Diadenosine 5′,5″-P1,P4-tetraphosphate (Ap4A) controls the timing of cell division in *Escherichia coli*

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Abstract

**Background:** The timing of the cell division in *Escherichia coli* is highly regulated, but its mechanism has not been identified. Previously we have found that the *cfcA1* mutation uncouples DNA replication and cell division and elevates the frequency of cell division. We further analysed the structure and the role of the *cfc* genes of *cfcA11*, a derivative of *cfcA1*, and another *cfc* mutant, *cfcB1*.

**Results:** The *cfc* mutants divided prior to the ordinary stage of cell division and produced many small cells with nucleoid. However, the cells grew exponentially, and the length of a cell cycle and the initiation mass for chromosome replication were not altered by the *cfc* mutations. These properties resulted from a reduction of the period between the nucleoid division and the cell division in a cell cycle, and a compensatory lengthening of the period between the cell division and the initiation of the next round of DNA replication. *CfcA11* has a mutation in *glySa* which encodes the α-subunit of glycyl-tRNA synthetase, and *cfcB1* has an IS2 insertion in *apaH* which encodes Ap4A hydrolase. The *cfc* properties of both *cfc* mutants were suppressed by a multicopy plasmid carrying *apaH*+, and the intracellular level of Ap4A in *cfcA1* was 15-fold higher, and *cfcB1* was 100-fold higher than their parent. Experiments using wild-type cells showed that a high level of Ap4A caused early cell division, and that a low level of Ap4A caused delayed cell division.

**Conclusion:** Ap4A is a signal for the induction of cell division. High levels of Ap4A are responsible for the initiation of cell division. The *glyS* mutation permits an efficient synthesis of Ap4A.

Introduction

During the cell cycle of *E. coli*, several events such as DNA replication and cell division take place through strictly periodic processes, and two identical daughter cells are produced. Therefore, the cell must have a mechanism(s) for coordinating each event. The cell division, for example, occurs after a constant period (20 min) at 37°C following the termination of replication under various growth conditions (Helmstetter & Cooper 1968; Pierucci & Helmstetter 1969; Dix & Helmstetter 1973). Cells lacking DNA are remarkably rare in cultures of wild-type strains (Howe & Mount 1975), and the inhibition of DNA replication usually arrests cell division (Inouye 1972; Burton & Holland 1983; Jaffe et al. 1986; Nishimura 1989). The variation in cell size at the time of cell division is very small, about 10% (Schaechter et al. 1962). These facts suggest that *E. coli* must have a mechanism that triggers the initiation of cell division at a specific cell size that is coordinated with DNA replication.

The cell division at a particular stage of a cell cycle may be initiated by a signal(s) that promotes the reaction, or suppresses a function which inhibits it. Approaches to determining if such a signal(s) exists are mainly based on the following three hypotheses. First, cell division of *E. coli* requires protein synthesis at the completion of chromosome replication (Jones & Donachie 1973), and FtsA was proposed as the putative
termination protein (Tormo et al. 1985). Second, a single flux of calcium ions triggers cell cycle events, including cell division in E. coli (Norris et al. 1988; Holland et al. 1990). This speculation is based upon the following observation: the level of free calcium ions is highly regulated (Gangola & Rosen 1987), amounts of which are significantly greater in dividing cells (Chang et al. 1986) and cell division is sensitive to inhibitors of calmodulin, which is often involved in calcium-mediating action in eukaryotes (Holland et al. 1990). Third, a certain internal metabolite, that may be synthesized and accumulated to a critical threshold level, induces cell division (D’Ari et al. 1990). Such a mechanism, which is called the ‘metabolic clock’, was speculated to exist from the observation that a nutritional shift-up or nutritional pulse causes a cell division delay and that FtsZ is involved in the induced division delay (Kepes & D’Ari 1987). It also reminded us of the former proposal of Inouye & Pardee (1970), that the molar ratio of putrescine to spermidine is a critical factor for cell division. Young & Srinivasan (1972) showed that addition of putrescine to a mutant, conditionally incapable of synthesizing putrescine, first causes the stimulation of protein synthesis. Thus they suggest that the primary effect of the ratio of putrescine to spermidine is on the synthesis of proteins that subsequently lead to cellular division.

We isolated the novel mutant cfcA1 that shows uncoupling between DNA replication and cell division, and high frequency of cell division (Nishimura 1989). In this paper, we analysed cfcA11, a derivative of cfcA1, and another cfc mutant, cfcB1. We found that Ap4A accumulates in cfcA11, a glyS mutant, and in cfcB1, an Ap4A hydrolase mutant, and conclude that the over-production of Ap4A causes early division in a cell cycle, uncoupling of cell division and DNA replication.

Results

Effect of cfc mutations on cell cycle
First, we examined the effects of the cfc mutations on cell division and growth. We used cfcA11—which is a derivative of cfcA1—and which regained a rapid growth rate and better viability at 30°C, but retained other phenotypes of cfcA1. Another mutant, named as cfcB1 in this paper, was one of four mutants described in a previous paper (Nishimura 1989) which showed similar cell division and growth phenotypes to cfcA11. CfcA11, cfcB1, and their cfc+ parent, also possess a temperature sensitive mutation for DNA replication, dnaBts. Cultures of these bacteria growing at 30°C were diluted to give an OD660 of 0.02, and continued to be incubated for 4 h. The values of OD660 of these cultures increased exponentially with the same doubling time (Fig. 1). It was also found that the cell number of each culture increased exponentially, with similar doubling times of 65, 72 and 70 min for cfc–, cfcA11 and cfcB1, respectively. However, the average numbers of cells per
\[ \text{OD}_{660} \text{ in the cfcA11 and cfcB1 cultures were about } 40\% \text{ higher than in the cfc}^+ \text{ culture. We found similar results in the cultures of the dnaB}^+ \text{ transductants of the cfc}^- \text{ and cfc}^+ \text{ strains. The above observation was not influenced by the dnaB}^\text{ts} \text{ mutation, although we used the dnaB}^\text{ts} \text{ strains throughout this work, unless otherwise stated. We also found that the amount of protein in each culture was proportional to the } \text{OD}_{660} \text{ value. Therefore, the } \text{OD}_{660} \text{ values represent the protein mass, and accordingly, the total cell mass.}

We examined the viability of cells by diluting each culture into a soft agar medium and incubating on a glass slide for 5 h, followed by microscope observation, as described by Tomizawa (1960). The observations showed that 97%, 91% and 95% of the original cells of cfc\(^+\), cfcA11 and cfcB1 formed colonies. Therefore, the cfc mutations affect cell viability very little.

From these results, we conclude that although the length of the cell cycle was not significantly altered by the mutations, the average cell size of both mutants was smaller than that of the parent. The reduction of cell size due to the mutations was demonstrated by the microscope observation of cells from the cultures, as shown in Fig. 2. Coulter counter measurements showed that the relative cell volumes of cfcA11 and cfcB1, compared to cfc\(^+\) were 78% and 71%. The values were a little higher than those expected from the \( \text{OD}_{660} \) ratio per cell number or from microscope observation. However, this discrepancy is not in conflict with other results, because the cell volume determined by Coulter counter analysis is judged by putting together various data such as cell shape and cell surface structure and is finally converted into an average volume of gloves. The microscope observation that all cells in either of the cultures had one or two nucleoids also suggested that the cell division of the cfc mutants did not take place more than once per single nucleoid division, the same as in the cfc\(^+\) strain. We also found that the fraction of cells with two nucleoids in cfc cultures was apparently low compared with that in cfc\(^+\) cultures; roughly 21% for cfc\(^+\), 6% for cfcA1 and 5% for cfcB1. This means that in the cfc cultures, the fraction of cells in the binucleoid stage is small. When cells were grown to a stationary phase, the sizes of most cfc cells were about 80% of those of the cfc\(^+\) parent (data not shown). It is known that \textit{E. coli} cells stop growing just after cell division when the cultures enter the stationary phase (Holland 1987). Taking these results together, we conclude that the mutant cells divided at a size smaller than that of the dividing parental cells due to the shortening of the period between the nucleoid division and the cell division.

These findings are summarized as follows. The cfc mutants grew exponentially, and the duration of the binucleoid stage was shortened, and yet the length of a cell cycle was not altered. The most likely explanation for these observations is that the mutant cells divide at the small size, but the subsequent cell cycle contains a compensatory elongation period during which the divided mutant cells grow to the size same as the parental cell when next cycle of DNA replication begins (Fig. 3a).

In the normal process of a cell cycle, chromosome synthesis initiates when a cell grows to a certain fixed mass, termed the ‘initiation mass’ (Donachie 1968). To examine the initiation mass of the cfc\(^-\) and cfc\(^+\) strains by directly observing the stained cells microscopically, cells were grown for 4 h in the presence of chloramphenicol (250 \( \mu \text{g/mL} \)) to inhibit protein synthesis. By this treatment, the initiation of a new round of replication is inhibited while allowing ongoing replication to be completed. The distribution of cell sizes (Fig. 4) showed that, although the average cell sizes in the total population of cfc mutants were smaller than...
that of their cfc\(^+\) strain, the sizes of both of the largest cells with a single nucleoid and smallest cells with a double nucleoid were not altered by the cfc mutations (3.0–3.2 \(\mu\)m). These results indicate that the initiation mass is little altered by these mutations.

These results support the conjecture that the cfc mutations cause early cell division, but they do not affect other processes of the cell cycle such as DNA replication or protein synthesis. The increase in cell number by cfc mutations is due to a shortening of the length from nucleoid division to cell division, by compensatory elongation of the period from cell division to the initiation of chromosome synthesis, as shown in Fig. 3a. As shown in Fig. 3b, the time from nucleoid division to cell division in cfc\(^-\) is \(\approx 2\) min against that in cfc\(^+\), which is \(\approx 20\) min.

**CfcA11 has a mutation in glySa gene**

The *glyA* gene, the cosmid clone E4105 (Tabata et al. 1989), which complemented the *glyA* mutation (Fig. 5a), was used. We obtained subclones of the cosmid that had a deletion at various regions, and then tested for their ability to complement cfcA11 growing at 41 °C for 3 h. Under these conditions, the cfc\(^+\) cells formed a population of long filamentous cells due to the *dnaB* mutation.

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Figure 3  (a) Cell cycles of cfc mutants and their parent. (b) Theoretical amounts of cell number during the division cycle. The full line shows cell number and dotted line shows OD\(_{660}\) as a function of time during the division cycle. C is the period for a round of chromosome replication, D is the period between nucleoid division and cell division, and B is the period between cell division and initiation of the next round of DNA replication (Cooper & Helmstetter 1968). Lengths of the periods of each strain are calculated roughly as follows. In the normal case, the length of the periods C and D are constant at 40 min and 20 min (Cooper & Helmstetter 1968; Helmstetter & Cooper 1968), and length of the cell cycle of cfc\(^+\) was 65 min. Therefore, the length of the B period of cfc\(^+\) is calculated as being 5 min. For the cfc mutants, the length of the cell cycle was about 70 min and length of the C period (which was not affected by the cfc mutations) was 40 min. Therefore, the D + B period should be 30 min. To affect a 40% increase in cell number, integral amounts of cell number of the cfc mutants during the cell cycle should be 40% higher (corresponding to the shaded region) than that of the cfc\(^+\) strain. Thus the length of the B period should be about 28 min, and the remaining 2 min is accounted for by the D period.
mutation, and cfcA11 formed shorter cells, as mentioned in a previous paper (Nishimura 1989). The results shown in Fig. 5b indicate that the 4 kb EcoRV segment carried by pNA4V can complement cfcA11, suggesting that the segment includes the cfcA gene. When cfcA11 was transformed by the plasmid pNA2E carrying a 2.2 kb EcoRI segment, 13 out of 15 transformants showed the Cfc\(^{+}\) phenotype. One

**Figure 4** The initiation mass of the cfc mutants and their parent. The cfc and the parent cells were grown 4 h in the presence of chloramphenicol (250 \(\mu\)g/mL) to inhibit protein synthesis. By this treatment, the initiation of a new round of replication is inhibited, while allowing ongoing replication to be completed. Phase-fluorescence micrographs were taken after staining chromosomal DNA by DAPI, and the cell size was measured. The size distribution of the total number of cells is shown by bars. The shaded bars show the size distribution of the cells which had one nucleoid.

**Table 2** Fine mapping of the \(\delta B1\) mutation by P1 transduction

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Selective marker</th>
<th>Mutations involved in co-transduction*</th>
<th>Co-transduction frequency</th>
<th>Distance (min)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 : ME8781 × cfcB1</td>
<td>Tc(^{+})</td>
<td>thr::Tn10 (\delta B)</td>
<td>0.08 (2/24)</td>
<td>1.15</td>
</tr>
<tr>
<td>P1 : ME8782 × cfcB1</td>
<td>Tc(^{-})</td>
<td>car-96::Tn10 (\delta B)</td>
<td>0.53 (38/72)</td>
<td>0.39</td>
</tr>
<tr>
<td>P1 : ME8783 × cfcB1</td>
<td>Tc(^{-})</td>
<td>zac3051::Tn10 (\delta B)</td>
<td>0.21 (15/71)</td>
<td>0.81</td>
</tr>
<tr>
<td>P1 : ME8783 × cfcB1</td>
<td>Tc(^{-})</td>
<td>zac3051::Tn10 leu</td>
<td>0.93 (66/71)</td>
<td>0.05</td>
</tr>
<tr>
<td>P1 : ME8783 × YM101</td>
<td>Tc(^{-})</td>
<td>zac3051::Tn10 ileS</td>
<td>0.03 (3/88)</td>
<td>1.35</td>
</tr>
</tbody>
</table>

* The temperature-sensitive phenotype (\(dnaB42\)) of recombinants was tested, and the unselected marker \(\delta B\) was identified as follows: recombinants were grown in broth at 41 °C for 3 h to inactivate the DnaB protein. Under these conditions, the \(\delta B^{+}\) recombinants showed a uniformly long filamentous cell population, and the \(\delta B1\) recombinants showed short filamentous cells. However, the cell size of the \(\delta B^{+}\) recombinants appeared to be slightly shorter than that of the cfc\(^{+}\) parent.

Reversion frequencies of mutations involved in co-transduction were less than 10\(^{-8}\).

‡ Map distances between selected and unselected mutations involved in co-transduction were calculated by Wu’s equation (Wu 1966): map distance in min = \(\phi(1-3\iota/\iota)\), where \(\iota\) = co-transduction frequency, and \(\phi = 2\) for the P1 phage.
A possible explanation for these two exceptions is that the 2.2 kb segment that was subcloned with a high copy number plasmid is toxic, resulting in the solution of a suppressor mutation.

To analyse the structure of the cfcA gene, the 2.2 kb EcoRI segment was cloned into the EcoRI site of M13 DNA and sequenced. The EcoRI segment contained the same sequence as glySa (Webster et al. 1983) which encodes an α-subunit of glycyl-tRNA synthetase. The restriction map of the 4 kb EcoRV segment of E4105, which complemented the cfcA11 mutation, agreed completely with the segment that contained no genes or open reading frame (ORF) other than glyS (Keng et al. 1982).

To localize the site of the cfcA11 mutation, the genomic DNA of cfcA11 was isolated and the EcoRV segment of the glyS region was cloned into the SmaI site of M13 DNA. Sequencing analysis showed that 143A in glySa was substituted by C in cfcA11; this corresponds to the amino acid substitution of 48Glu by Ala.

CfcB1 has a mutation in the apaH gene

The cosmid carrying glyS⁺ did not complement cfcB1. Therefore, cfcB1 was located at a different site from glyS. We mapped cfcB1 by conjugation for 60 min with the Hfr strain ME8300, which transfers chromosomal DNA starting from thr in the clockwise direction. The cfc⁺ was found in 61% of thr⁺-str⁺ recombinants. These results suggest that cfcB1 was in the proximity of thr. Fine
mapping by P1-phage-mediated transduction showed that the \(cfcB1\) allele was assigned to 1.15 min on the \(E.\ coli\) genetic map (Table 2).

To subclone \(cfcB1\) we used \(pLC14-37\), which complemented \(cfcB1\), from the Clark–Carbon library (Clark & Carbon 1976). We obtained subclones of \(pLC14-37\) that deleted at various regions and tested their ability to complement \(cfcB1\) growing at 41\(^\circ\)C for 3 h. The results shown in Fig. 6 suggest that the 1.9 kb \(Pvu\)II–\(Eco\)RI segment carried by \(pNB2\) contained the \(cfc\) gene. To sequence the 1.9 kb \(Pvu\)II–\(Eco\)RI segment, the segment from \(pNB2\) was cloned into the \(Sma\)I–\(Eco\)RI site of \(M13\) DNA. Sequencing analysis showed that this fragment contained the \(apaG–apaH\) gene, but no other genes and ORFs.

Figure 6 Physical map around \(cfcB1\) and complementation analysis. (a) The physical map of the 1 min region and location of known genes. Abbreviations: \(P\) shows the promoter; \(B\), \(Bam\)HI; \(E\), \(Eco\)RI; \(V\), \(Eco\)RV; \(P\), \(Pst\)I; \(Pv\), \(Pvu\)II. (b) Chromosomal segments carried by the plasmids. The complementation of the \(cfcB1\) mutation by each plasmid is indicated at the right. The plasmid \(pBR322\) was used as the vector.

Figure 7 Two-dimensional thin-layer chromatography of cellular nucleotides of the \(cfc\) mutants and their parent. Cultures of \(cfcA11\), \(cfcB1\) and \(cfc^+\) were grown in a low-phosphate medium containing disodium hydrogen \([\text{32P}]\)orthophosphate at 41\(^\circ\)C for 1 h. Formic acid soluble fractions were isolated and chromatographed. The buffer containing 1.75 m Morpholine, 0.1 m bolic acid, 1.4 m HCl (pH 8.7) was used for first direction, and the buffer containing 3 m (\(\text{NH}_4\)\(\text{SO}_4\), 2% sodium EDTA (pH 5.5) was used for second direction. The standard Ap4A solution was co-chromatographed and visualized by UV260 absorbance. The arrows indicate Ap4A spots. Amounts of Ap4A was determined as relative counts of 32P-Ap4A for total 32P-compounds.
To isolate the cfcB1 mutation site, the genomic DNA of cfcB1 was isolated and a \( \text{Pvu} \) II–Eco RI segment which hybridized to pNB2 \( \text{D} \) \( \text{Pvu} \) II was cloned into M13 RFI DNA. Sequencing analysis showed that an IS2 was inserted just before the 40th G of the \( \text{apaH} \) coding region of cfcB1. From the results of complementation and sequencing analysis, we concluded that cfcB1 has a mutation in \( \text{apaH} \).

Cellular level of Ap4A in both cfcA11 and cfcB1 is high
The \( \text{apaH} \) encodes the diadenosine–tetraphosphate (Ap4A) hydrolase (Mechulam et al. 1985) and Ap4A is synthesized in vitro by certain aminoacyl-tRNA synthetases (Zamecnik et al. 1966). Complementation analysis showed that the plasmid encoding \( \text{apaH}^+ \) partially complemented cfcA11 (Table 3). The results suggest that cfcA11, a mutant of glycyl-tRNA synthetase, can synthesize Ap4A in vivo and that high levels of Ap4A can be a signal for the early cell division of the mutant.

To examine the cellular level of Ap4A in cfcA11, cfcB1 and their parent, we isolated \( ^{32} \text{P} \)-labelled compounds in formic acid-soluble fractions of these strains and analysed them by two-dimensional thin layer chromatography (Fig. 7). The results showed that cfcA11 produced about 15-fold more Ap4A than the parent and that cfcB1 produced about 100-fold more. Ap4A was not detected in the \( \text{apaH}^+ \) transformants of cfcA11 or cfcB1 (data not shown). These results suggest that the accumulation of Ap4A causes early cell division, both under the conditions of \( \text{glySa}^- \) and \( \text{apaH}^- \).

High levels of Ap4A in a wild-type cell causes early cell division
To examine the cause of the Cfc\(^-\) phenotype, various strains were constructed using the \( \text{dnaB}^+ \) strain MG1655. The cfc phenotype of these strains was analysed as shown in Figs 8a and 9. The cfcB1 transductants of the wild-type strain (Fig. 8a–2, Fig. 9)

### Table 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative cell number (per OD at 41 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{cfc}^+ ) parent ( \text{dnaB}^+ )</td>
<td>1</td>
</tr>
<tr>
<td>cfcA11 ( \text{dnaB}^+ \text{cfcA11} )/pBR322</td>
<td>4.2</td>
</tr>
<tr>
<td>cfcA11 ( \text{dnaB}^- \text{cfcA11} )/pBR322 ( \text{apaH}^- )</td>
<td>1.8</td>
</tr>
<tr>
<td>cfcB1 ( \text{dnaB}^+ \text{cfcB1} )/pBR322</td>
<td>3.1</td>
</tr>
<tr>
<td>cfcB1 ( \text{dnaB}^- \text{cfcB1} )/pBR322 ( \text{apaH}^- )</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Figure 8 Cfc phenotype of various transformants and transductants delivered from MG1655, a wild-type strain, at 30 °C. MG1655 \( \text{cfcB1} \) was made by co-transduction with \( \text{car}^+ \) from cfcB1 to MG1655 \( \text{car}-96: \text{Tn10} \). MG1655 \( \text{cfcA11} \) was made by co-transduction with \( \text{yph}^+ \) from cfcA11 \( \text{yph}^+ \) to MG1655 \( \text{yph}^- \). The plasmid \( \text{apaH}^+ \) carries the 2 kb EcoRI–PvuI segment from pLC14–37, and the \( \text{glySa}^+ \) carries a 2.2 kb EcoRI segment from the genomic DNA of cfcA1.

To localize the cfcB1 mutation site, the genomic DNA of cfcB1 was isolated and a \( \text{Pvu} \) II–EcoRI segment which hybridized to pNB2\( \Delta \text{PvuII} \) was cloned into M13 RFI DNA. Sequencing analysis showed that an IS2 was inserted just before the 40th G of the \( \text{apaH} \) coding region of cfcB1. From the results of complementation and sequencing analysis, we concluded that cfcB1 has a mutation in \( \text{apaH} \).
showed the Cfc⁻ phenotype, producing characteristic small cells when transductants were purified by single colony isolation and cultivated in a liquid medium for several generations. However, the phenotype was lost after overnight cultivation. Transformation of apaH⁺ into the wild-type strain (Fig. 8a-3, Fig. 9) caused a reduction of the frequency of cell division for the first few generations in a liquid medium after the single colony isolation of transformants—producing longer rod cells—but the phenotype was lost after overnight cultivation. On the other hand, cfcB1 transductants of the parent strain continuously demonstrated a Cfc⁻

Table 1  Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAT42</td>
<td>F⁻; thr-1 leu-6 thi-1 argH1 thyA his-1 trp-1</td>
<td>Hirota et al. 1968</td>
</tr>
<tr>
<td></td>
<td>str-9 xyl-7 mtl-2 met lacYI Δ101 malA1/N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tonA2 supE44 dnaB⁺</td>
<td></td>
</tr>
<tr>
<td>cfcA11</td>
<td>derivative of PAT42 cfcA1</td>
<td>this work</td>
</tr>
<tr>
<td>cfcB1</td>
<td>PAT42 cfcB1</td>
<td>this work</td>
</tr>
<tr>
<td>ME8781</td>
<td>MG1655 thr-34::Tn10</td>
<td>Singer et al. 1989</td>
</tr>
<tr>
<td>ME8782</td>
<td>MG1655 car-96::Tn10</td>
<td>Singer et al. 1989</td>
</tr>
<tr>
<td>ME8783</td>
<td>MG1655 Zac-3051::Tn10</td>
<td>Singer et al. 1989</td>
</tr>
<tr>
<td>MG1655</td>
<td>wild-type</td>
<td>Singer et al. 1989</td>
</tr>
</tbody>
</table>
phenotype. From these results, we conclude that an increase in the cellular concentration of Ap4A causes early cell division, and a decrease causes delayed cell division. To maintain these phenotypes, some genetic background, which is lost by the cultivation of the cfcB1 transductants or the apaH+ transformants is needed.

Transduction of the cfcA11 mutation into wild-type cells (Fig. 8b–2, Fig. 9) caused the Cfc− phenotype. This phenotype did not change, even after three cycles of overnight cultivation. The plasmid carrying glySa also caused the Cfc− phenotype (Fig. 8b–3, Fig. 9). Thus glySa− allows the continuous expression of the Cfc− phenotype.

Discussion

The cfc mutants divide before they grow to the size of dividing cfc+ cells and produce many small cells with nucleoid. The mutations affect the timing of the cell division but not the initiation mass for chromosome replication. These mutant properties result from a reduction of the period between the nucleoid division and the cell division in the cell cycle, and a compensatory elongation of the period between the cell division and the initiation of the next round of DNA replication. The mutations relieve a cell from division arrest due to the inhibition of DNA replication (Nishimura 1989). Thus the cfc mutants are defective in the coupling between DNA replication and cell division. We studied the mechanism of induction of cell division and conclude that Ap4A is a signal molecule for this process.

The biochemical analysis showed that the cellular level of Ap4A in cfcA11 was about 15-fold higher than the parental cfc+ strain, and that in cfcB1 it was about 100-fold higher. The genetic study showed that the cfcA11 mutation is in the glyS gene, while cfcB1 has a mutation in apaH, which encodes Ap4A hydrolase. The Cfc− properties and high cellular level of Ap4A of both cfc mutants are suppressed by a multicopy plasmid encoding apaH+ These results show that the degradation of Ap4A by an overproduction of the apaH+ gene product abolishes not only the effect of the mutation in the allelic cfcB1 gene, but also that of the mutation in the nonallelic cfcA11 gene. We conclude that the concentration of Ap4A affects the initiation of cell division, and that this concentration is controlled by glySa and apaH.

While the effect of the apaH mutation on the level of Ap4A is straightforward, the effects of the glyS mutations are complex. It has been shown in vitro that glycol adenylate intermediates which are bound to glycytRNA synthetase (Led et al. 1983), or some other aminoacyl-tRNA synthetases (Zamecnik et al. 1966) donate AMP to ATP, yielding Ap4A. However, the existence of such reactions in vivo has not been demonstrated; neither aminoacyl-tRNA synthetase mutations, inhibitory treatments of aminoacylation, nor structural mutations of tRNA are known to produce Ap4A in vivo (Bochner et al. 1984). On the contrary, glycyl-tRNA synthetase also catalyses the degradation of Ap4A to ADP in the presence of PPi (Led et al. 1983). Thus the increase in Ap4A concentration by the cfcA11 mutation is caused either by an enhanced synthesis of Ap4A, or by the inhibition of degradation. However, it is evident that the enzyme can catalyse different reactions depending on the cellular environment and can thus create widely different Ap4A concentrations.

The biological significance of Ap4A was first demonstrated by Rapaport & Zamecnik (1976). They showed that the intracellular level of Ap4A changes in a cell cycle dependent manner in mammalian cells growing in tissue culture (reviewed by Zamecnik 1983). Furthermore, the addition of Ap4A to permeabilized resting cells of the baby hamster kidney induced multiple replication eyes (Grummt 1978). Apparently contrary results showed that Ap4A causes cell contact inhibition (Segal & Le Pecq 1986). We observed that the elevation of Ap4A concentration by cfc mutations did not affect the length of DNA replication, the DNA/mass ratio (data not shown), or the initiation mass, but that a particular concentration of Ap4A induces cell division. One possible explanation of how Ap4A affects cell division is that Ap4A binds to a particular protein essential for the cell division and alters its function. This speculation comes from our observations that an increase in the cellular level of Ap4A subsequent to transduction of the cfcB1 mutation causes earlier cell division, and that its decrease subsequent to the transformation of a plasmid carrying apaH causes delayed cell division. However, these phenotypes are lost after overnight cultivation. The loss of these phenotypes may be explained by some suppressor mutation or physiological change that may alter the binding of Ap4A to the putative binding protein—in view of the fact that Ap4A can bind many cellular proteins (Johnstone & Farr 1991; Grummt et al. 1979). The cfcA11 and allelic glySa− mutations allowed a continuous expression of the Cfc− phenotype in wild-type cells. Because a multi-copy plasmid carrying glySa+ inhibited the cell division of wild-type cells producing filament cells under normal growth conditions (data not shown), we
speculate that GlySa may have an important role in the normal cell cycle, in conjunction with Ap4A.

**Experimental procedures**

**Bacterial strains and bacteriological procedures**

The *E. coli* strains used are listed in Table 1. Cosmid E4105 (Tabata et al. 1989) was kindly supplied by A. Higasitani. LB medium contains 10 g/L bacto-tryptone (Difco Laboratories), 5 g/L bacto-yeast extract (Difco Laboratories), 5 g/L NaCl, 1 g/L glucose, and 40 mg/L thymine, and adjusted to pH 7.0 with NaOH. In order to label intracellular nucleotides with 32P at glucose, and 40 mg/L thymine, and adjusted to pH 7.0 with 5 g/L bacto-yeast extract (Difco Laboratories), 5 g/L NaCl, 1 g/L medium contains 10 g/L bacto-tryptone (Difco Laboratories), et al. (Tabata et al. 1989) were centrifuged for 1 min at 4°C Blackwell Science Limited Genes to Cells (1997) were centrifuged for 10 min at 4°C. OD660 nm. Before acid extraction, the labelled cultures were diluted 10-fold into fresh low phosphate medium containing disodium [32P]orthophosphate (30 μCi/mL 7.4 GBq/mmol) and aerated at 41°C for 3 h. Identical parallel cultures lacking 32P were also inoculated, and their growth was monitored spectrophotometrically at 660 nm. Before acid extraction, the labelled cultures were concentrated to 10-fold as follows: 800 μL of labelled cultures were centrifuged for 1 min at 4°C in a microfuge tube and 700 μL of supernatants were removed. The remaining 100 μL of concentrated cultures were mixed rapidly with 10 μL of cold 11 N formic acid. After 30 min, the formic acid suspensions were centrifuged for 10 min at 4°C. 80 μL of the supernatant extracts were stored at −20°C until use.

**Sequencing analysis and cloning of genomic DNA**

Fragments to be sequenced were recloned into M13mp18. Overlapping deletions of the cloned insert were constructed using the Kilo-Sequencing Deletion Kit (Takara, Kyoto). The sequencing reactions were carried out using Sequenase version 2.0 (Toyobo, Osaka). Mutant genes were cloned and sequenced as follows. Total cellular DNA from overnight culture was digested with the appropriate restriction enzymes. DIG-labeled phages were detected by plaque hybridization (Nishimura et al. 1992). DNA sequences were determined by using a series of synthetic oligonucleotide primers. Chromosomal DNA was isolated as described by Davis et al. (1980). The polymerase chain reaction was done as described by Innis et al. (1988).

**Determination of intracellular levels of Ap4A**

Cultures of cfc mutants and their parent were grown, labelled, and extracted as described by Bochner & Ames (1982). Overnight cultures were diluted into fresh LB medium to give an OD660 of 0.02, and aerated at 30°C until the logarithmic phase. The cultures were diluted 10-fold into fresh low phosphate medium containing disodium hydrogen [32P]orthophosphate (30 μCi/mL 7.4 GBq/mmol) and aerated at 41°C for 3 h. Identical parallel cultures lacking 32P were also inoculated, and their growth was monitored spectrophotometrically at 660 nm. Before acid extraction, the labelled cultures were concentrated to 10-fold as follows: 800 μL of labelled cultures were centrifuged for 1 min at 4°C in a microfuge tube and 700 μL of supernatants were removed. The remaining 100 μL of concentrated cultures were mixed rapidly with 10 μL of cold 11 N formic acid. After 30 min, the formic acid suspensions were centrifuged for 10 min at 4°C. 80 μL of the supernatant extracts were stored at −20°C until use.

Polyethyleneimine (PEI)-cellulose thin-layer sheets were obtained commercially, (Merck) and prior to the beginning of the chromatographic development they were soaked in distilled water for 30 min, then dried at room temperature. Acid extracts (2 ~ 5 μL) were applied as a spot on TLC plates and developed as described by Bochner & Ames (1982). For the two-dimensional chromatography to separate the Ap4A, the solution for the first direction was 1.75 M Morpholine, 0.1 M boric acid, 1.4 M HCl (pH 8.7), and for the second direction was 3 M (NH4)2SO4, 2% disodium EDTA (pH 5.5) as described by Lee et al. (1983). In co-chromatography experiments, 0.5 μL aliquots of 100 mm standard solution were mixed with 10 μL radioactive samples before application to the TLC plates. Locations of the standard were determined by UV260 absorbance. 32P-labelled compounds were visualized by autoradiography. The amount of Ap4A was determined as relative counts of 32P-Ap4A for total 32P-compounds by the method described by Cashel (1969). Total counts of 32P-compounds were calculated from counts obtained with aliquots (2 μL) of acid extracts spotted on TLC plates, rinsed in Methanol, dried, and counted.

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