Regulatory Interactions between Phospholipid Synthesis and DNA Replication in *Caulobacter crescentus*

BRENDA LOEWY,[†] GREGORY T. MARCZYNSKI, ANDREW DINGWALL, AND LUCILLE SHAPIRO*

Department of Developmental Biology, Beckman Center, Stanford University School of Medicine,
Stanford, California 94305

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Several *Caulobacter crescentus* mutants with lesions in phospholipid biosynthesis have DNA replication phenotypes. A *C. crescentus* mutant deficient in glycerol 3-phosphate dehydrogenase activity (*gpsA*) blocks phospholipid synthesis, ceases DNA replication, and loses viability in the absence of a glycerol phosphate supplement. To investigate the interaction between membrane synthesis and DNA replication during a single cell cycle, we moved the *gpsA* mutation into a synchronizable, but otherwise wild-type, strain. The first effect of withholding supplement was the cessation of synthesis of phosphatidylglycerol, a major component of the *C. crescentus* membrane. In the absence of glycerol 3-phosphate, DNA replication was initiated in the stalked cell at the correct time in the cell cycle and at the correct site on the chromosome. However, after replication proceeded bidirectionally for a short time, DNA synthesis dropped to a low level. The cell cycle blocked at a distinct middivision stalked cell, and this was followed by cell death. The “glycerol-less” death of the *gpsA* mutant could be prevented if the cells were treated with novobiocin to prevent the initiation of DNA replication. Our observations suggest that the processivity of *C. crescentus* replication requires concomitant phospholipid synthesis and that cell death results from incomplete replication of the chromosome.

Caulobacter crescentus is a useful organism in which to study the regulation of DNA synthesis. A complete physical map of the chromosome and its correlation with the genetic map (11, 12) allows a clear analysis of replication functions. The single chromosome has one bidirectional origin of replication (10, 20). *C. crescentus* cell cultures can be easily synchronized, and DNA replication can be studied as a function of its well-defined developmental cycle. This bacterium undergoes one round of replication per cell cycle and then divides to produce two different progeny cells: a sessile stalked cell that immediately initiates DNA replication and a motile swarmer cell in which chromosome replication is delayed until transition of the motile cell into the stalked cell later in the cell cycle (Fig. 1; 9, 22). The stalked cell follows a temporally regulated program that includes a round of DNA replication in addition to the synthesis of a new flagellum, chemotaxis methylation proteins, pili, and DNA bacteriophage receptor sites at the cell pole opposite the stalk. The predivisional cell thus has an inherent asymmetry which yields two different daughter cells upon division (for reviews, see references 22 and 26).

Analysis of several mutants that are auxotrophic for lipid precursors suggested that DNA replication is dependent on membrane phospholipid synthesis (6, 16, 17, 19, 27). Termination of phospholipid synthesis by starvation of a glycerol 3-phosphate auxotroph (*gpsA* mutant) for glycerol phosphate resulted in the preferential inhibition of DNA synthesis, although both RNA and protein syntheses continued (6). When lipid synthesis was inhibited by starvation of a fatty acid auxotroph (*fatB* mutant), there was a sharp decrease in DNA synthesis, but only after enough time had elapsed for a round of replication to be completed (17). *fatB* mutant

strains cannot initiate a new round of DNA replication unless exogenous fatty acids are supplied.

In this study, the interaction between membrane biogenesis in a *gpsA* mutant strain and chromosome replication was analyzed during a single cell cycle. The *gpsA505* mutation causes glycerol phosphate auxotrophy by reducing the activity of *sn*-glycerol 3-phosphate dehydrogenase 10-fold below wild-type levels (6). We have reported previously that glycerol phosphate starvation of asynchronous mutant cultures causes a block in phospholipid synthesis followed by a decline in the rate of DNA synthesis and a loss of viability (8). We report here that in the absence of phospholipid synthesis, the initiation of DNA replication occurs in the stalked cell at the correct time in the cell cycle and at the correct site on the chromosome but that most initiated chromosomes are unable to complete a round of chromosome replication or to reinitiate a new round of replication. Cell death, which occurred in the absence of glycerol phosphate, was prevented by blocking the initiation of chromosome replication early in the cell cycle.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are shown in Table 1. *C. crescentus* strains were grown at 30°C in peptone-yeast extract (PYE) or in minimal glucose (0.2%) medium (BMG) (7) supplemented when appropriate with 1 mM glycerol and 1 mM glycerol 3-phosphate or 0.3 mM adenosine. *C. crescentus* wild-type CB15N and derivative strains were synchronized following the separation of swarmer cells in a Ludox density gradient by the method of Evinger and Agabian (14) as modified by Mansour et al. (21).

Construction of strain AE9203 (*gpsA*). The original *gpsA505* mutant strain (AE5168) was isolated by Contreras et al. (6), and the mutant allele was moved into a wild-type CB15 genetic background (AE5512) by Hodgson et al. (16). Since neither strain can be synchronized, we moved the *gpsA505* mutant allele into the synchronizable wild-type

* Corresponding author.

[†] Present address: Department of Biology, Adelphi University, Garden City, NY 11530.

TABLE 1. List of strains

Strain	Genotype	Derivative or source (donor × recipient) ^a
<i>C. crescentus</i>		
CB15N	Wild type, synchronizable	Evinger and Agabian (14)
CB15N-R	CB15N, Rif ^r	Spontaneous Rif ^r
AE5000	CB15, wild type	B. Ely
AE5091	AE5000(RP4)	Contreras et al. (6)
AE5168	<i>gps-505</i>	NTG of AE5091
AE5512	<i>gpsA505</i>	Hodgson et al. (16)
SC1090	<i>purA::Tn5 str</i>	B. Ely
AE9200	<i>gpsA505 purA::Tn5</i>	AE5512 × SC1090T
AE9201	<i>gpsA505 purA::Tn5(pVS1)</i>	AE7000 × NC9412C
AE9202	<i>gpsA505 purA::Tn5, Rif^r</i>	AE8001 × CB15N ^{rifC}
AE9203	<i>gpsA505</i> , synchronizable	AE5512 × AE9202T
<i>E. coli</i>		
NC9412 (C600)	<i>hsdR hsdM supE44 thr leuB6 lacY tonA21 thi-1(pVS1)</i>	Barrett et al. (3)
NC9298 (AEE101)	<i>pro met</i> RP4	

^a T, Transduction with ϕ Cr30; C, conjugation (RP4 mediated); NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

background of CB15N (14). This was accomplished via ϕ Cr30-mediated general transduction (13) and by RP4-mediated conjugation (2, 3). We took advantage of the genetic linkage between *purA* and *gpsA* to obtain a selectable marker for the *gpsA* gene. A Tn5-marked *purA* gene (2) was transduced from strain SC1090 into strain AE5512. The resulting transductant, strain AE9200, was auxotrophic for adenosine and glycerol 3-phosphate and resistant to kanamycin. The plasmid pVS1 (a kanamycin-sensitive derivative of RP4) was then moved from *Escherichia coli* NC9412 into strain AE9200 (*gpsA505 purA::Tn5*) by conjugation. The resulting strain, AE9201, was mated with a spontaneous rifampin-resistant mutant of CB15N, and the recombinants were selected on PYE plates containing kanamycin and rifampin. The Km^r Rif^r progeny were screened for glycerol phosphate and adenosine auxotrophy and for the ability to be synchronized. Strain AE9202 exhibited all the correct phenotypes. However, the strain had a long generation time. Thus, a wild-type *purA*⁺ locus was transduced from strain AE5512 into strain AE9202 (*gpsA505 purA::Tn5*, CB15N), selecting for restoration of adenosine prototrophy. One of the resulting transductants, strain AE9203 (*gpsA505*, CB15N), exhibited the glycerol phosphate auxotrophy attributed to the *gpsA505* mutation, was synchronizable, and had an appropriate doubling time of ~3 to 3.5 h in minimal medium supplemented with glycerol and glycerol 3-phosphate.

Lipid synthesis. Synchronous swarmer cells were suspended in minimal medium with or without glycerol phosphate and allowed to proceed through the cell cycle. At various times, 2-ml samples were pulse-labeled with 2 μ Ci of [¹⁴C]acetate (1.7 mCi/mmol; New England Nuclear Corp.) for 10 min. A 0.5-ml sample of the labeled culture was precipitated with an equal volume of cold 20% trichloroacetic acid (TCA), and the radioactivity was counted in Betafluor. Lipids were extracted from the remaining 1.5 ml of cells with methanol-chloroform by the method of Bligh and Dyer (5) as modified by Ames (1). Portions of the chloroform extracts were also counted in Betafluor. The lipids were separated by two-dimensional paper chromatography as described by Contreras et al. (7). The region corresponding to phosphatidylglycerol was excised, and the amount of incorporated label was determined in a liquid scintillation counter.

Macromolecular synthesis. The syntheses of DNA and

RNA were analyzed as described by Contreras et al. (6). Cultures were either pulse-labeled for 10 min or continuously labeled with 20 μ Ci of [8-³H]guanosine (4 Ci/mmol; ICN Pharmaceuticals, Inc.) per ml. To measure incorporation of counts into DNA, the labeled samples were hydrolyzed in 5 N NaOH at 60°C and then neutralized with 5 N HCl and precipitated with 20% TCA. To measure the amount of labeled nucleotide incorporated into RNA, equal parts of the labeled samples were directly precipitated with 20% TCA. The counts incorporated into DNA were then subtracted from the total counts incorporated.

Analysis of chromosome replication by pulsed-field gel electrophoresis. Synchronous swarmer cells were diluted to an optical density at 660 nm of ~0.2 to 0.4 in minimal medium and incubated at 30°C. The synchronized cultures were either labeled continuously or pulsed for 5 min at various times in the cell cycle with 5 μ Ci of [8-³H]deoxyguanosine (15 Ci/mmol; ICN) per ml. Labeled DNA was prepared from samples of cell culture as described by Dingwall and Shapiro (10). The DNA embedded in agarose plugs was digested for 7 h at 37°C with 33 U of restriction enzyme *Dra*I (Boehringer Mannheim Biochemicals) or *Ase*I (New England BioLabs, Inc.) and separated on a 1% agarose pulsed-field gel (12). The gels were stained with ethidium bromide, photographed, and later treated with En³Hance (New England Nuclear Corp.), dried, and exposed to X-ray film (Kodak XAR-5).

RESULTS

Morphogenesis and viability of the *gpsA* auxotroph as a function of the cell cycle. When log-phase cultures of a *gpsA* mutant are deprived of glycerol phosphate, cell death commences at approximately 1 generation time (6). To determine if cell cultures become blocked at a specific stage in the cell cycle under these conditions, synchronized swarmer cells were suspended in minimal medium in the presence or absence of glycerol phosphate. As the cells proceeded through the cell cycle, the number of viable cells was measured at the times shown in Fig. 1. The morphologic development of *gpsA* mutant cells was followed microscopically, and the results are shown schematically in Fig. 1. In the presence of supplement, the cells proceeded through the cell cycle in a manner indistinguishable from that of the wild-type strain, with a generation time of approximately 220

FIG. 1. (A) Schematic of *C. crescentus* *gpsA* mutant cell cycle in the presence of glycerol and glycerol 3-phosphate (+GG) and in the absence of supplement (-GG). Wild-type and supplemented *gpsA* mutant cells (+GG) progress identically through the cell cycle. Flagellated swarmer cells eject their flagella and differentiate into stalked cells. Coincident with this morphogenic change, the chromosome initiates replication. The oval, theta, and figure-eight drawings inside each cell represent the *C. crescentus* chromosome during progressive phases of DNA replication. Wild-type and supplemented *gpsA* mutant cells (+GG) complete DNA replication and asymmetric cell division. The generation time is 220 min. In contrast, the unsupplemented *gpsA* mutant cells (-GG) begin the developmental cell cycle, but they arrest at a distinct mid-division stalked-cell morphology. (B) Effect of glycerol starvation on viability. Synchronized swarmer cells were allowed to proceed through the cell cycle in the presence (□) or absence (♦) of glycerol and glycerol 3-phosphate. At the times indicated, viable cells were measured as CFU per milliliter in the presence of glycerol and glycerol 3-phosphate. In the presence of supplement, the cells divided at 220 min (arrow). In the absence of supplement, the cells blocked at the late stalked-cell stage shown in panel A.

min. The stalked cells elongated, developed into predivisional cells, gained motility, and completed division. However, in the absence of glycerol phosphate, the viable count began to decrease between 120 and 150 min (between 0.6 and

FIG. 2. Effect of glycerol starvation on lipid synthesis in *gpsA* mutant cultures. Synchronous swarmer cells of the *gpsA* mutant strain AE9203 were suspended in minimal medium with (□) or without (♦) glycerol and glycerol 3-phosphate supplement. At the indicated times in the cell cycle, samples were pulse-labeled with [14 C]acetate (1 μ Ci/ml). Lipids were extracted from the cells as described in Materials and Methods. Portions of the extracts were counted in Betafluor. (A) Incorporation of label into total extractable lipid. The lipids in the remaining extract were separated by two-dimensional paper chromatography as described in Materials and Methods. The region corresponding to a phosphatidylglycerol standard was cut out, and the incorporated label was counted in Betafluor. (B) Incorporation of label into phosphatidylglycerol as a function of the cell cycle.

0.7 cell division units) and the cells arrested at the late stalked-cell stage.

Lipid synthesis in the *gpsA* mutant. The effect of the *gpsA* lesion on lipid synthesis was analyzed as a function of the cell cycle. Synchronized swarmer cells were suspended in medium in the presence or absence of glycerol phosphate. At the cell cycle times shown in Fig. 2, samples were pulse-labeled with [14 C]acetate and total extractable lipids were prepared as described in Materials and Methods. Acetate was incorporated into the fatty-acid chains attached to the glycerol 3-phosphate backbone of phosphatidic acid to form the phospholipids, as well as into sphingolipids, glycolipids, and neutral lipids. The total extractable lipids were separated by two-dimensional paper chromatography, allowing the measurement of both total lipid synthesis (Fig. 2A) and phosphatidylglycerol synthesis (Fig. 2B). The major phospholipids in *C. crescentus* are phosphatidylglycerol and cardiolipin, and this organism lacks phosphatidylethanolamine and phosphatidylserine (7).

The incorporation of label into total extractable lipid in the

FIG. 3. Effect of glycerol 3-phosphate starvation on DNA synthesis in *gpsA* mutant cultures. Synchronous populations of swarmer cells were suspended in minimal medium with (□) or without (◆) glycerol and glycerol 3-phosphate supplement (GG). DNA synthesis was examined by pulse-labeling samples with [^3H]guanosine (20 $\mu\text{Ci}/\text{mmol}$) at the indicated times throughout the cell cycle. To measure incorporation of label into DNA, the labeled samples were treated with alkali and precipitated with TCA as described in Materials and Methods. A schematic of the cell cycle is shown above the graph.

absence of glycerol phosphate was significantly lower than in the presence of glycerol phosphate (Fig. 2A). Examination of the incorporation of label into a specific phospholipid demonstrated a more dramatic difference between supplemented and starved cultures (Fig. 2B). In the absence of supplement, very little, if any, label was incorporated into phosphatidylglycerol. Control experiments showed that starved *gpsA* cells incorporated [^{14}C]acetate into total TCA-precipitable material at only a slightly lower level than that in the supplemented culture (data not shown), suggesting that the uptake and metabolism of acetate were not affected in the *gpsA* mutant. Rather, as would be expected, starvation of the *gpsA* mutant for glycerol phosphate specifically affected the incorporation of [^{14}C]acetate into phospholipid.

DNA replication during the cell cycle of the *gpsA* mutant. To gain insight into the relationship between the inhibition of phospholipid synthesis and the pattern of cell cycle arrest and cell death, we studied the replication of the chromosome as a function of the cell cycle. The rate of DNA replication was assayed in two ways: (i) by the incorporation of pulse-labeled precursor into acid-precipitable counts and (ii) by pulse-labeling or continuous labeling of DNA followed by resolution of newly replicated large restriction fragments by pulsed-field gel electrophoresis.

Synchronized swarmer cells were suspended in minimal medium with or without glycerol phosphate, and samples were pulse-labeled with [^3H]guanosine (Fig. 3). In the presence of supplement, DNA synthesis occurred in the periodic fashion that has been reported for the cell cycle of wild-type *Caulobacter* cells and is described in the legend to Fig. 1 (9, 21a). DNA replication was initiated during the transition

FIG. 4. Chromosome replication in AE9203 in the absence or presence of supplement. Synchronized swarmer cells were allowed to proceed through the cell cycle in the presence of [^3H]deoxyguanosine and in the presence (+, lanes 1 through 4) or absence (–, lanes 1 through 5) of glycerol and glycerol 3-phosphate supplement. Samples of cells were removed at 40, 65, 90, and 110 min (lanes 1 through 4) and at 30, 40, 65, 90, and 110 min (lanes 5 through 9). They were then embedded in agarose plugs, and the DNA was digested in situ with endonuclease *DraI*. The restriction fragments were resolved by pulsed-field gel electrophoresis using 25-s pulses, as described in Materials and Methods, and subjected to autoradiography. The sizes of the various labeled restriction fragments are indicated at the left of the autoradiograph (in kilobases).

from swarmer cell to stalked cell between 30 and 40 min into the cell cycle, as has been observed in wild-type cultures. There was a short postsynthetic period followed by a reinitiation of DNA replication in the progeny stalked cells. Although DNA synthesis initiated at approximately the same time and at approximately the same rate in the unsupplemented and supplemented cultures, the rate of DNA synthesis in the unsupplemented cultures decreased significantly following the initiation of replication (Fig. 3). These starved cultures were unable to reinitiate DNA replication, and the cells failed to divide. This is in contrast to the supplemented culture, in which the progeny stalked cells immediately reinitiated a new round of DNA replication and the progeny swarmer cells entered G_1 phase. As additional metabolic controls, RNA and protein syntheses were measured under both conditions (data not shown). The rates of RNA and protein syntheses in the starved cultures decreased gradually compared with rates in the supplemented cultures. Even after 10 h of glycerol starvation, the RNA and protein biosynthetic rates still remained approximately 50% of their nonstarved levels, while the DNA synthetic rate dropped to background level.

To directly assay the effect of glycerol phosphate starvation on chromosome replication, we took advantage of the fact that physical maps of the *C. crescentus* chromosome have been constructed from very large restriction fragments generated by *DraI* (12) or *AseI* digestion (11). Analysis of restriction digests separated by pulsed-field gel electrophoresis has been used to determine the origin, rate, and direction of DNA replication in synchronized wild-type cultures of *C. crescentus* (10). The *gpsA* mutant strain AE9203 was synchronized and allowed to proceed through one cell cycle in the presence of [^3H]deoxyguanosine and in the presence or absence of glycerol phosphate. Samples of cells were removed and embedded in agarose plugs at the times indicated in Fig. 4. The DNA was digested in situ with

endonuclease *DraI*, and restriction fragments were separated by pulsed-field gel electrophoresis (Fig. 4). In the presence of glycerol phosphate, the time of replication initiation and the order of labeling of the large restriction fragments were the same as those described for the wild-type *C. crescentus* chromosome (10). In wild-type cells, the first two restriction fragments to be labeled were the 305- and 105-kilobase (kb) bands. The position of the origin of replication has recently been localized to a specific region of the 305-kb fragment (G. T. Marczyński, unpublished data). In both supplemented and starved cultures of AE9203, the pattern of labeled restriction fragments at 40 min was the same (Fig. 4). The 305- and 105-kb DNA fragments were the first labeled, suggesting that the starved mutant cells initiated chromosomal replication at the correct location and at the correct time in the cell cycle. The early incorporation of label into both the 105- and 305-kb restriction fragments showed that in both cases replication initiated in a bidirectional manner (10). However, at subsequent times there was a marked decrease in label incorporated into the various labeled restriction fragments in the unsupplemented culture (Fig. 4). These results suggest that a large fraction of the initiated chromosome was unable to complete a round of replication. The results of this experiment support the results shown in Fig. 3; i.e., the initial rate of DNA replication was the same in supplemented and unsupplemented cultures. For one experiment (Fig. 3), the rate of replication was determined by pulse-labeling cultures with [3 H]guanosine at several times in the cell cycle. However, in another experiment (Fig. 4), the cultures were assayed in the continuous presence of [3 H]deoxyguanosine and there was a larger decrease in incorporation of label into DNA later in the cell cycle than was observed in the pulse-label experiment shown in Fig. 3. It is difficult to make quantitative conclusions about the rates of DNA synthesis in the continuously labeled cultures at late times in the cell cycle because [3 H]deoxyguanosine is significantly depleted with time because of its incorporation into RNA (9). The experiment whose results are shown in Fig. 5 analyzed pulse-labeled cultures and allowed a direct assay of DNA replication using the pulsed-field gel electrophoresis technique at the most critical times in the cell cycle.

Synchronized swarmer cells were incubated in the presence or absence of glycerol phosphate, and at 30, 160, and 600 min, cell samples were pulse-labeled with [3 H]deoxyguanosine (Fig. 5). The cells were then embedded in agarose, the chromosomal DNA was digested with *AseI*, and the resulting DNA fragments were separated by pulsed-field gel electrophoresis. The restriction enzyme *AseI* produces an early-replicating 250-kb DNA fragment that is easily resolved from the rest of the chromosome and therefore serves as a diagnostic band for early-replicating DNA. The ethidium bromide-stained gel is presented above its fluorogram in Fig. 5. The arrow points to the 250-kb *AseI* fragment. This band is preferentially labeled at 30 min (lane 1) in both cultures. In fact, the patterns of chromosome labeling in these cultures are almost identical, indicating that glycerol starvation does not perturb the transition from swarmer cell to stalked cell and subsequent initiation of DNA synthesis. However, the two cultures presented very different patterns of DNA labeling at 160 min (lane 2). The restriction fragments were labeled at a significantly lower intensity in the unsupplemented culture. This result supports the conclusion drawn from continuously labeled synchronized cultures (Fig. 4) that the initiation of chromosome replication occurs in the absence of supplement. The decrease in the replication

FIG. 5. Initiation of DNA replication in AE9203. Synchronized swarmer cells were incubated in the presence (+) or absence (-) of glycerol and glycerol 3-phosphate. Samples were taken at 30, 160, and 600 min (lanes 1, 2, and 3, respectively) and pulse-labeled with [3 H]deoxyguanosine for 10 min. The generation time of this culture was 220 min. DNA preparations were cut with *AseI* and resolved by the orthogonal pulsed-field gel technique as described in Materials and Methods, using 25-s pulses. The gel stained with ethidium bromide (EB) is presented above its fluorograph. The arrow points to an early-replicating 250-kb band that is easily separated from other late-replicating bands. Lane 4, Unlabeled CB15N DNA prepared from stationary-phase cells and digested with *AseI*; lane 5, 60-kb plasmid RP4, digested with *AseI* as a size marker.

rate later in the cell cycle agrees with the rate of incorporation of pulse-labeled precursor shown in Fig. 3. These experiments clearly show that a significant fraction of initiated chromosomes were unable to complete replication and that reinitiation failed to occur late in the cell cycle. Finally, we observed that the glycerol-starved cells labeled at 10 h (lane 3) showed only a trace of [3 H]deoxyguanosine incorporation into chromosomal DNA compared with incorporation in the 10-h control, which showed a uniform (i.e., asynchronous) labeling pattern. After 10 h of starvation for glycerol phosphate (when most of the cells have lost viability), the ethidium bromide-stained *AseI*-digested DNA bands remained sharp and intact. Therefore, major chromosomal degradation could not account for this dramatic cell death.

Effect of novobiocin on viability of the *gpsA* mutant. A possible cause of cell death in unsupplemented cultures of the *gpsA* mutant might be the inability to complete a round of DNA replication. In an attempt to rescue the cell death phenotype, we prevented the initiation of DNA replication with novobiocin early in the cell cycle of starved AE9203 cultures. Novobiocin specifically inhibits DNA gyrase activ-

FIG. 6. (A) Effect of novobiocin on DNA replication in the *gpsA* mutant strain AE9203. A synchronized swarmer cell population was suspended in minimal medium under four different conditions: presence of glycerol and glycerol 3-phosphate (GG; $\square$), absence of glycerol and glycerol 3-phosphate ($\diamond$), presence of novobiocin (Nov) and absence of glycerol and glycerol 3-phosphate ($\bullet$), and presence of glycerol, glycerol 3-phosphate, and novobiocin ($\circ$). The effect on DNA synthesis was examined by continuously labeling each culture with [³H]deoxyguanosine. At various times in the cell cycle, incorporation of label into DNA was measured as described in the legend to Fig. 3. (B) Effect of novobiocin on the viability of glycerol-starved *gpsA* mutant cells. Synchronized swarmer cells were suspended in minimal medium ($\diamond$), minimal medium to which novobiocin was added ($\bullet$), or minimal medium supplemented with glycerol and glycerol 3-phosphate ($\square$). Viable cells per milliliter were measured at the indicated times in the absence of novobiocin.

ity in *E. coli*, thereby inhibiting initiation of DNA replication (15). The addition of novobiocin to a synchronized swarmer cell population of wild-type *Caulobacter* cultures completely inhibited DNA synthesis, although RNA and protein syntheses continued (K. Amemiya, unpublished data). The effect of novobiocin on DNA replication in synchronized cultures of the *gpsA* mutant strain AE9203 is shown in Fig. 6A.

The ability of unsupplemented cultures to initiate but not maintain normal levels of DNA synthesis in continuously labeled cells (Fig. 6A) confirms the results obtained with pulse-labeled cultures shown in Fig. 3. As expected, the initiation of DNA synthesis in both the supplemented and unsupplemented cultures was completely inhibited by the presence of novobiocin (Fig. 6A). To determine if novobiocin could rescue *gpsA* mutant cells from "glycerol-less" death, the number of viable cells was measured at the indicated times as the cells proceeded through the cell cycle in the presence of novobiocin (Fig. 6B). The *gpsA* mutant maintained viability in the presence of glycerol phosphate,

but in its absence, the viable cell count began to decrease at 120 to 150 min (as was also shown in Fig. 1). However, the viable cell count remained constant throughout the cell cycle when cells were starved for glycerol in the presence of novobiocin (Fig. 6B). Therefore, the inhibition of the initiation of DNA replication at the beginning of the cell cycle rescued the starved *gpsA* mutant from cell death. This result argues that DNA replication that is initiated, but not completed, contributes to the glycerol-less death observed in the *gpsA* mutant strain.

DISCUSSION

In an effort to define the mechanisms that contribute to the cell cycle control of DNA replication in *C. crescentus*, we have analyzed mutants with known biochemical defects. Among these are mutants with defects in phospholipid metabolism. Phospholipid biosynthesis in *C. crescentus* was reported by O'Neill and Bender (24) to occur at two defined

periods in the cell cycle. The first period occurs when DNA replication is about two-thirds complete and continues until the end of DNA replication. The second period occurs in the progeny swarmer cell and continues until the initiation of DNA replication during the transition from swarmer cell to stalked cell. It has also been demonstrated that the pattern of phospholipid synthesis differs in the progeny swarmer and stalked cells (21). Phospholipid precursors that are not detected in the stalked cell are detected in the swarmer cell. Therefore, both phospholipid biosynthesis and chromosome replication are tightly controlled cell cycle events. In addition, we found that mutants in phospholipid metabolism affect the replication of the chromosome (6, 17, 27).

One of these mutants, AE9203 (*gpsA*), is deficient in *sn*-glycerol 3-phosphate dehydrogenase (6). The *gpsA* gene has been mapped and the multiple phenotypes have been shown to be due to a single genetic lesion (16). In the absence of glycerol phosphate, there is a block in both phospholipid and DNA syntheses. A unique feature of this lipid mutant is that attempted growth in the absence of supplemented leads to cell death (6). In this report we have examined the phenotype of the *gpsA* mutant in synchronized cells in an effort to understand the interaction between phospholipid biosynthesis and the replication of the chromosome. The first effect of withholding supplement from synchronized swarmer cells was the cessation of synthesis of phosphatidylglycerol, the major phospholipid component of the *C. crescentus* membrane (7). In the absence of phospholipid synthesis, DNA replication was initiated, but the rate of replication soon dropped significantly and reinitiation failed to occur later in the cell cycle. The evidence that supports this conclusion includes the following. (i) The incorporation of [³H]guanosine into DNA in mutant cultures in the absence of supplement, using either continuous or pulse-labeling, started at the same time in the cell cycle as in supplemented mutant cultures and wild-type cultures. (ii) The initial rate of DNA replication was the same in pulse-labeled cultures of synchronized cells grown in the presence or absence of supplement (Fig. 3). The rate of incorporation then declined in the unsupplemented cultures and failed to increase later in the cell cycle when supplemented cultures reinitiated DNA replication. (iii) A direct physical assay of DNA replication, in which large restriction fragments were labeled in the order of their replication and separated by pulsed-field gel electrophoresis, revealed that the first labeled fragments were the same in the absence or presence of supplement. Physical maps of the *C. crescentus* chromosome have been generated with two different restriction enzymes, *Dra*I and *Ase*I (11, 12). Chromosomal DNAs from cultures labeled continuously or in short pulses were digested with either restriction enzyme, and the results showed that the initiation of DNA replication in the absence of phospholipid synthesis started at the correct time in the cell cycle and at the single origin of replication. The level of incorporation of label into the restriction fragment that contained the origin of replication was the same in both supplemented and unsupplemented cultures (Fig. 4 and 5). Only a small percentage of the initiated chromosomes completed replication (Fig. 5), and it appears that a large percentage of the initiated chromosomes stop replication at random sites. This is shown diagrammatically in Fig. 1. Although cell death began near the end of the first cell cycle, there was no apparent degradation of chromosomal DNA. (iv) We previously demonstrated that plasmid pVS1 is able to initiate DNA replication throughout the cell cycle, including at times when the chromosome is normally unable to replicate (21a). We found that in the *gpsA*

mutant, pVS1 was able to initiate replication in the swarmer cell in the absence of supplement; however, by the middle of the cell cycle, pVS1 was no longer able to replicate.

The glycerol-less death exhibited by the *gpsA* mutant appears to be related to the inability of the chromosome to complete replication. This conclusion is based on two observations. First, the cell death phenotype was rescued by using novobiocin to prevent the initiation of replication early in the cell cycle. We found that if novobiocin was added to unsupplemented cultures of *gpsA* swarmer cells, they did not lose viability. Second, cell death was prevented when the *gpsA* mutant allele was placed in a *fatB* genetic background. In the absence of an oleic acid supplement, *fatB* mutants are unable to initiate DNA replication and retain viability (19, 27). Analysis of a *fatB gpsA* double mutant showed that *fatB* was epistatic to *gpsA* because oleic acid starvation prior to glycerol phosphate starvation prevented cell death (17). One interpretation of this result is that the *fatB* lesion rescues the cultures from glycerol-less death by preventing the initiation of replication.

The interaction between chromosome replication and membrane biogenesis in *E. coli* has been investigated by Pierucci and Rickert (25) and Joseleau-Petit et al. (18). In those studies the glycerol auxotroph BB26-36 (4) was used to analyze the relationships among phospholipid synthesis, DNA replication, and cell division. The auxotrophy of this mutant is due to a defect in glycerol-3-phosphate acyltransferase, which exhibits an apparent K_m for glycerol phosphate that is approximately 14-fold higher than that in the wild type. Glycerol starvation of this mutant resulted in an immediate cessation of phospholipid synthesis. Both groups observed that the inhibition of phospholipid synthesis prevented the initiation of new rounds of replication and cell division. However, if initiation was allowed to occur, the loss of phospholipid synthesis had no effect on the completion of ongoing rounds of replication and cell division. Like the *C. crescentus fatB* mutant, but in contrast to the *C. crescentus gpsA* mutant, the absence of supplement did not cause cell death of this *E. coli* mutant. Thus, it appears that cell death results if replication is initiated but not completed.

Chromosomal replication does not necessarily require phospholipid synthesis to be continuously ongoing throughout the replication cycle. There may be critical points in the cell cycle at which active phospholipid synthesis is required in order to confer upon the cell the abilities to replicate DNA and to properly segregate the replicating chromosome. It is now important to define this critical execution point for the requirement of phospholipid synthesis to complete chromosomal replication.

How might an alteration in phospholipid metabolism prevent the completion of DNA replication? One possible explanation is that an inhibitor of replication accumulates or that the starved cells deplete an activated form of a replication protein(s), thus interfering with the elongation reaction. Another possibility is based on the hypothesis that replicating chromosomes or plasmids are attached to membrane and that membrane growth plays a role in chromosome separation (23). In that case, a block in the synthesis of phosphatidylglycerol, a major component of the *C. crescentus* membrane, would prevent membrane growth.

The interaction between membrane lipid synthesis and DNA replication is obviously a complex one that occurs at several levels. The specific link identified by the *gpsA* lesion is unique in that the *gpsA* gene is essential for viability. To gain a better understanding of this intricate regulation, we are attempting to isolate mutants that partially correct the

pleiotropic defect in an effort to select for second-site suppressors that uncouple this coordinate regulation.

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