replication. To confirm the length of the C and D periods for the B/rK strain we have monitored the progression of the DNA replication forks through the *ftsZ* gene by measuring *ftsZ* DNA levels in one of the experiments shown in Figure 4. The *ftsZ* gene is located at 2.5 min in the *E.coli* genetic map (Bachmann, 1990) and, hence, at 18.5 map min from the origin of replication. From these data it can be deduced that the *ftsZ* gene is duplicated ~16 min after initiation of DNA replication, i.e. one round occurring 2 min after cell division and the next one at 44 min. Figure 4 shows that the amount of *ftsZ* DNA follows stepwise kinetics with doublings close to 2 and 44 min after cell division as expected.

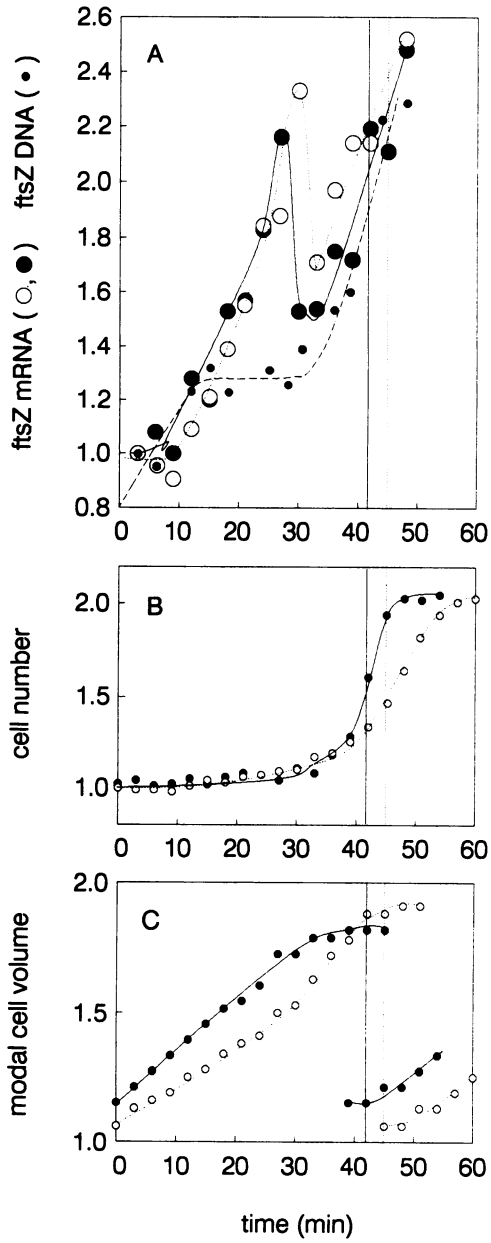

Fig. 4. *ftsZ* mRNA levels oscillate during the *E.coli* cell cycle. Newly divided B/rK cells growing in M9+glucose were used to monitor cell growth, cell number and *ftsZ* mRNA levels at short time intervals during a single division cycle. Results obtained in two separate experiments with slightly different generation times, 42 min (closed symbols) and 45 min (open symbols), are shown. Vertical lines indicate the time at which half of the cells had divided for each experiment. (**A**) Total RNA samples were obtained to perform transcription titration assays with a fixed amount of competitor cRNA. The *ftsZ* mRNA levels (large circles) are plotted as molar mRNA/cRNA ratios relative to that obtained at the first time point. The *ftsZ* DNA levels (small circles) are plotted as molar chromosomal/competitor DNA ratios relative to that obtained at the first time point. (**B**) Cell numbers are plotted relative to that obtained at the first time point. (**C**) Modal cell volumes are plotted relative to the theoretical volume just after cell division, which was calculated from the mean cell volume of exponentially growing cultures as described by Donachie *et al.* (1976).

Maximal levels of ftsZ mRNA are attained at the time of DNA replication initiation

To correlate the oscillating pattern of transcription of the *ftsZ* gene with either initiation or termination of DNA replication, we measured the amount of *ftsZ* mRNA in synchronous populations during a nutritional shift-up. Under these conditions, cell division in the first cycle is delayed, while successive rounds of DNA replication are initiated following the timing imposed by the new growth rate (Maaloe and Kjeldgaard, 1966). Newly divided cells grown in M9+glycerol were subjected to a nutritional shift-up by addition of glucose and casamino acids, and samples were taken to monitor growth and cell number and to measure *ftsZ* mRNA levels by the transcription titration assay (Figure 5). Compared with the cells kept in the poorer medium, the amount of *ftsZ* mRNA in the richer one does not increase until 20 min after formation of daughter cells and shows a second sudden increase between 40 and 44 min after formation of daughter cells. A nutritional shift-up causes general mRNA synthesis to increase immediately to meet the demands of the new growth rate (Maaloe and Kjeldgaard, 1966; Jensen and Pedersen, 1990). Yet, the *ftsZ* mRNA does not increase during the first 20 min after formation of daughter cells, indicating that a cell cycle dependent control is exerted on *ftsZ* transcription. Taking into account that the mass doubling times in the poorer and the richer medium were 56 and 22 min respectively, new rounds of DNA replication are initiated under these conditions every ~22 min after formation of daughter cells. Thus, our data indicate that *ftsZ* mRNA levels increase at the time when new rounds of DNA replication are to initiate. On the other hand, *ftsZ* mRNA levels in the unshifted culture show a minimum value 20 min after formation of daughter cells and reach a maximum level at cell division, when cells are also initiating new rounds of DNA replication.

Cell-cycle dependent transcription of ftsZ is due to the two proximal promoters

As there are no transcription terminators within the *ftsQAZ* region, the *ftsZ* transcription rate is not only due to the

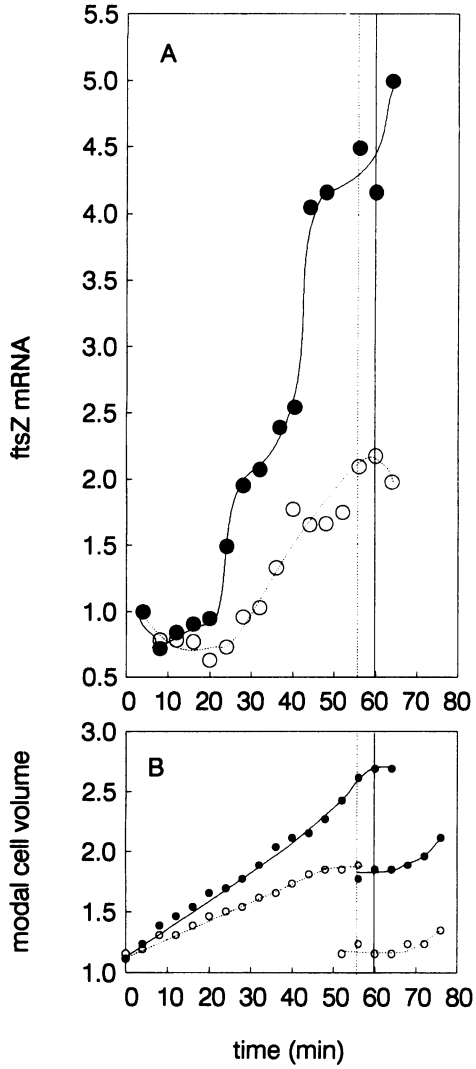

Fig. 5. *ftsZ* mRNA levels are induced at about the time DNA replication is to initiate. B/rK newly divided cells grown in M9+glycerol were used to monitor cell growth, cell number and *ftsZ* mRNA levels at short time intervals during a single division cycle both under the same growth conditions (open symbols) and during a shift-up after the addition of glucose and casamino acids (closed symbols). Vertical lines indicate the time at which half of the cells had divided for each growth condition. (**A**) Total RNA samples were obtained to perform transcription titration assays with a fixed amount of competitor cRNA. The *ftsZ* mRNA levels are plotted as molar mRNA/cRNA ratios relative to that obtained at the first time point. (**B**) Modal cell volumes are plotted relative to the theoretical volume just after cell division as in Figure 4C.

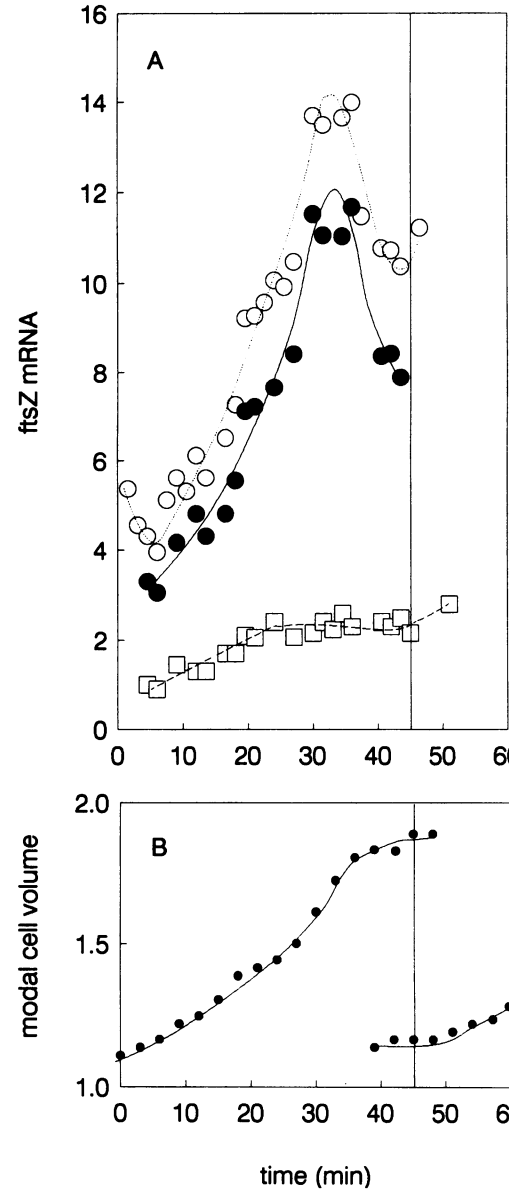

Fig. 6. Cell-cycle regulated transcription of *ftsZ* is due to the two proximal promoters. B/rK newly divided cells growing in M9+glucose were used to monitor cell growth, cell number and *ftsZ* mRNA levels at short time intervals during a single division cycle. Vertical lines indicate the time at which half of the cells had divided. (**A**) Total RNA samples were obtained to perform transcription titration assays with a fixed amount of competitor cRNA. Total *ftsZ* mRNA levels (open circles) and those that originated upstream from the two proximal promoters (open squares) were measured by using TG2 and MA9 respectively as forward primers during the amplification steps. The *ftsZ* mRNA levels due to the two proximal promoters (closed circles) were calculated by subtracting the values obtained with MA9 from those obtained with TG2. Plotted values are all expressed as molar mRNA/cRNA ratios relative to that obtained with MA9 at the first time point. (**B**) Modal cell volumes are plotted relative to the theoretical volume just after cell division as in Figure 4C.

activity of the four *ftsZ* promoters located in the upstream gene, *ftsA*, but also to that of the two *ftsQ* promoters located further upstream. Assuming that the half-lives of the different messages at their 5′ end are similar, the contribution of the different promoters to the total rate of transcription of *ftsZ* can be evaluated by the transcription titration assay when different forward primers are used (Figure 1). Thus, while primer TG2 quantifies all messages entering *ftsZ*, primer MA9 only quantifies those messages that originate upstream from the *ftsZp1* and *ftsZp2* promoters. In addition, primer MA10 can be used to quantify messages that originated at the *ftsQ* promoters. By using this approach we have found that, in B/rK cells growing exponentially in M9+glucose, only 21% of the messages covering the *ftsZ* open reading frame originate upstream from the two proximal promoters, *ftsZp1* and *ftsZp2*. Under the same conditions, messages that originated further upstream at the *ftsQ* promoters account for only 10% of the total *ftsZ* messages. Since the half-life of the messages measured with TG2 as the forward primer is already very short (1.5 min, see above), we conclude that transcription due to *ftsZp1* and *ftsZp2*, the two proximal promoters, can be as high as 79% of the total *ftsZ* transcription.

Table I. Expression from the *ftsZ* promoters does not depend on DnaA

	AB1157 (*dnaA*$^+$)			GC2018 (*dnaA46* ts)		
Phage	30°C	42°C	Ratio	30°C	42°C	Ratio
λTGV14	279	298	1.07	245	297	1.21
λTGV15	174	172	0.99	196	189	0.96
λTGV17	226	208	0.92	216	217	1.01

Strains AB1157 and GC2018 lysogenized with λTGV14, λTGV15 or λTGV17 were grown exponentially at 30°C in LB and incubated at 30 or 42°C for 90 min. Values shown are β-galactosidase units which were corrected by subtracting the corresponding background levels in AB1157 (30 and 23 units at 30 and 42°C, respectively) and GC2018 (19 units at both 30 and 42°C).

We used the same approach to evaluate the contribution of the different promoters to the cell-cycle dependent transcription of *ftsZ*. Figure 6 shows the *ftsZ* mRNA levels measured with TG2 or MA9 as forward primers during a cell cycle of B/rK growing in M9+glucose. Contrary to the oscillating pattern shown by the total *ftsZ* mRNA as measured with TG2, transcripts that originated upstream from the two proximal promoters show stepwise kinetics very similar to those observed for the *ftsZ* DNA increase (see above and Figure 4). As the generation time for this experiment was 45 min, the *ftsZ* gene would be duplicated at about 5 and 50 min after formation of daughter cells. Then, we conclude that transcription that originates from the two distal *ftsZ* promoters and the *ftsQ* promoters is not cell cycle regulated. The transcription levels measured with MA9 have been subtracted from those measured with TG2 in Figure 6 to obtain the amounts of mRNA originated at the two *ftsZ* proximal promoters. It can be seen that transcription initiated at these promoters is activated 4-fold 15 min before division.

Transcription of ftsZ is not regulated by DnaA

We have investigated the possibility that *ftsZ* expression could be regulated by the DnaA protein, as it may regulate gene expression (Messer *et al.*, 1988) and some putative DnaA boxes have been found, based on sequence homologies, in the *ftsQAZ* region (Masters *et al.*, 1989). For this purpose three phages carrying different *lacZ* transcriptional fusions under the control of the *ftsZ* promoters were used (see Materials and methods). Phage λTGV17 contains the four *ftsZ* promoters fused to *lacZ* but only contains one of the proposed DnaA boxes. Phage λTGV15 contains more DNA upstream from the *ftsZ* promoters and includes the three putative DnaA boxes. Finally, phage λTGV14 carries a larger piece of DNA extending further upstream to include the two *ftsQ* promoters. The expression levels driven by the *fts* promoters in these three phages were determined in the presence of wild-type DnaA or the temperature-sensitive DnaA46, both at 30 and 42°C (Table I). Independently of the phage used, our results show no major effect of the DnaA protein on the transcriptional activity of the *ftsZ* promoters. In addition, overexpression of *dnaA* driven by the *tac* promoter did not affect the β-galactosidase activity in cells carrying λTGV17 or λTGV14 (Table II).

FtsZ is rate-limiting for cell division

FtsZ has been found to be essential for cell division (Dai and Lutkenhaus, 1991; Pla *et al.*, 1991). Both reports studied a strain with a disrupted chromosomal copy of the *ftsZ* gene and a wild-type copy of the gene cloned in a temperature-sensitive replicon. In these constructions cell length increased as the FtsZ levels dropped below those of wild-type cells, suggesting that total FtsZ levels must be strictly regulated to attain a proper cell division frequency.

Table II. DnaA overproduction does not affect expression from the *ftsZ* promoters

Phage	Plasmid	−IPTG	+IPTG	Ratio
λTGV14	pLSK5(*ptac-dnaA*)	249	208	0.84
λTGV17	pLSK5 (*ptac-dnaA*)	188	170	0.90
λTGV17	pJF119EH (control)	191	160	0.84

Strain RYC1000 containing phages λTGV14 or λTGV17 and plasmids pJF119EH or pLSK5 was grown exponentially in LB at 37°C and incubated with or without 1 mM IPTG for at 37°C. Values shown are in β-galactosidase units.

None of the above systems permitted the evaluation of whether FtsZ is rate-limiting for cell division in steady-state conditions. To determine this directly, we uncoupled the *ftsZ* gene from its natural promoter sequences in a strain, VIP205, that contains the chromosomal structural *ftsZ* gene under the control of the *tac* promoter (see Materials and methods). As no other copy of the *ftsZ* gene is present in VIP205, this strain depends for growth on externally added IPTG. We first evaluated the transcriptional activity of the *tac* promoter fused to *lacZ* in λMAV108 at different IPTG concentrations. This fusion produced ~200 β-galactosidase units at 10 μM IPTG, which is about the level produced by the four *ftsZ* promoters when fused to *lacZ* in λTGV17 (see Table II). Accordingly, preliminary experiments indicated that VIP205 could only grow on plates in the presence of IPTG concentrations ranging from 10 to 30 μM (data not shown). The effect of varying IPTG concentrations on the growth and morphological parameters of VIP205 was then tested by growing this strain exponentially with 15 or 30 μM IPTG and transferring the cells to the same growth medium with various IPTG concentrations. Figure 7 shows that cell volume is clearly dependent on the FtsZ cellular contents. Both lower or higher IPTG concentrations produced filamentation and, as expected from previous studies (Ward and Lutkenhaus, 1985), minicells were observed at higher than wild-type FtsZ levels (data not shown). Filaments did not show any sign of constriction, ruling out the possibility of indirect effects due to lower expression levels of *envA*, the next and last downstream gene identified in the operon. Since *ftsZ* transcripts terminate immediately downstream from *envA* (Ghelardini *et al.*, 1991), no other gene will be affected by transcription from the *tac* promoter in VIP205. Moreover, the *envA* promoter is several-fold more active than the *ftsZ* promoters (Ghelardini *et al.*, 1991) and is still

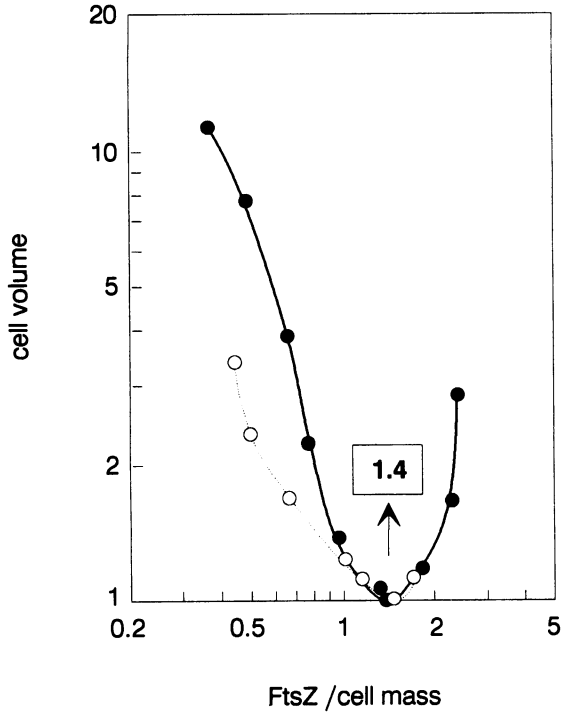

Fig. 7. FtsZ is rate-limiting for cell division. VIP205 cells, which contain the chromosomal *ftsZ* gene under control of the *tac* promoter, were grown exponentially in LB with 15 μM (closed symbols) or 30 μM (open symbols) IPTG and transferred to the same medium with IPTG concentrations ranging from 3 to 300 μM. After five or six generations of exponential growth samples were taken to monitor cell volume and FtsZ contents per cell mass as described in Materials and methods. Cell volumes and FtsZ contents per cell mass were made relative to those of the parental strain, MC1061, growing under the same conditions.

present in VIP205. Thus, we conclude that FtsZ is indeed rate-limiting for cell division. On the other hand, VIP205 cell volume medians (Figure 7) and distributions (data not shown) were indistinguishable from those of the parental strain only when the FtsZ contents was 40% higher than the wild-type level. Conversely, at FtsZ levels equal to the wild-type ones, VIP205 cells were 20–40% larger than the parental strain. Thus, cell cycle dependent expression of *ftsZ* driven by its own promoters is required for the correct timing for division, but it may be overcome by the production of higher levels of the FtsZ protein. At these higher levels the cell cycle dependent expression of the gene is not strictly required for correct timing, suggesting the existence of additional rate-limiting factors for cell division.

Discussion

The FtsZ protein is a key element controlling cell division in *E.coli*. It has an essential role in division (Dai and Lutkenhaus, 1991; Pla *et al.*, 1991) and is the target of a number of endogenous cell division inhibitors (de Boer *et al.*, 1990). Its ability to form a ring-like structure at the future site of division (Bi and Lutkenhaus, 1991), for which a GTPase activity may be essential (de Boer *et al.*, 1992; RayChaudhuri and Park, 1992), suggests that FtsZ may be a cytoskeletal element that could initiate cell division when a sufficient number of molecules are assembled to complete the ring. Thus, expression of the *ftsZ* gene may be one key point among the regulatory mechanisms of cell division.

We have used a transcription titration assay (Becker-André and Hahlbrock, 1989) and the membrane elution synchronization technique (Helmstetter, 1969) to measure the transcription levels of the *ftsZ* gene during the cell cycle of *E.coli*. Our results show that transcription of the *ftsZ* gene is not constant during the cell cycle but oscillates, reaching a maximum at about the time when a new round of DNA replication is initiated. We have repeatedly observed this pattern of expression, even when cells are subjected to a nutritional shift-up. When newly divided *E.coli* cells are shifted up to grow faster, RNA and protein synthesis rates increase almost immediately (Jensen and Pedersen, 1990), whereas DNA replication progressively adapts to the new growth rate by initiating new rounds of replication every time a multiple of the initiation mass is reached (Donachie, 1968). Our results show that *ftsZ* transcription does not increase immediately after a shift-up, indicating that this gene is under cell cycle dependent control and that transcription from the *ftsZ* promoters is not limited only by the availability of RNA polymerase and/or RNA precursors. More importantly, *ftsZ* transcription increases abruptly at the time when new rounds of replication are to be initiated. We have shown that the *ftsZ* mRNA is of relatively low abundance (on average ~3 molecules/cell) and has a short half-life (1.5 min), indicating that a transcriptional control can be an essential component in the overall regulation of the *ftsZ* gene expression.

By using different forward primers in our transcription titration assays we have shown that the two proximal *ftsZ* promoters, *ftsZ1p* and *ftsZ2p*, account for ~80% of the overall transcription of *ftsZ* and, more importantly, that these two promoters are responsible for the observed oscillatory pattern of transcription of the *ftsZ* gene during the *E.coli* cell cycle. Previous attempts to characterize the mode of expression of the *ftsZ* gene during the cell cycle were done with a transcriptional fusion to *lacZ* that only contained the two distal promoters, *ftsZ3p* and *ftsZ4p* (Dewar *et al.*, 1989; Robin *et al.*, 1990), for which we have found no cell cycle dependency. The data provided by Robin *et al.* (1990) indicate that transcription driven by *ftsZ3p* and *ftsZ4p* doubles at ~20 min after division, which coincides with the initiation of replication under the experimental conditions used. The authors state that termination of replication coincides with the next initiation. However, a careful look at their results (Figure 4 in Robin *et al.*, 1990) rather indicates that termination takes place 25 min after initiation of the following period of replication. Taking this into account, the doubling in the rate of synthesis could be interpreted as a direct result of the replication of the lambda attachment locus, where the transcriptional fusion is found. The data provided by Dewar *et al.* (1989) are not conclusive enough and could be interpreted in similar terms. A previous report on the absence of periodic proteins in *E.coli* (Lutkenhaus *et al.*, 1979) was based on the study of cells fractionated according to their size; this procedure yields homogeneous populations only for small cells, not for larger ones. An oscillation in the synthesis of a protein occurring near the time of cell division, specially a drop like the one we observe in Figures 4 and 6, would be obscured in those experiments by the heterogeneity of these fractions. The FtsZ mean half-life, when measured by immunodetection procedures, extends over more than a generation time (M.Sánchez and M.Vicente, unpublished) so that the total amount of FtsZ

present in the cell may not change dramatically throughout the cell cycle. However, the periodic assembly and disassembly of an FtsZ ring may suggest that a substantial proportion of the total protein could be inactivated; periodic bursts of *ftsZ* transcription would replenish the pool of active protein without increasing the total amount of protein over levels detectable with the relatively insensitive methodologies used for protein quantification. Models postulating that continuous accumulation is sufficient to explain the triggering of the division process (Cooper, 1991) do not take these possibilities into account, and should be revised to accommodate the recent evidence derived from molecular genetics.

The oscillatory pattern of transcription of *ftsZ* is very similar to that reported for the *nrd* operon (Sun and Fuchs, 1992), which codes for ribonucleotide reductase, an enzyme involved in the biosynthesis of DNA precursors. Transcription levels driven by the *nrd* promoter were found also to increase 3- to 4-fold at the time of initiation of DNA replication. The *nrd* promoter is subject to both positive and negative controls but their contribution to the cell-cycle regulation has not been evaluated yet (Sun and Fuchs, 1992). Further work on the regulatory mechanisms operating upon the *nrd* and *ftsZ* promoters will be invaluable in determining how cyclic gene expression is accomplished in a prokaryotic organism.

Expression of *ftsZ* can be uncoupled from its natural promoter sequences rendering its transcription dependent on the *tac* promoter. A strain carrying this construction, VIP205, is only viable in a narrow range of IPTG concentrations and its cell volume is clearly dependent on the FtsZ contents. Our observations demonstrate that FtsZ is rate-limiting for cell division. However, independently of the FtsZ contents, VIP205 cells were never smaller than the parental cells, suggesting that FtsZ may not be the only rate-limiting element controlling cell division. The *min* gene products may form part of this control because a strain containing a deletion in the *min* genes divides at smaller sizes than normal when *ftsZ* is overexpressed (Bi and Lutkenhaus, 1990). VIP205 cells growing in the presence of IPTG concentrations that result in the production of FtsZ in a 40% excess over the wild-type levels, show volume distributions that are indistinguishable from those corresponding to the parental strain. These results indicate that the oscillatory mode of transcription of *ftsZ* is not sufficient to explain the accurate timing of cell division which, in turn, points again to the existence of additional rate-limiting elements which could also be regulated in a cell cycle dependent manner. The fact that FtsZ assembles into a ring-like structure (Bi and Lutkenhaus, 1991) suggests that *E.coli* cells require a critical FtsZ level at a particular cell age, i.e. during cell septum initiation. This critical level could be achieved either by cell cycle regulated or unregulated promoters, but the latter would produce an inevitable surplus of FtsZ at stages other than cell septum initiation. This is actually found in VIP205 cells, which show this 40% increase in the FtsZ contents when their volume distributions are indistinguishable from those obtained with the parental strain under the same growth conditions.

The DnaA protein is a key element regulating initiation of DNA replication (Lobner-Olesen *et al.*, 1989), although other rate-limiting elements may also be involved (Hwang and Kornberg, 1990). In contrast to a previous report (Masters *et al.*, 1989) on transcription driven by the distal *ftsZ* promoters, *ftsZ3p* and *ftsZ4p*, we have found that transcriptional activities of different fragments containing the four *ftsZ* promoters do not depend on DnaA. Our results indicate that there is no effect of the DnaA protein on the putative DnaA boxes that could modify *ftsZ* expression. Transcription of *ftsZ* would not be linked to the initiation of DNA replication; this agrees with the findings that the DnaA protein is only essential as a cell cycle protein for the initiation of DNA replication (Bernarder *et al.*, 1991) and that cell division is not triggered by chromosome replication (Bernarder and Nordström, 1990).

The possibility that the *E.coli* cell cycle is the result of the coordination of multiple independent processes (Nordström *et al.*, 1991) is supported by our results on *ftsZ* expression. The two proteins FtsZ and DnaA, which are rate-limiting for cell division and DNA replication respectively, would not exert a regulatory role on each other to coordinate the two processes sequentially. Instead, the activity of the two proteins would be regulated by different mechanisms which would in turn be temporarily coordinated at about the time DNA replication is to be initiated. In this respect, an attractive parallelism to the eukaryotic cell cycle may be established. A eukaryotic organism such as *Saccharomyces cerevisiae* is committed to cell division by the activation of the Cdc28 protein kinase by G_1 cyclins at a time termed START, after which several distinct processes are initiated, among them DNA replication and bud emergence (see Reed, 1992, for a review). Similarly, a prokaryotic organism such as *E.coli* would coordinate the initiation steps of DNA replication and cell division at a point where a threshold for DnaA activity is reached and, on the other hand, transcription from the *ftsZ* promoters is activated to initiate the accumulation of FtsZ up to a critical level which, later in the cycle, will trigger cell division.

Materials and methods

Media and growth conditions

Luria broth and Luria agar were supplemented with antibiotics when required (50 μg/ml ampicillin, 50 μg/ml kanamycin). Minimal medium was M9 (Miller, 1972) diluted 2-fold and supplemented with 0.2% glucose or 0.4% glycerol as carbon sources. Casamino acids were used at a final concentration of 0.2%. All incubations were at 37°C, unless stated otherwise. Exponentially growing cultures were routinely obtained by 200-fold dilution of standing overnight cultures and incubation with shaking at 225 r.p.m. for six to eight generations at the desired temperature. Synchronic cultures of *E.coli* B/rK were obtained as described by Helmstetter (1969). Cell concentrations and volume distributions were determined using a Coulter Counter ZM (30 μm, Coulter Electronics) and a Channelyzer C1000 (Coulter Electronics).

Plasmid DNA isolation and cloning procedures

Plasmid DNA isolation, cloning techniques and transformation procedures were done as described by Sambrook *et al.* (1989). Lysozyme and RNase A were purchased from Sigma Chemical Co. All other enzymes were obtained from and used as recommended by Boehringer Mannheim. RNase-free agarose (D2 Pronarose) was a gift from Hispanagar, SA. Primer oligonucleotides were synthesized in a Gene Assembler Plus (Pharmacia).

Bacterial strains, plasmids and phages

E.coli B/rK (C.Helmstetter) was used for synchronization experiments. Strain RYC1000 [K12, *araD139*, *Δ(lac)U169*, *rpsL*, *relA*, *thiA*, *recA56;* F.Moreno] was lysogenized with phages carrying transcriptional fusions to *lacZ*. Strains AB1157 (K12, *thr1*, *leu6*, *proA2*, *hisG4*, *thi1*, *argE3*, *lacY1*, *galK2*, *ara14*, *xyl5*, *mtl1*, *tsx33*, *rpsL31*, *supE44*; C.Lark) and GC2018 (same as AB1157 but *dnaA46ts*; A.Jaffé) were used to analyze DnaA-dependent transcription. Strain MC1061 [*araD139*, *Δ(ara-leu)7697*, *Δ(lac)X74*, *galU*, *galK*, *rpsL*; R.Gourse] was used to construct VIP205 with pMAV115 (see below) by gene replacement techniques.

A suitable regulatable-expression cassette containing the kanamycin resistance gene, four copies of a strong transcriptional terminator, the *lacI*^{q} gene and the *tac* promoter was constructed by inserting a 1.5 kb *Eco*RI–*Nru*I fragment from pJF119EH into pRS551 digested with *Eco*RI. *Eco*RI ends were filled in before ligation and a plasmid was selected in which the *tac* promoter was in the same orientation as the *lacZ* gene. The resulting plasmid, pMAV108, was used to transfer the whole construction to λRS45 as described by Simons *et al.* (1987). The phage carrying the pMAV108 construct, λMAV108, was lysogenized into RYC1000 and used to determine the *tac* promoter expression levels at different IPTG concentrations.

To uncouple the *ftsZ* gene from its natural promoters we first constructed a plasmid, pMAV113, by inserting a 0.6 kb *Hin*dIII–*Nru*I fragment from pZAQ into the *Hin*dIII and *Eco*RV sites of pBR322. This 0.6 kb *Hin*dIII–*Nru*I fragment contains the flanking sequences to the *ftsA*–*ftsZ* junction. Then, a 4.2 kb *Aat*II–*Bam*HI fragment from pMAV108 containing the regulatable-expression cassette was inserted into the *Mst*II site at the *ftsA*–*ftsZ* junction of pMAV113. All ends were filled in before ligation and a plasmid, pMAV114, was selected in which *ftsZ* sequences were found downstream from the *tac* promoter. The whole construction in pMAV114, regulatable-expression cassette and flanking *fts* sequences, was finally transferred as a 5.1 kb *Hin*dIII–*Sph*I fragment to the pMAK700 thermosensitive replicon. The resulting plasmid, pMAV115, was used to perform a two-step gene replacement in strain MC1061 as described by Pla *et al.* (1991). During the excision steps of the plasmid, and as soon as the *ftsZ* chromosomal gene was to be under control of the *tac* promoter, cells were grown in the presence of 10 μM IPTG to ensure cell survival. This IPTG concentration had been shown to derepress the *tac* promoter up to a level such that a similar transcriptional activity to that of the four *ftsZ* promoters was obtained. After excision steps, the resulting plasmid was first confirmed to contain the wild-type *ftsA*–*ftsZ* junction and thereafter cured by growth at the restrictive temperature for the thermosensitive replicon. Finally, the presence of the regulatable-expression cassette at the *ftsA*–*ftsZ* chromosomal junction in the resulting strain, VIP205, was confirmed by Southern blot hybridization.

Overproduction of DnaA driven by the *tac* promoter was accomplished by using plasmid pLSK5 (Messer *et al.*, 1988). The transcriptional fusion of the four *ftsZ* promoters to *lacZ* in phage λTGV17 has been described (Aldea *et al.*, 1990). Phages λTGV14 and λTGV15 also contain the four *ftsZ* promoters but carry additional upstream sequences. Phage λTGV14 contains a 3 kb *Pst*I–*Eco*RI fragment from pZAQ that includes the two *ftsQ* promoters. Phage λTGV15 carries a smaller 2.3 kb *Eco*RI fragment that does not include the *ftsQ* promoters. Both phages were obtained by first subcloning the respective fragments into pRS550 and transferring the transcriptional fusions to λRS45 as described by Aldea *et al.* (1989). Plasmids pZAQ (Ward and Lutkenhaus, 1985), pRS550 and pRS551 (Simons *et al.*, 1987) and pJF119EH and pMAK700 (Pla *et al.*, 1991) have been described elsewhere.

DNA-free RNA and chromosomal DNA isolations

A simple miniprep method for obtaining DNA-free total RNA from *E.coli* cells was developed by combining the acid-phenol extraction described by Kedzierski and Porter (1991) with standard methods for total RNA preparation. Briefly, 0.5 ml samples (5×10^5 to 5×10^6 cells) of cultures in half-strength M9 were added to 50 μl of an ice-cold solution of 10 mg/ml lysozyme in 20 mM EDTA, pH 8, and immediately frozen in a dry ice–ethanol bath. Samples were then thawed at 37°C for 2 min and frozen again for 2 min in dry ice–ethanol, and this cycle was repeated once more. To the frozen samples 10 μl of 1 μg/μl yeast tRNA, 50 μl of 10% SDS, 20 μl of 10% acetic acid, 70 μl of 2 M sodium acetate, pH 4, and 0.5 ml of water-saturated phenol were quickly added. After 2 min at 37°C, samples were vigorously vortexed for 1 min and incubated at 0°C for 10 min. Cold mixtures were spun at 12 000 *g* for 15 min in the cold and 350 μl of the supernatant were taken very carefully to a new tube to avoid DNA contamination from the interface. Two volumes of ethanol were then added and, after 30 min at 0°C, total RNA was collected by centrifugation at 12 000 *g* for 15 min in the cold, washed with 70% ethanol, dried and resuspended in 100 μl TE (10 mM Tris–HCl, 0.1 mM EDTA, pH 8). We found it very important to use M9 medium diluted 2-fold to avoid salt precipitation by ethanol. This method was even more efficient than DNase treatment for the removal of chromosomal DNA. On the other hand, total RNA yields were comparable to those obtained with standard methods (Sambrook *et al.*, 1989).

Chromosomal DNA was obtained by a similar procedure to that described for total RNA isolations, except that acetic acid was omitted and phenol–chloroform (Sambrook *et al.*, 1989) was used instead of water-saturated phenol.

DNA and cRNA competitors for the transcription titration assay

An internal *ftsZ* deletion was used as a source for both DNA and cRNA competitors. This deletion was carried out in pMAV116, a pZAQ derivative that already lacks a 1.75 kb *Pst*I–*Bgl*II fragment comprising the whole *ftsQ* gene and the N-terminal sequences of *ftsA*. Then, a 210 bp *Eco*RI–*Kpn*I fragment containing internal *ftsZ* sequences was deleted from pMAV116 to obtain pMAV117. The *ftsZ* deletion carried by this plasmid still contains the sites for all primers used in the transcription titration assay (see Figure 1). A 1.7 kb *Hin*dIII–*Sph*I fragment from pMAV117 was isolated by electrophoresis and used as DNA competitor to quantify chromosomal *ftsZ* DNA levels.

To obtain the cRNA competitor, the 2.7 kb *Pvu*II–*Bam*HI fragment from pMAV117 containing the internally deleted *ftsZ* gene was subcloned in pSPTBM21 (Boehringer Mannheim) digested with *Eco*RV and *Bam*HI. The resulting plasmid, pTGV20, was linearized with *Bam*HI and used as template for T7 RNA polymerase *in vitro* transcription. The cRNA obtained was acid-phenol extracted (Kedzierski and Porter, 1991) to eliminate the DNA template, ethanol precipitated and resuspended in TE buffer. This DNA-free cRNA was checked for integrity by electrophoresis and quantified by measuring its absorbance at 260 nm.

Transcription titration assays and chromosomal DNA quantifications

We have used the transcription titration assay described by Becker-André and Hahlbrock (1989) with minor modifications. Briefly, 10 μl aliquots of total RNA samples were mixed with 10 μl of double strength reverse transcriptase buffer (Boehringer Mannheim) containing 0.8 mM of each dNTP, 4 μM TG3 primer (5′-TAGCACAAAGAGCCTCGA-3′, starting 43 nt downstream from the *ftsZ* termination codon), 0.2 units/μl of RNase inhibitor and the appropriate amount of cRNA competitor. After incubation at 65°C for 5 min, primer annealing was performed at room temperature for 5 min and RNase inhibitor (5 units) and M-MLV reverse transcriptase (10 units) were added to perform cDNA synthesis at 42°C for 60 min. Finally, reverse transcriptase was inactivated by heating at 95°C for 5 min and quickly chilling on ice.

Amplification was performed by adding 5 μl of the cDNA samples to 50 μl of amplification buffer (Boehringer Mannheim) containing 0.2 mM of each dNTP, 0.25 μM each of the forward and reverse primers and 1 unit of *Taq* DNA polymerase. In all assays MA8 (5′-GCCTCGAAAC-CCAAATTCCAGTCA-3′, starting at 32 nt downstream from the *ftsZ* termination codon) was used as the reverse primer. MA8 overlaps TG3, the primer used for cDNA synthesis, by 7 nt and provided the assay with a higher specificity for the expected fragments. Depending on the purpose of the assay, different oligonucleotides were used as forward primers. Total *ftsZ* mRNA levels were assayed with TG2 (5′-CCGACGATGATTACG-GCCTCAGGC-3′, starting 49 nt upstream from the *ftsZ* initiation codon) as forward primer. Transcripts originated upstream from the two *ftsZ* proximal promoters were measured with MA9 (5′-GAAGTGCCGAGCG-TAGGTGGTCGT-3′, starting 498 nt upstream from the *ftsZ* initiation codon) as forward primer. Finally, transcripts originated from the *ftsQ* promoters and further upstream were quantified with MA10 (5′-CGTTGTGGGCT-GAAAGTTGACCAA-3′, starting 795 nt upstream from the *ftsZ* initiation codon) as forward primer. After 1 min incubation at 94°C, samples were subjected to 35 cycles of amplification (30 s at 94°C, 1 min at 66°C and 3 min at 72°C), followed by a final incubation at 72°C for 8 min. The annealing temperature was 60°C for MA10, and the number of amplification cycles was raised to 40 when using MA9 or MA10 as forward primer. The reaction products were resolved in 1% agarose gels run at 5 V/cm and quantified by laser densitometry.

A minor drawback to the transcription titration assay used is that the final reaction products contain a significant proportion of hybrid molecules formed by complementary strands of the competitor and wild-type molecules (Becker-André and Hahlbrock, 1989). We have evaluated the proportion of hybrid molecules in our system in two different ways: (i) by digestion with *Kpn*I, since only wild-type molecules are sensitive to this restriction enzyme, and (ii) by resolving the hybrid in 1.5% agarose gels run at 1.7 V/cm. This preliminary work indicated that the hybrid molecule migrated with or very close to the wild-type molecule under standard conditions, i.e. 1% agarose gels at 5 V/cm (see Figure 2), and that its proportion depended on the relative amounts of wild-type and competitor molecules present in the reaction. An error of up to 30% may be introduced when quantifying absolute mRNA levels if hybrid molecules are not considered apart from the wild-type ones. However, an error of less than 10% is introduced when only relative values are required. Thus, we only took into account the proportion of hybrid molecules in those experiments dedicated to determine the absolute *ftsZ* mRNA levels per cell.

Chromosomal *ftsZ* DNA quantifications were performed essentially by the amplification procedure described for the transcription titration assays. Oligonucleotides TG2 and MA8 were used as the forward and reverse primers, respectively, and an appropriate amount of DNA competitor was added to the amplification reactions before the thermal cycling steps. cRNA obtained by *in vitro* transcription and total RNA samples was always checked for DNA contamination by this assay.

Enzyme assays and immunoblotting procedures

β-galactosidase activities were measured by the method of Miller (1972), being referred to cell density and expressed as Miller units. Immunoblotting procedures were done as described by Aldea *et al.* (1990) and Pla *et al.* (1991). FtsZ levels were always referred to the EF-Tu protein levels, which were measured on the same blots using polyclonal antisera raised against this protein as described by Pla *et al.* (1991).

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