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Cloning and Cell Cycle-Dependent Expression of DNA Replication Gene *dnaC* from *Caulobacter crescentus*

NORIKO OHTA, MALTHI MASUREKAR,† AND AUSTIN NEWTON*

Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544-1014

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Chromosome replication in the asymmetrically dividing bacteria *Caulobacter crescentus* is discontinuous with the new, motile swarmer cell undergoing an obligatory presynthetic gap period (G1 period) of 60 min before the initiation of DNA synthesis and stalk formation. To examine the regulation of the cell division cycle at the molecular level, we have cloned the DNA chain elongation gene *dnaC* from a genomic DNA library constructed in cosmid vector pLAFR1-7. To ensure that the cloned sequence corresponded to *dnaC*, we isolated the gene by genetic complementation of the temperature-sensitive allele *dnaC303* on a DNA fragment that contained a Tn*5* insertion element tightly linked by transduction to *dnaC*. The size of the *dnaC* gene was estimated to be 1,500 bp or less based on the pattern of complementation by subcloned restriction and BAL 31 deletion fragments. Nuclease S1 assays were used to map the transcription start site and to determine the pattern of *dnaC* expression in the cell cycle. Large amounts of the *dnaC* transcript began to accumulate only in the late G1 period of the swarmer cell and then peaked early during chromosome replication. We confirmed that the gene is periodically transcribed by monitoring the rate of β-galactosidase synthesis directed by a *dnaC* promoter-*lacZ* fusion in a synchronous cell culture. *dnaC* is the first *C. crescentus* cell cycle gene whose regulation has been reported, and the discontinuous pattern of its expression suggests that the DNA synthetic period in these dimorphic bacteria is regulated in part by the stage-specific expression of DNA replication genes.

Caulobacter crescentus is an asymmetrically dividing bacterium which produces two different cell types, a motile swarmer cell and a nonmotile stalked cell, at division. The two cells inherit different developmental programs, and this difference is reflected in the patterns of their DNA synthesis (4). DNA synthesis is initiated in the stalked cell immediately after cell division and continues for 90 min (S period) in minimal medium; this DNA synthetic period is followed by a 30-min postsynthetic gap (G2 period). DNA synthesis in the swarmer cell, on the other hand, is delayed for ca. 60 min. During this presynthetic gap (G1 period) the cell loses its flagellum, forms a stalk, and initiates chromosome replication. The new stalked cell then undergoes the same S-G2 periods described above for the sibling stalked cell (4).

Conditional mutants defective in DNA replication have been identified among temperature-sensitive cell division cycle (*cdc*) mutants of *C. crescentus* that grow and divide normally at 30°C and form filamentous cells at 37°C (nonpermissive temperature [29]). Mutations were classified originally as being defective in either DNA synthesis (*dna*) or cell division (*div*). Strains with mutations in the DNA chain initiation gene *dnaA* and DNA chain elongation genes *dnaB*, *dnaC*, and *dnaD* were all defective in DNA synthesis at 37°C. Strains with mutations in cell division were shown to undergo DNA synthesis normally at the nonpermissive temperature (30). This set of conditional mutations was used in reciprocal shift experiments to demonstrate that the cell cycle steps are organized into two dependent pathways, a DNA synthetic pathway and a cell division pathway (21, 30).

Chromosome replication is a central event in the *C. crescentus* cell cycle. It controls steps required for both initiation and completion of the cell division pathway (30), as well as the periodic expression of flagellar genes in the cell cycle (33) and formation of the polar DNA bacteriophage receptors (12). Therefore, the timing of cell cycle and developmental events at one level appears to require the activity of DNA synthetic genes. To begin to address the question of how the activity of *cdc* genes is controlled in these developing bacteria, we have cloned a DNA chain elongation gene from *C. crescentus* and examined its regulation.

We describe here the use of genetic complementation to identify *dnaC* in a genomic library of *C. crescentus* DNA constructed in cosmid vector pLAFR1-7 (J. Kitajewsky and A. Newton, unpublished data). The location of the transcription start site was mapped, and the accumulation of *dnaC* mRNA in synchronous cell cultures was measured by using nuclease S1 assays. The results of these and similar experiments in which promoter activity was measured in a *dnaCp-lacZ* fusion showed that *dnaC* is transiently expressed at the end of the G1 period in swarmer cells and at the beginning of the S period. The discontinuous expression of *dnaC* suggests that the DNA synthetic cycle in *C. crescentus* is regulated in part by the stage-specific expression of genes required for DNA replication.

MATERIALS AND METHODS

Media and cell growth. Cells were grown routinely in M2 medium or PYE medium supplemented when necessary with kanamycin (150 μg/ml in M2 and 50 μg/ml in PYE) or tetracycline (2 μg/ml) (27). *Escherichia coli* strains were grown in ML medium (27).

Isolation of linked Tn*5* insertions. The preparation and use of pooled or "orgy" bacteriophage lysates in *C. crescentus* was adapted from procedures described for use in *Salmonella typhimurium* (15). A collection of approximately 2,000 independently isolated kanamycin resistant (Km^r) colonies were obtained by mutagenesis of *C. crescentus* CB15 with transposon Tn*5*, collected in PYE medium, and used to

* Corresponding author.
† Present address: Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry, Newark, NJ 07103.

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Source or reference
C. crescentus		
CB15	Wild type	ATCC 19089
PC1040	*divC307*	29
PC2076	*dnaA301*	29
PC2153	*dnaD304*	29
PC2179	*dnaC303*	29
SC1403	*aroF rif-163*	B. Ely
PC7236	*dnaC303 rif-163*	φ(PC2179) × SC1403
PC6010	*dnaC*$^+$ *zja-306*::Tn*5*	orgy φ(Tn*5*) × PC7236
PC6035	*dnaC*$^+$ *zja-306*::Tn*5*	φ(PC6010) × PC7236
CM5256	*rec-526*	28
PC7068	*rec-526* Sm^r	This study
PC7070	*rec-526* Sm^r *zbe*::Tn*5*	orgy φ(Tn*5*) × PC7068
PC7159	*dnaC303 rec*$^+$ *rif-163*	φ(PC7236) × SC1403
PC7162	*dnaC303 rec-526 zbe*::Tn*5*	φ(PC7070) × PC7159
E. coli		
HB101		2
JM107		37

prepare a pooled or orgy φCr30 (13) lysate [orgy φ(Tn*5*)]. Since insertion of the Tn*5* transposon is random in the *C. crescentus* genome to a first approximation (7), we assume that DNA fragments with Tn*5* insertions representing most of the genome are present in the lysate. The orgy lysate was then used to transduce temperature-sensitive *dnaC303* strain PC7236 (Table 1), and Km^r recombinants were selected on PYE agar supplemented with kanamycin and screened for growth at the nonpermissive temperature (37°C) by replica printing. Linkage of the Tn*5* insertions to *dnaC* was confirmed by backcrossing the Km^r marker into the original temperature-sensitive strain. Transposon *zja-306*::Tn*5* was shown to be approximately 90% linked to *dnaC*, and the *dnaC*$^+$-*zja-306*::Tn*5* strain, PC6035, isolated in this cross was used for cloning *dnaC*$^+$. Relevant genotypes of other strains used in these studies are listed in Table 1.

O'Neill et al. (28) have described a recombination-deficient allele of *C. crescentus rec-526* that confers a sensitivity to UV light and deficiency in Weigle reactivation similar to that of *recA* alleles in *Escherichia coli*. We isolated the Tn*5* insertion *zbe*::Tn*5*, which is 75% linked to *rec-526*, from orgy φ(Tn*5*) as described above. Lysates prepared on strain PC7070 (Table 1) were used to transfer the *rec* mutation to strain PC7159 carrying the *dnaC303* allele.

Cloning vectors and construction of a cosmid library. Vectors used for the initial cloning and complementation studies were all derivatives of pRK290 (5). Cosmid vector pLAFR1-7 was constructed (Kitajewsky and Newton, unpublished) by inserting the polylinker from pUC7 (36) into the *Eco*RI site of pLAFR1 (9). This vector can accommodate approximately 20 kb of insert DNA when packaged in vitro and, like its parent plasmid, pRK290, can be transferred to and replicates stably in *C. crescentus* cells (27). Plasmid pRK2L1 was constructed by cloning the polylinker from pUC18 at the *Eco*RI site of pRK290 (20).

A genomic library was constructed in cosmid vector pLAFR1-7 by partially digesting DNA from strain PC6035 with serially diluted *Mbo*I to give a maximum yield of 20- to 40-kb DNA fragments, which were then ligated to the *Bam*HI site of the vector and packaged in vitro (11). Cosmid clones were transfected in *E. coli* HB101 (2) and screened as described in Results.

Subcloning of BAL 31 deletion fragments and complementation tests. The 3.3-kb *Bgl*II-*Bam*HI fragment (see Fig. 4) containing the *dnaC*$^+$ gene subcloned in pUC18 (37) was recloned as the *Bam*HI-*Hin*dIII fragment in M13tg130 (Amersham Corp.) for BAL 31 digestion. The hybrid phage DNA was linearized at the *Bam*HI site and digested with BAL 31 to various extents. The deleted *Caulobacter* DNA fragments were then cloned into pRK2L1 for complementation analysis (see Results). The temperature-sensitive phenotype of *dnaC303* is characterized by the failure of cells to divide and the formation of long filamentous cells at 37°C. The *dnaC303* mutants are also unable to form colonies when replica printed at 37°C, and the ability of the *dnaC* subclones to complement the *dnaC* defect was judged by their ability to restore normal cell division and growth in this test. Cells were also examined for morphology by light microscopy.

Construction of a transcriptional fusion. The 480-bp *Sma*I(a)-*Sma*I(b) fragment (fragment b in Fig. 2C), which contains the *dnaC* promoter, was first cloned into pUC18 to place the *Kpn*I site at the 5′ end and the *Xba*I site at the 3′ end of the fragment. The resulting *Kpn*I-*Xba*I fragment was then cloned in promoter fusion vector pANZ5 (N. Ohta, L.-S. Chen, D. A. Mullin, and A. Newton, submitted for publication) to obtain plasmid pANZ421 with *dnaCp-lacZ* fusion.

Nuclease S1 assay. RNA isolation and nuclease S1 assays were performed as described previously (26). Hybridizations were performed at 60°C. Partially protected fragments were visualized by electrophoresis on a 5% polyacrylamide–8 M urea gel and exposure to X-ray film.

Radioimmunoassay. Synchronous culture of swarmer cells grown in M2 medium was pulse-labeled with [^{35}S]methionine (15 μCi/ml) for 12 min. Labeled cells were harvested, lysed, and incubated with Omnisorb cells (Calbiochem) to remove material which binds nonspecifically. After centrifugation,

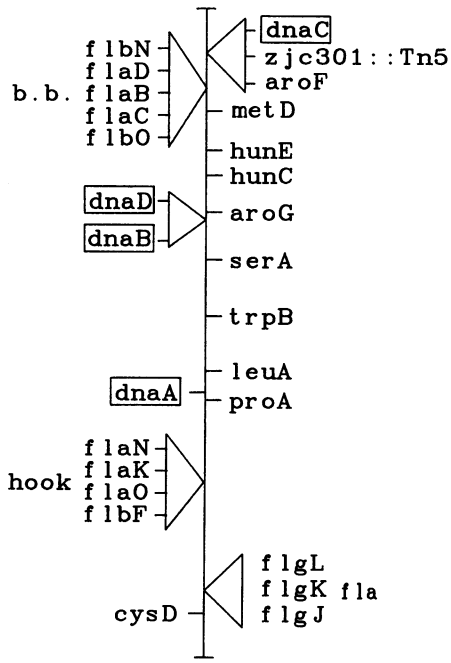

FIG. 1. Location of *dna* genes on the genetic map of *C. crescentus*. DNA replication genes are boxed. Major flagellar gene clusters, the basal body gene cluster (b.b.), the hook gene cluster (hook), and the α-flagellin gene cluster (fla) are shown in addition to auxotrophic markers linked to *dna* genes. Not all *fla* genes in each of the clusters is indicated.

each supernatant fraction was divided into two parts. One part was incubated for 3 h with monoclonal anti-β-galactosidase antibody (Promega Corp.), and the second part was incubated for 3 h with polyclonal immunoglobulin G raised against the 70-kDa hook protein (33). Omnisorb cells were then added to each sample to precipitate the specific antigens. The 27- and 25-kDa flagellins were also detected in the assay with anti-hook protein immunoglobulin G, because components of flagellar filament associated with the hook structure are immunoprecipitated by the antibody. The procedures used to visualize labeled proteins by autoradiography have been described previously (33).

TABLE 2. Complementation of cell division cycle mutants

Strain	Genotype	Phenotype[a]	Plasmid[b]			
			pMA2	pMA3	pMA4	pLAFR1-7
CB15	Wild type		+	+	+	+
PC7159	*dnaC*	DNAe	+	+	−	−
PC2153	*dnaD*	DNAe	−	−	−	−
PC2076	*dnaA*	DNAi	−	−	−	−
PC1040	*divC*	CS	−	−	−	−

[a] DNAi, DNA chain initiation; DNAe, DNA chain elongation; CS, cell separation.
[b] + indicates growth and − indicates failure to divide at 37°C.

RESULTS

Selection and mapping of Tn*5* insertion linked to *dnaC*. Temperature-sensitive mutations were identified in the DNA chain initiation gene *dnaA* and DNA chain elongation genes *dnaB*, *dnaC*, and *dnaD* (29, 30), and these genes were subsequently mapped on the *C. crescentus* chromosome by conjugation and transduction (Fig. 1) (A. Newton, L. Kulick, and M. Bucuk, unpublished data). *dnaC*, which is linked by transduction to *aroF* (Fig. 1), was selected for cloning because the *dnaC303* allele shows a particularly tight phenotype at 37°C and thus provides a good genetic background for the identification of complementing clones. To provide a selectable marker that could be used to enrich for the region of the chromosome containing this *dna* gene, Tn*5* transposon *zja-306*::Tn*5*, which is 90% linked to *dnaC*, was isolated from an orgy φCr30 lysate (see Materials and Methods). Previous studies with genes in the hook cluster, where correlation between genetic and physical distances are known (27), have indicated that genes with 90% linkage by transduction are separated by 10 kb or less (F. Chang, N. Ohta, and A. Newton, unpublished data).

Isolation and identification of *dnaC*. The strategy for cloning *dnaC* was to isolate hybrid cosmid clones that carried the Km^r marker of *zja-306*::Tn*5* from *C. crescentus* PC6035 (Table 1) and to screen the Km^r clones for the closely linked *dnaC*$^+$ gene by their ability to complement the temperature-sensitive *dnaC303* allele at the restrictive temperature. Km^r Tc^r (cosmid marker) transformants were selected from the genomic DNA library prepared from strain PC6035 in pLAFR1-7 (see Materials and Methods), and 17 of the

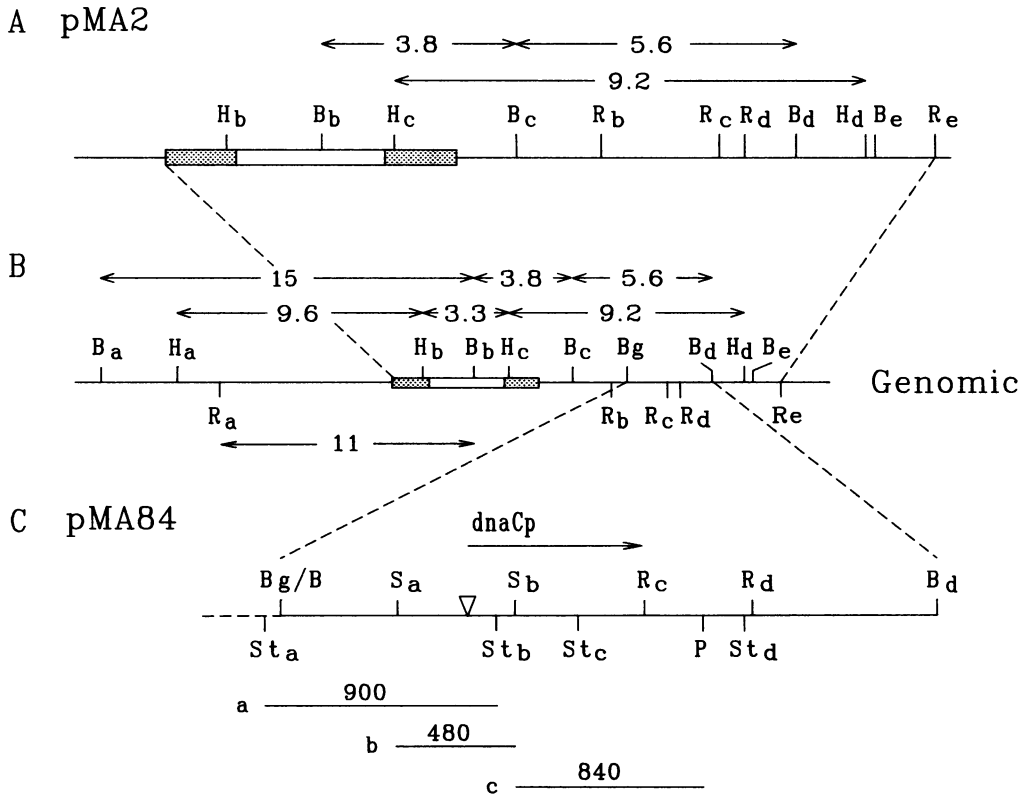

FIG. 2. Restriction maps of the *dnaC* clone and genomic sequence. (A) Partial restriction map of the cosmid clone pMA2. (B) Genomic restriction map of PC6035 deduced from Southern analysis with Tn*5* and the 9.2-kb *Hin*dIII(c)-*Hin*dIII(d) fragment as probes (Fig. 3). (C) Detailed map of subclone pMA84. DNA fragments a, b, and c used for S1 nuclease assays, the approximate location of the start site (▽), and the direction of transcription are shown. Boxes in panels A and B represent the Tn*5* sequence with IS*50* shaded. Restriction sites: B, *Bam*HI; Bg, *Bgl*II, H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sma*I; St, *Sst*I. Distances are shown in kilobases.

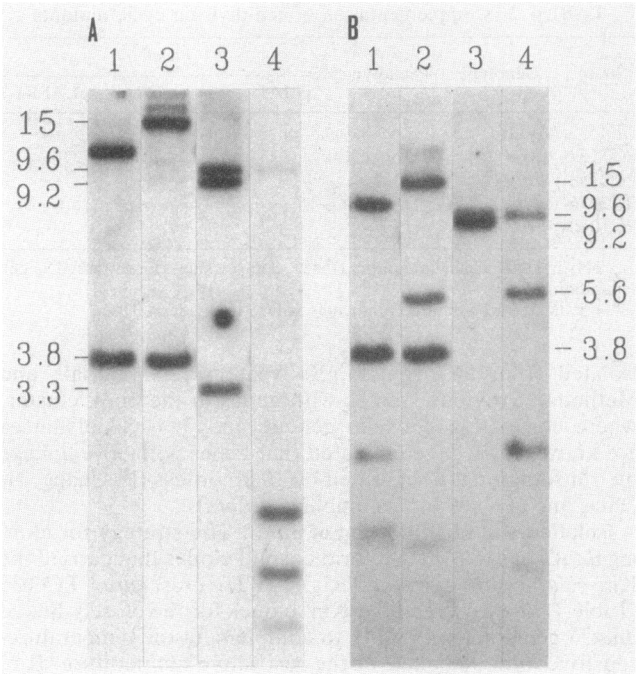

FIG. 3. Southern analysis of the strain PC6035 genome. DNA from PC6035 was digested with *Bam*HI plus *Eco*RI (lane 1), *Bam*HI (lane 2), *Hin*dIII (lane 3), and *Bam*HI plus *Hin*dIII (lane 4). Probes are pSUP10141(Tn*5*) (A) and the 9.2-kb *Hin*dIII(c)-*Hin*dIII(d) fragment (B) (Fig. 2).

transformants were individually transferred to the *dnaC* mutant strain PC7159 by triparental crosses (27). The Km^r Tc^r transconjugants were then screened for their ability to complement the *dnaC* allele for growth and cell division at 37°C. Three cosmids, pMA2, pMA3, and pMA11, which complemented the mutation, and pMA4, which did not, were used for further studies. None of the clones complemented other cell cycle mutations in DNA replication genes *dnaA* (PC2076), *dnaB* (PC1042), and *dnaD* (PC2153) (Table 2), all of which are unlinked to *dnaC* (Fig. 1). Hybrid plasmids were also transferred to the wild-type CB15 cells to show that increased dosage of the gene (five to six copies per cell) does not have deleterious effects on cell growth or division (Table 2).

Physical mapping of *dnaC* clones. The DNA inserts from cosmid clones pMA2, pMA3, and pMA11 were subjected to restriction analysis and analyzed for overlapping sequences. Although some rearrangement was apparent in the sequences flanking the Tn*5* insertion on the left, the three complementing clones contained sequences with overlapping restriction fragments immediately to the right of the Tn*5* element, which is shown for cosmid pMA2 (Fig. 2A). This result suggested that the *dnaC* gene might be located in this region of the insert, a conclusion that was confirmed by complementation results (see below).

Physical mapping of genomic sequences. The organization of genomic DNA in strain PC6035 adjacent to Tn*5* insertion *zja-306*::Tn*5* was examined by using the 9.2-kb *Hin*dIII fragment (Fig. 2A) and pSUP10141 carrying Tn*5* (34) as DNA probes on Southern blots. Probing with the Tn*5* sequence revealed a 9.6-kb *Hin*dIII fragment, an 11-kb *Bam*HI-*Eco*RI fragment, and a 15-kb *Bam*HI fragment that extend from sites within the insertion into genomic sequences to the left (Fig. 2B and 3A). It also identified the

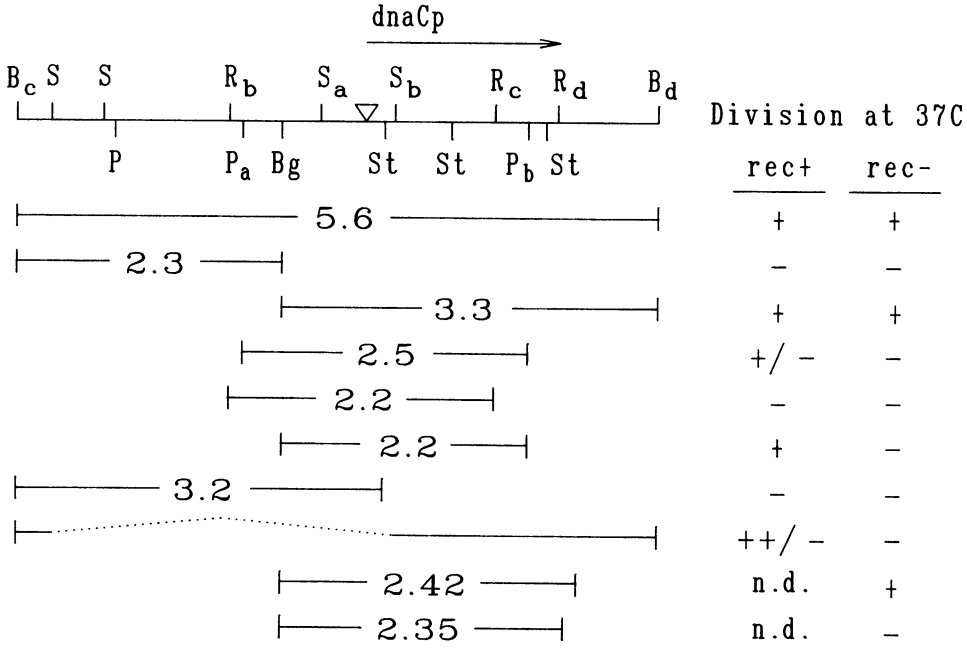

FIG. 4. Complementation analysis. The restriction map shows the 5.6-kb *Bam*HI(c)-*Bam*HI(d) fragment (Fig. 2) and various subclones. The 2.42- and 2.35-kb subclones were obtained by BAL 31 digestion of the *Bgl*II-*Bam*HI(d) fragment from the *Bam*HI(d) site. These subclones were transferred to *dnaC303* strains PC7159 (*recA*$^+$) and PC7162 (*recA*) and scored for the growth and division at 37°C. Symbols: +, growth and cell division comparable to the wild-type strain CB15; −, lack of growth; ++/− and +/−, intermediate response by the test described in Materials and Methods. n.d., Not determined. Abbreviation of the restriction sites are as described in the legend to Fig. 2.

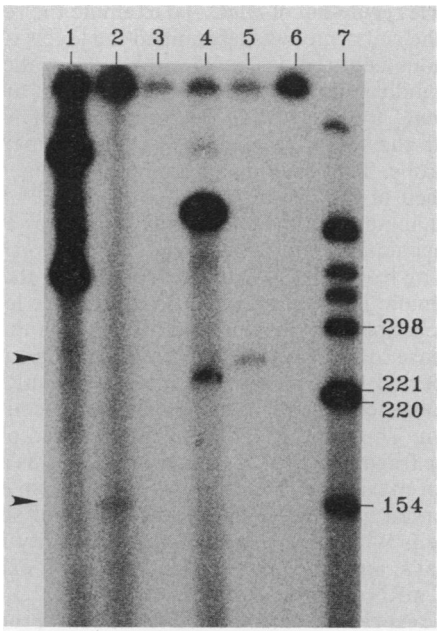

FIG. 5. Nuclease S1 mapping of the transcription start site of *dnaC*. Total RNA (100 μg) prepared from *C. crescentus* CB15 was used in each nuclease S1 protection assay at 60°C as described previously (26). ^{32}P-double-end-labeled probes were as follows: lanes 1 to 3, 900-bp fragment a (Fig. 2C); lanes 4 to 6, 480-bp fragment b (Fig. 2C). Nuclease S1 was present in lanes 2, 3, 5, and 6. Lanes 1 and 4 contained probe alone; lanes 2 and 5 also contained CB15 RNA; lanes 3 and 6 contained no RNA. Lane 7 shows size standards indicated in bases. Arrowheads indicate the protected fragments.

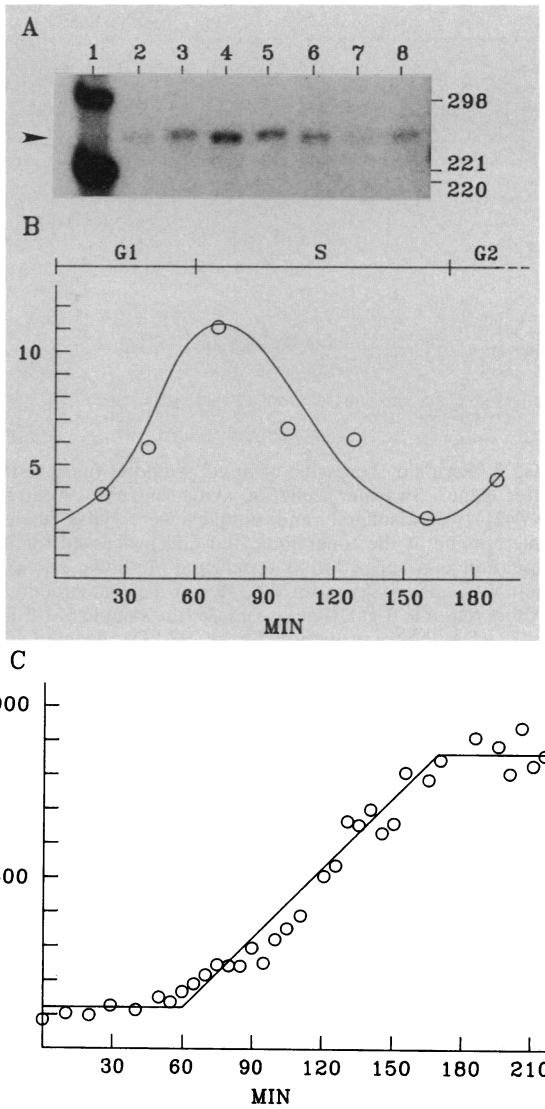

FIG. 6. Cell cycle regulation of the *dnaC* transcript. RNA was isolated from a synchronous culture of the density variant *C. crescentus* CB15F at the times indicated. A 40-μg portion of RNA from each sample was used in the S1 nuclease assays and probed with double-end-labeled *Sma*I(a)-*Sma*I(b) fragment b (Fig. 2C). (A) Autoradiogram. Lane 1 shows size standards; the other lanes represent nuclease S1 assays on RNA from cells collected at 20 min (lane 2), 40 min (lane 3), 70 min (lane 4), 100 min (lane 5), 130 min (lane 6), 160 min (lane 7), and 190 min (lane 8). (B) Quantification of partially protected DNA fragments. Bands marked by the arrowhead in panel A were traced by using a Bio-Rad model 620 video densitometer and quantified. The *y* axis is in arbitrary units. (C) DNA synthesis was followed by the incorporation of [^{3}H]guanosine into acid-precipitable, alkali-stable material as described previously (30); 25 μCi of [^{3}H]guanosine per ml and 10 μg of cold guanosine per ml were added to the culture at 0 min, and accumulation of label was monitored by taking a 25-μl sample at the times indicated.

9.2-kb *Hin*dIII and 3.8-kb *Bam*HI fragments that are present in both genomic and cloned sequences and extend to the right of the insertion (Fig. 2A and B). The restriction map to the right of the Tn*5* insertion was established by probing the genomic digests with the 9.2-kb *Hin*dIII fragment (Fig. 2A). The 5.6-kb *Bam*HI-*Bam*HI and other fragments also present in the restriction map of pMA2 were detected by this Southern analysis (Fig. 3B). Since the 9.2-kb probe contains one of the IS*50* inverted repeat elements of Tn*5*, it also hybridized to all fragments containing left-hand-side IS*50* (Fig. 2), including the 9.6-kb *Hin*dIII fragment, the 11-kb *Bam*HI-*Eco*RI fragment, and the 15-kb *Bam*HI fragment. These results revealed no detectable rearrangements in the approximately 10-kb sequence cloned to the right of the Tn*5* insertion.

Subcloning and identification of *dnaC* by complementation. To locate *dnaC* in cosmid clone pMA2, the 9.2-kb *Hin*dIII(c)-*Hin*dIII(d) fragment (Fig. 2A) was initially subcloned in plasmid vector pRK2L1 and shown to complement the *dnaC303* allele in strain PC7159 (data not shown). We subsequently cloned the 5.6-kb *Bam*HI(c)-*Bam*HI(d) fragment in the same vector and showed that it also complemented strain PC7159 (Fig. 4).

To define the minimum DNA sequence required for *dnaC* complementing activity a series of seven different restriction fragments were prepared from the 5.6-kb *Bam*HI(c)-*Bam*HI(d) fragment, subcloned in pRK2L1, and tested for complementation of *dnaC303*. The analysis was carried out initially with recombination-deficient strain PC7162 (*rec*; see Materials and Methods) to avoid false-positive results produced by recombination between the genome and subcloned fragments of the gene. The shortest DNA fragment complementing *dnaC303* in these initial experiments was the 3.3-kb *Bgl*II-*Bam*HI(d) restriction fragment (Fig. 4). The boundaries of *dnaC* were defined more accurately by making BAL 31 deletions (see Materials and Methods) of the 3.3-kb *Bgl*II-*Bam*HI(d) fragment from the *Bam*HI(d) site and test-

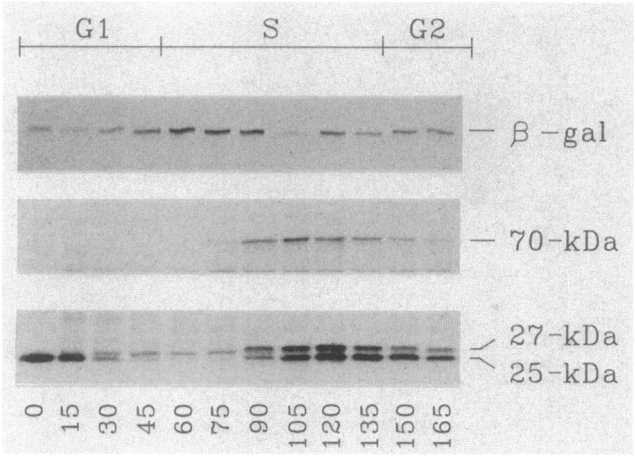

FIG. 7. Periodic expression of *dnaC* promoter fused to the *lacZ* reporter gene. Swarmer cells of synchronizable strain CB15F (pANZ421) were isolated, and samples were pulse-labeled with [^{35}S]methionine at the times indicated and processed for immune precipitation as described in Materials and Methods. The same cell extracts were also assayed for the rates of flagellar protein synthesis. Abbreviations: β-gal, β-galacosidase (the sample at 105 min was lost); 70-kDa, hook protein; 27-kDa, 27-kDa flagellin; 25-kDa, 25-kDa flagellin.

ing them for complementation. These results suggested that *dnaC* is bounded by the *Bgl*II site on the left and by a site between the BAL 31 endpoints of the 2.35- and 2.42-kb fragments on the right (Fig. 4).

The restriction fragments were also examined for restoration of cell division at 37°C in the rec$^+$ *dnaC303* mutant, PC7159. Three fragments which did not show complementation in the *rec* mutant background gave recombinants in the *rec*$^+$ background (Fig. 4). Comparison of the results with those for the 2.5-kb *Pst*I(a)-*Pst*I(b) and the 2.2-kb *Eco*RI(b)-*Eco*RI(c) fragments in the *rec*$^+$ and *rec* mutant backgrounds (Fig. 4) suggested that the *dnaC303* allele must be located within the *Eco*RI(c)-*Pst*(b) fragment.

The physical proximity of the DNA sequence complementing *dnaC303* to Tn*5* insertion *zja-306*::Tn*5*, which is genetically linked to *dnaC* (A. Newton, L. Kulick, and M. Bucuk, unpublished data), indicated that the sequence cloned in pMA2 is in fact the *dnaC* gene and not an extragenic suppressor. This conclusion is strengthened by the recombination results discussed above (Fig. 4).

Mapping of the *dnaC* transcription start site. Three DNA fragments spanning the *Sst*I(a)-*Pst*I sequence (Fig. 2C, fragments a, b, and c) were used as probes in nuclease S1 protection assays to determine the transcription start site as described previously (26). The double-end-labeled 900-bp *Sst*I(a)-*Sst*I(b) fragment (Fig. 2C, fragment a) gave a partially protected fragment of approximately 155 nucleotides (Fig. 5, lane 2). Because only the *Sst*I(b) site is in the *C. crescentus* DNA and the *Sst*I(a) site is in the plasmid polylinker, we concluded that the direction of transcription is from left to right starting approximately 155 nucleotides to the left of the *Sst*I(b) site (Fig. 2C). This conclusion was confirmed by probing with the double-end-labeled 480-bp *Sma*I(a)-*Sma*I(b) fragment (Fig. 2C, fragment b), which gave a longer partially protected fragment of approximately 260 nucleotides (Fig. 5, lane 5). The 840-bp *Sma*I(b)-*Pst*I fragment, fragment c, did not give a partially protected fragment (data not shown).

Cell cycle regulation of *dnaC*. To examine the regulation of *dnaC* in the cell cycle, we determined the levels of mRNA in synchronous cultures. Cells were taken from the culture at the times indicated (Fig. 6), RNA was purified, and the level of the *dnaC* transcript was determined by a nuclease S1 assay with the 480-bp fragment (Fig. 2C, fragment b) as the labeled probe. The resulting autoradiogram (Fig. 6A), which is quantified in Fig. 6B, and the pattern of DNA synthesis measured in the same culture (Fig. 6C) showed that the mRNA accumulates in a stage-specific fashion in the cell cycle, rising from relatively low levels early in the G1 period to a maximum level early in the S period. The levels of the *dnaC* transcript then decrease sharply later in the S period. The increase in the level of *dnaC* transcript observed late in the G2 period could reflect an increase in mRNA in the predivisional cells or in the stalked cells produced at the time of division. The same RNA samples were also probed with the 900-bp fragment (Fig. 2C, fragment a). In this experiment the time of maximum synthesis was 10 to 15 min earlier than that observed with the 480-bp probe (data not shown); this difference is within the experimental variability inherent in these assays, and the result confirms the early expression of the *dnaC* mRNA synthesis in the cell cycle.

Periodic expression of *lacZ* fused to the *dnaC* promoter. Cell cycle regulation of the *dnaC* promoter was also examined in a transcription fusion to the *lacZ* reporter gene. Plasmid pANZ421 carrying the *dnaC* promoter on the 480-bp *Sma*I(a)-*Sma*I(b) fragment (Fig. 2) fused to *lacZ* was constructed as described in Materials and Methods. The plasmid was introduced into strain CB15F, and the rate of the synthesis of β-galactosidase in synchronous culture was determined by radioimmunoassay of cells pulse-labeled with [^{35}S]methionine (Fig. 7). The same cell extracts were also assayed for flagellar gene expression. Consistent with the results of S1 nuclease assays, the rate of the β-galactosidase synthesis was periodic, with the maximum rate occurring at the beginning of the S period. The maximum rates of hook protein synthesis occurred after the mid-S period; this was followed in sequence by synthesis of the 27-kDa flagellin and finally by the 25-kDa flagellin (Fig. 7). Since the periodic expression of the three flagellar proteins late in the *Caulobacter* cell cycle is well established (26, 33), their assay in parallel with *dnaC* expression provides convincing evidence for the early transcription from the *dnaC* promoter.

DISCUSSION

To investigate the control of the cell cycle and the regulation of the *cdc* genes during asymmetric cell division in *C. crescentus*, we have cloned and analyzed the DNA chain elongation gene *dnaC* (29). We report the use of complementation assays to identify the gene in a genomic library and subcloning experiments to delimit its maximum size to approximately 1,500 bp. Measurements of gene expression in synchronous cell cultures by using nuclease S1 assays and expression of the *dnaC* promoter fused to a *lacZ* reporter gene showed that *dnaC* is transiently expressed at the end of the G1 period in swarmer cells and the beginning of the S period (Fig. 6 and 7). Therefore, it seems probable that the periodic expression of *dnaC* is regulated transcriptionally and, as discussed below, that the discontinuous pattern of *dnaC* expression accounts in part for the observed pattern of DNA synthesis in the *C. crescentus* cell cycle.

dnaC was originally defined as a DNA chain elongation gene because the *dnaC303* allele results in the immediate cessation of DNA synthesis after shifting cells to the non-

permissive temperature (29, 30). The biochemical nature of the defect has not been determined, and the mutation could be in a gene encoding a protein subunit directly required for DNA chain elongation or, alternatively, in a gene encoding a protein required for biosynthesis of a DNA precursor (see below for a discussion of yeast cell cycle genes). Therefore, possible explanations for the differential regulation of DNA synthesis in the two newly divided *C. crescentus* cells (4) are the presence of the DNA synthetic machinery and precursors in the stalked cell that enters the S period, but not in the swarmer cell that enters an extended G1 period. Consistent with this model for the control of DNA synthesis are the observations that little if any replication of the IncP plasmid pRK290 can be detected in swarmer cells during the G1 period (17; N. Ohta and A. Newton, unpublished data) and that the replication of this plasmid requires an active *dnaC* gene product in the host cell (Ohta and Newton, unpublished).

Periodic gene expression is not a general feature of gene regulation in *C. crescentus*. Transcription of *rrnA* operon from the p1 promoter was recently shown to be continuous (1), and synthesis of the major surface array protein is also not cell cycle regulated (8). Earlier studies have shown that most of the soluble proteins (3, 18), as well as membrane proteins (18; M. Clancy, Ph.D. thesis, Princeton University, Princeton, N.J., 1980), were synthesized throughout the cell cycle. These same studies revealed, however, that 10 to 15% of the *C. crescentus* proteins examined were synthesized periodically or in a cell-type-specific fashion. This last result is in sharp contrast to conclusions of work with *E. coli* that have provided no evidence for the discontinuous synthesis of cellular proteins (16).

By far the largest number of genes that have been examined in *C. crescentus* are flagellar genes, and all of those that have been described are periodically expressed in the cell cycle (reviewed in references 22 and 23). The analysis of the promoter sequences (19, 20, 25) and the assay of promoter activity in transcription fusions support the idea that the flagellar genes are regulated at the level of transcription initiation (24, 38). The present results now add one DNA replication gene to the set of cell-cycle-regulated genes in these bacteria, and it is tempting to speculate that *dnaC* will prove to be one of a larger set of *cdc* genes whose expression is under developmental regulation in the cell cycle.

Isolation of *dnaC* on a DNA fragment that contained a Km^r Tn*5* insertion element tightly linked by transduction had two advantages. First, the initial selection of hybrid plasmids with the Tn*5* insertion reduced the number of *C. crescentus* transconjugants that must be screened, and second, the genetically linked Tn*5* element provided assurance that the DNA sequence cloned by complementation corresponds to the cell cycle gene and not to an unlinked suppressor locus. This conclusion was confirmed by physical mapping of Tn*5* and flanking DNA sequences in the genome (Fig. 2 and 3). Cosmid vector pLAFR1-7 has also been used to isolate genes by direct complementation from genomic libraries without prior selection of linked drug resistance markers. We have isolated several cell division genes in this way (unpublished data), and Schoenlein et al. have recently reported the use of similar pLAFR1-7 libraries for cloning flagellar and nutritional genes (32). Thus, cosmid pLAFR1-7 is generally useful cloning vector in *C. crescentus*.

An important feature of the complementation analysis of *dnaC* described here was the use of rec^+ and *rec* strains (Fig. 4). The results demonstrated that misleading results can be obtained if recombination and complementation cannot be distinguished and that rec^+ and *rec* strains are useful in locating the mutation in cloned segments of *C. crescentus* DNA. The patterns of complementation and recombination in Fig. 4 show that *dnaC303* is located within the *Eco*RI(c)-*Pst*I(b) fragment. Therefore, the mutation is approximately 5.5 kb from the end of the Tn*5* insertion (Fig. 2), to which it is 90% linked by transduction.

There has been no information available on the cell cycle regulation of *cdc* gene expression in bacteria. DNA replication and cell division genes in *C. crescentus*, as shown previously for *Saccharomyces cerevisiae* (10), are organized in separate, dependent pathways (30). It is not clear to what extent these *cdc* functions, which are expressed in a tightly ordered sequence in the cell cycle, are controlled at the level of gene expression or posttranslationally. In *S. cerevisiae* several genes required for DNA synthesis, including DNA polymerase I (14), DNA ligase (31), thymidylate synthase (35), and ribonucleotide reductase (6), are cell cycle regulated, and the results presented here show that at least one of the DNA replication genes in *C. crescentus* is under cell cycle control. It will be important to examine other *dna* (Fig. 1) and *div* genes in these bacteria to determine whether this is a common feature of cell cycle gene regulation.

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LITERATURE CITED

1. **Amemiya, K.** 1989. Conserved sequence elements upstream and downstream for the transcription initiation site of the *Caulobacter crescentus rrnA* gene cluster. J. Mol. Biol. **210**:245–254.
2. **Boyer, H. B., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
3. **Cheung, K. K., and A. Newton.** 1977. Patterns of protein synthesis during development in *Caulobacter crescentus*. Dev. Biol. **56**:417–425.
4. **Degnen, S. T., and A. Newton.** 1972. Chromosome replication during development in *Caulobacter crescentus*. J. Mol. Biol. **64**:676–680.
5. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
6. **Elledge, S. J., and R. W. Davis.** 1990. Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase. Genes Dev. **4**:740–751.
7. **Ely, B., and R. H. Croft.** 1982. Transposon mutagenesis in *Caulobacter crescentus*. J. Bacteriol. **149**:620–625.
8. **Fisher, J. A., J. Smit, and N. Agabian.** 1988. Transcriptional analysis of the major surface array gene of *Caulobacter crescentus*. J. Bacteriol. **170**:4706–4713.
9. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18**:289–296.
10. **Hereford, L. M., and L. H. Hartwell.** 1974. Sequential gene function in the initiation of *Saccharomyces cerevisiae* DNA synthesis. J. Mol. Biol. **84**:445–461.
11. **Hohn, B., and K. Murray.** 1977. Packaging recombinant DNA molecules into bacteriophage particles *in vitro*. Proc. Natl. Acad. Sci. USA **74**:3259–3263.

12. **Huguenel, E. D., and A. Newton.** 1982. Localization of surface structures during procaryotic differentiation: role of cell division in *Caulobacter crescentus*. Differentiation **21**:71–78.
13. **Johnson, R. C., and B. Ely.** 1977. Generalized transduction in *Caulobacter crescentus*. Genetics **87**:391–399.
14. **Johnston, L. H., J. H. M. White, A. L. Johnson, G. Lucchini, and P. Pleviani.** 1987. The yeast DNA polymerase I transcript is regulated in both the mitotic cell cycle and in meiosis and is also induced after DNA damage. Nucleic Acids Res. **15**:5017–5030.
15. **Kleckner, N., J. Roth, and D. Botstein.** 1977. Genetic engineering *in vivo* using translocatable drug-resistant elements. New methods in bacterial genetics. J. Mol. Biol. **116**:125–129.
16. **Lutkenhaus, J. F., B. A. Moore, M. Master, and W. D. Donachie.** 1979. Individual proteins are synthesized continually throughout the *Escherichia coli* cell cycle. J. Bacteriol. **138**:352–360.
17. **Marczynski G. T., A. Dingwall, and L. Shapiro.** 1990. Plasmid and chromosomal replication and partitioning during the *Caulobacter crescentus* cell cycle. J. Mol. Biol. **212**:709–722.
18. **Milhausen, M., and N. Agabian.** 1981. Regulation of polypeptide synthesis during *Caulobacter* development: two-dimensional gel analysis. J. Bacteriol. **148**:163–173.
19. **Minnich, S. A., and A. Newton.** 1987. Promoter mapping and cell cycle regulation of flagellin gene transcription in *Caulobacter crescentus*. Proc. Natl. Acad. Sci. USA **84**:1142–1146.
20. **Mullin, D. A., and A. Newton.** 1989. Ntr-like promoters and upstream regulatory sequence *ftr* are required for transcription of a developmentally regulated *Caulobacter crescentus* flagellar gene. J. Bacteriol. **171**:3218–3227.
21. **Newton, A.** 1984. Temporal and spatial control of *Caulobacter* cell cycle, p. 51–75. *In* P. Nurse and E. Streiblova (ed.), The microbial cell cycle. CRC Press, Inc., Boca Raton, Fla.
22. **Newton, A.** 1989. Differentiation in *Caulobacter*: flagellum development, motility and chemotaxis, p. 199–220. *In* D. A. Hopwood and K. F. Chater (ed.), Bacterial diversity. Academic Press, Inc. (London) Ltd., London.
23. **Newton, A., and N. Ohta.** 1990. Regulation of the cell division cycle and differentiation in bacteria. Annu. Rev. Microbiol. **44**:689–719.
24. **Newton, A., N. Ohta, G. Ramakrishnan, D. A. Mullin, and G. Raymond.** 1989. Genetic switching in the flagellar gene hierarchy requires both positive and negative regulation of transcription. Proc. Natl. Acad. Sci. USA **86**:6651–6655.
25. **Ninfa, A. J., D. A. Mullin, G. Ramakrishnan, and A. Newton.** 1989. *Escherichia coli* sigma RNA polymerase recognizes *Caulobacter crescentus flbG* and *flaN* flagellar gene promoters in vitro. J. Bacteriol. **171**:383–391.
26. **Ohta, N., L.-S. Chen, E. Swanson, and A. Newton.** 1985. Transcriptional regulation of a periodically controlled flagellar gene operon in *Caulobacter crescentus*. J. Mol. Biol. **186**:107–115.
27. **Ohta, N., E. Swanson, B. Ely, and A. Newton.** 1984. Physical mapping and complementation analysis of transposon Tn*5* mutations in *Caulobacter crescentus*: organization of transcriptional units in the hook gene cluster. J. Bacteriol. **158**:897–904.
28. **O'Neill, E. A., R. H. Hays, and R. Bender.** 1985. Recombination deficient mutant of *Caulobacter crescentus*. Mol. Gen. Genet. **197**:399–402.
29. **Osley, M. A., and A. Newton.** 1977. Mutational analysis of developmental control in *Caulobacter crescentus*. Proc. Natl. Acad. Sci. USA **74**:124–128.
30. **Osley, M. A., and A. Newton.** 1980. Temporal control of the cell cycle in *Caulobacter crescentus*: roles of DNA chain elongation and completion. J. Mol. Biol. **138**:109–128.
31. **Peterson, T. A., L. Prakash, S. Prakash, M. A. Osley, and S. I. Reed.** 1985. Regulation of *CDC9*, the *Saccharomyces cerevisiae* gene that encodes DNA ligase. Mol. Cell. Biol. **5**:226–235.
32. **Schoenlein, P. V., L. M. Gallman, and B. Ely.** 1988. Use of transmissible plasmids as cloning vectors in *Caulobacter crescentus*. Gene **70**:331–334.
33. **Sheffrey, M., and A. Newton.** 1981. Regulation of periodic protein synthesis in the cell cycle: control of initiation and termination of flagellar gene expression. Cell **24**:49–57.
34. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–790.
35. **Storms, R. K., R. W. Ord, M. T. Greenwood, B. Mirdamadi, F. K. Chu, and M. Belfort.** 1984. Cell cycle-dependent expression of thymidylate synthase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**:2858–2864.
36. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
37. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
38. **Xu, H., A. Dingwal, and L. Shapiro.** 1989. Negative transcriptional regulation in the *Caulobacter* flagellar hierarchy. Proc. Natl. Acad. Sci. USA **86**:6656–6660.