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Expression of *Caulobacter dnaA* as a Function of the Cell Cycle

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The initiation of DNA replication is under differential control in *Caulobacter crescentus*. Following cell division, only the chromosome in the progeny stalked cell is able to initiate DNA replication, while the chromosome in the progeny swarmer cell does not replicate until later in the cell cycle. We have isolated the *dnaA* gene in order to determine whether this essential and ubiquitous replication initiation protein also contributes to differential replication control in *C. crescentus*. Analysis of the cloned *C. crescentus dnaA* gene has shown that the deduced amino acid sequence can encode a 486-amino-acid protein that is 37% identical to the DnaA protein of *Escherichia coli*. The gene is located 2 kb from the origin of replication. Primer extension analysis revealed a single transcript originating from a sigma 70-type promoter. Immunoprecipitation of a DnaA′–β-lactamase fusion protein showed that although expression occurs throughout the cell cycle, there is a doubling in the rate of expression just prior to the initiation of replication.

Caulobacter crescentus divides asymmetrically into a sessile stalked cell and a motile swarmer cell, which later develops into a stalked cell (see Fig. 5B). The swarmer cell chromosome is unable to initiate replication until later in the cell cycle, while in the stalked cell, the initiation of chromosome replication is immediate (7, 9, 28, 29). The newly replicated chromosomes segregate randomly to these two cell types (28), at which point they behave very differently with respect to replicative ability and gene expression (37). As part of an effort to define the determinants of DNA replication initiation in *C. crescentus*, we have cloned and characterized the gene for the replication initiation protein, DnaA.

In *Escherichia coli*, DnaA is essential for the initiation of replication (21). The protein binds to recognition sequences (DnaA boxes) at the origin of replication, where it acts to unwind the DNA duplex so that a complex of proteins can build replication forks (2). When bound to ATP, but not ADP, DnaA is active in promoting replication initiation in vitro (40). Membrane fractions and membrane components can influence the change from inactive to active DnaA states (41, 48). DnaA forms large aggregates of as many as 40 molecules which are inactive in in vitro replication initiation assays (17). Both phospholipase and DnaK, a molecular chaperone, can disaggregate and thus activate DnaA (23). DnaA abundance, nucleotide binding state, cellular location, and aggregation state may be critical to the regulation of the chromosome replication pathway.

There is evidence that DnaA also functions in transcriptional regulation in *E. coli*. For example, there are DnaA boxes found near or in the promoters of several genes, such as *guaP* (46) and *uvrB* (47). There is also evidence that DnaA "fine-tunes" the expression of its own gene (1a, 4, 35). In this report, we describe the isolation of the *C. crescentus dnaA* gene, identify its promoter, examine its expression as a function of the *C. crescentus* cell cycle, and test whether it is rate limiting for replication initiation.

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MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains and plasmids used are described in Table 1. CB15N (renamed NA1000), a synchronizable derivative of the *C. crescentus* wild-type strain CB15 (13), was routinely grown in PYE medium (34) at 30°C. M2 minimal glucose medium (12) was used when cells were synchronized by Ludox density gradient centrifugation (13). *E. coli* strains were grown in LB medium at 37°C. Strains LS769 and LS770 were grown in PYE medium supplemented with 5 μg of tetracycline per ml and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Cloning of the *dnaA* gene. Cosmids (from a genomic library provided by G. Marczynski) that mapped to the earliest-replicating part of the chromosome were digested with *Bam*HI and transferred to nitrocellulose. Blots were hybridized at low stringency (6× SSC [1× SSC is 0.15 M NaCl and 0.015 M sodium citrate]–5× Denhardt solution–0.1% sodium dodecyl sulfate at 55°C; washed in 5× SSC at 37°C) to a *Pseudomonas putida dnaA* fragment probe provided by H. Ingmer. A single band of 3.8 kb was detected. A single *Xba*I restriction site was found, and 1.8- and 2.0-kb *Xba*I-*Bam*HI fragments were subcloned into these sites in the pBluescriptII-SK(+) plasmid vectors, making pGZ10 and pGZ11, respectively. pGZ11 was found to hybridize to the *P. putida dnaA* probe.

DNA sequencing. Single-stranded DNA templates from pBluescriptII-SK(+) phagemids served as templates and were prepared from various subclones as described by Short et al. (43). The entire sequence of the pGZ11 insert was completed on both DNA strands. pGZ10 was partially sequenced. The PAN facility of Stanford Beckman Center made the DNA sequencing primers and performed the DNA sequencing reactions.

Construction of a DnaA′–β-lactamase translation fusion. A DnaA′–β-lactamase translation fusion was made by inserting the 1.18-kb *Xba*I-*Sal*I fragment in front of the *bla* gene in pJAMY31. The resulting plasmid, pGZ12, was introduced by conjugation into *C. crescentus* NA1000, creating strain LS771.

Primer extension analysis. The *dnaA* transcriptional start site was mapped by primer extension analyses (18) by using two oligonucleotides, primer A (CCATGGCGCGACCGTT TT) and primer B (TGGCAACCCCGCCCTTCATG), as shown in the underlined sequences in Fig. 2. Total *C. crescen-*

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant properties	Source or reference
E. coli		
DH10B	*mcrA* Δ(*mrr-hsd* RMS-*mcr* BC)	Bethesda Research Laboratories
XL-1	F′ *proAB*, *lacZ*ΔM15 Tn*10*(Tet^r)	5
S17-1	*E. coli* 294::RP4-2(Tc::Mu) (Km::Tn*7*)	44
C. crescentus		
NA1000	Synchronizable derivative of the wild-type strain CB15	13
LS769	NA1000 carrying pGZ14	This report
LS770	NA1000 carrying pGZ13	This report
LS771	pGZ12 integrant of NA1000	This report
Plasmids		
pBluescriptII-SK(+)	Cloning and single-stranded phagemids	Stratagene
pBGST18T	Cloning and transduction; oriT and Kan^r	1
pJAMY31	For β-lactamase translational fusion; Kan^r	1
pJGZ290	pRK290-based plasmid that is maintained at 2–5 copies in *C. crescentus*	17a
pTac7	Source of P_{tac} and *lacI*^{q}	1
pGZ10	1.8-kb *Xba*I-*Bam*HI fragment of 3.8-kb *Bam*HI clone (Fig. 1A) in pBluescriptII-SK(+)	This report
pGZ11	2.0-kb *Xba*I-*Bam*HI fragment of 3.8-kb *Bam*HI clone (Fig. 1A) in pBluescriptII-SK(+)	This report
pGZ12	1.18-kb *Xba*I-*Sal*I fragment of pGZ10 in pJAMY31; in-frame translational fusion of *dnaA* to *bla*	This report
pGZ13	2.0-kb fragment of pTac7 including P_{tac} and *lacI*^{q} followed by the 1,661-bp *Nco*I-*Bam*HI fragment of pGZ10 in pJGZ290, so that the *lacZ* reporter follows the *dnaA* gene (Fig. 6A)	This report
pGZ14	2.0-kb fragment of pTac7 including P_{tac} and *lacI*^{q} followed by the 246-bp *Nco*I-*Sal*I fragment of pGZ10 in pJGZ290, so that the *lacZ* reporter follows the truncated *dnaA* gene (Fig. 6A)	This report
pGZ15	785-bp *Sal*I-*Xho*II fragment of pGZ10 in pBGST18T	This report
pGZ16	673-bp *Xho*I-*Bam*HI fragment of pGZ10 in pBGST18T	This report

tus RNA (25 μg) was annealed to a 5′-end γ-^{32}P-labeled oligonucleotide for 16 h at 45°C. Products extended by avian myeloblastosis virus reverse transcriptase were analyzed next to a DNA sequencing ladder generated by the same primer on an 8% polyacrylamide gel.

Labeling of cellular protein and DNA. Synchronized cultures were pulse-labeled with [^{35}S]methionine (3 μCi/ml) for 10 min at specific times in the cell cycle. β-Lactamase was immunoprecipitated from cell extracts with antibody to β-lactamase as described previously (18). The amount of protein extract used per immunoprecipitation was normalized to a specific amount of ^{35}S incorporation. The proteins were separated by electrophoresis through 12% polyacrylamide–sodium dodecyl sulfate gels. The labeled proteins were visualized by autoradiography following treatment of the gels with En^{3}Hance. Densitometry of the autoradiograms was performed by using a Molecular Dynamics machine and software. To measure the rate of DNA synthesis in synchronized cells, swarmer cells were isolated and resuspended in M2G medium at an optical density at 600 nm (OD_{600}) of 0.4 and allowed to proceed through the cell cycle. At 20-min intervals, 20 μCi of [^{3}H]dGTP was added to 1 ml of culture for a 5-min period. The rate of DNA synthesis was determined from 0.2-ml samples of these cells as previously described (7).

***dnaA* disruption experiment.** A 785-bp *Sal*I-*Xho*II fragment and a 673-bp *Xho*I-*Bam*HI fragment (restriction endonuclease sites shown in Fig. 2) were cloned into the polylinker in pBGST18T, a vector that cannot replicate in *C. crescentus*, creating pGZ15 and pGZ16, respectively. These plasmids were introduced into *C. crescentus* both by electroporation and by mating from the *E. coli* host strain S17-1. By either means of introduction and under equivalent conditions, antibiotic-resistant colonies transformed with pGZ16 appeared 400 times more frequently then those transformed with pGZ15. A South-

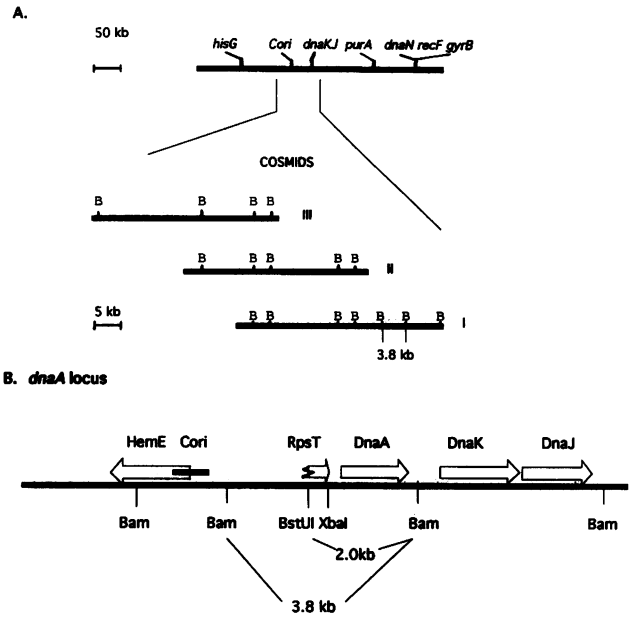

FIG. 1. Genetic and physical maps of the origin and *dnaA* gene. (A) Three overlapping cosmids spanning the origin of replication (*Cori*) were used to identify the *dnaA* gene (29). *Bam*HI sites are indicated by the letter B. The 3.8-kb *Bam*HI fragment hybridized to the *P. putida dnaA* gene probe. (B) Map of the origin and *dnaA* locus with neighboring genes, *hemE* (29), *rpsT* (this report), and *dnaKJ* (18).

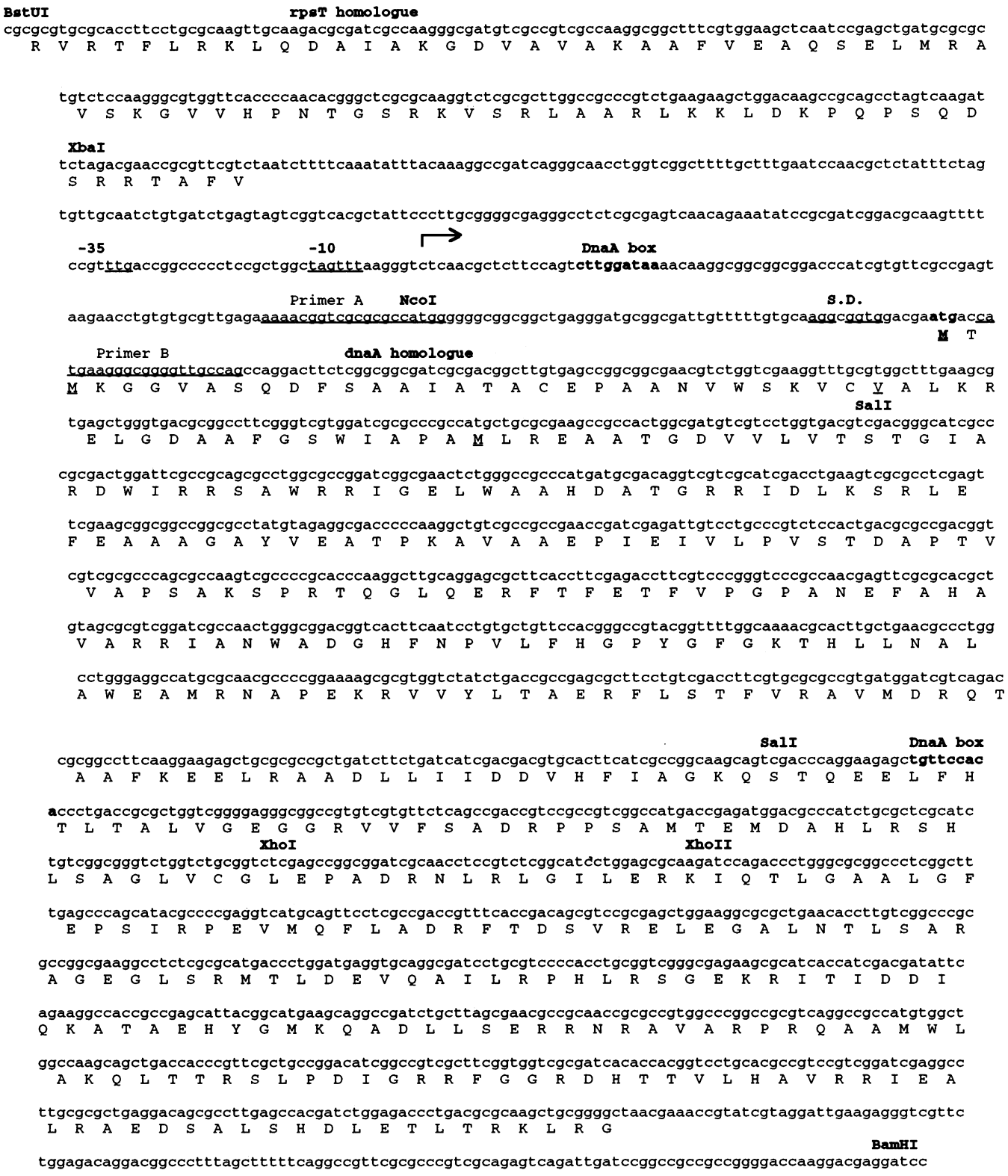

FIG. 2. Nucleotide and predicted amino acid sequences of the 2.0-kb *Bst*UI-*Bam*HI fragment shown in Fig. 1B. The nucleotide sequence that could encode the carboxy-terminal end of a protein with homology to RpsT is shown at the top. Following this sequence is the sequence of the *dnaA* gene. The *dnaA* promoter is underlined, and the DnaA boxes are in boldface. The Shine-Dalgarno sequence for the *dnaA* gene is underlined, as are potential initiating amino acids. The most likely translational start is in boldface. The open reading frame encodes a predicted protein of 486 amino acids.

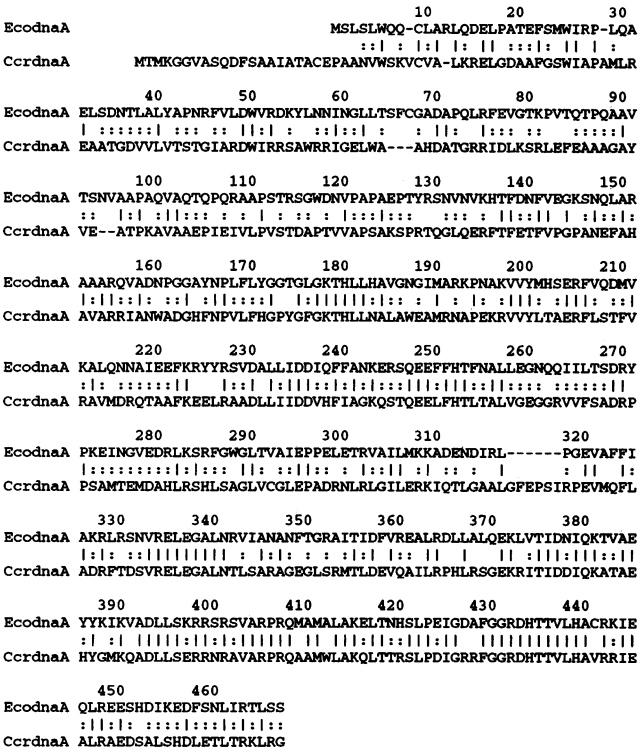

FIG. 3. Comparison of the predicted amino acid sequences of the *C. crescentus* DnaA protein (CcrdnaA) and the *E. coli* DnaA protein (EcodnaA) (19). The sequences were aligned by using the Tfasta program from the Genetics Computer Group package (8). Bars between the sequences represent identical residues, and colons represent similar residues. The two proteins show 37.3% identity and 79.9% similarity over a 468-amino-acid sequence.

ern blot of *Sal*I-cleaved DNA from three colonies that were transformed with pGZ15 was hybridized to a probe that spanned the *dnaA* gene. Autoradiography revealed that the native gene was intact and that integration of pGZ15 occurred outside the *dnaA* gene.

β-Galactosidase assay. *C. crescentus* LS769 and LS770 were grown in PYE medium containing 5 μg of tetracycline per ml and 1 mM IPTG to an OD_{660} of 0.5. The β-galactosidase assays and calculation of enzyme unit activities were performed as described by Miller (30).

Fluorescence-activated cell sorting (FACS) analysis. *C. crescentus* LS769 and LS770 were grown in PYE medium containing 5 μg of tetracycline per ml and 1 mM IPTG. Exponential-phase cultures of LS769 and LS770, grown at 30°C, were collected by centrifugation, resuspended in PYE without antibiotic, and grown to an OD_{600} of 0.1. When the cultures had grown to an OD_{600} of 0.4, they were treated with 25 μg of chloramphenicol per ml for 2 h at 30°C. They were then fixed by the addition of 100% ethanol to a final concentration of 70% and stored at 4°C. The cells were then collected by centrifugation in microcentrifuge tubes at 5,220 × *g*, resuspended in TMS (10 mM Tris-HCl [pH 7.2], 1.5 mM $MgCl_2$, 150 mM NaCl) containing 10 μg of chromomycin A3 per ml, and kept at 4°C in the dark overnight. The cells were analyzed on a Becton Dickinson FACStar Plus machine. Excitation was at 458 nm, and fluorescence was measured at 495 nm.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper was submitted to GenBank and assigned accession number U01667.

RESULTS

Isolation and identification of the *dnaA* gene. A *C. crescentus* *dnaA* homolog was identified by Southern blot hybridization of a *P. putida* *dnaA* gene to *Bam*HI-cleaved cosmids containing DNA that spans the *C. crescentus* origin of replication (29) (Fig. 1A). The 3.8-kb *Bam*HI fragment that hybridized to the *P. putida* *dnaA* probe was subcloned, and the 2.0-kb region shown in Fig. 1B was sequenced. The DNA sequence of the putative *dnaA* gene and a portion of an upstream gene, which is a homolog of the protein encoded by the *E. coli* *rpsT* gene (25), is shown in Fig. 2. The translation start site shown in Fig. 2 yields a DnaA homolog that has 37% identity and 80% similarity to the *E. coli* DnaA protein in a 467-amino-acid alignment (Fig. 3). Although the protein has not been sequenced and thus the actual start site is unknown, we assigned the first ATG (underlined in Fig. 2) as the start codon for two reasons. First, a protein made from the assigned ATG is encoded by a sequence that has the highest-percentage third-position G+C content. *C. crescentus* DNA has a 67% G+C content overall, and in open reading frames, the G+C content of codon third positions is over 90%. Second, the first ATG has an upstream sequence that best matches the ribosome binding sequences of several *C. crescentus* genes and the *C. crescentus* 16S rRNA complement (11). The sequence originating at the first ATG encodes an additional amino-terminal 20 to 21 amino acids, not observed in nine other reported bacterial *dnaA* sequences.

The predicted *C. crescentus* DnaA protein has the same pattern of conserved amino acids as do nine other DnaA homologs. It has a moderately conserved amino-terminal third of the protein and a highly conserved carboxy-terminal half that includes a proposed ATP binding site (42).

Gene organization in the origin region of the chromosome. Analysis of restriction maps of overlapping clones containing the origin of DNA replication (29) revealed that *dnaA* lies 2 kb from the origin (Fig. 1A). The direction of *dnaA* transcription relative to the origin sequence is shown in Fig. 1B. A partial open reading frame (Fig. 2) was found between the *dnaA* gene and the origin. A computer data base search revealed 41% identity of the predicted 61 amino acids with the carboxy-terminal end of the *E. coli* ribosomal protein S20, encoded by the *rpsT* gene. The *rpsT* gene of *E. coli* does not map near *dnaA* or the origin of replication (24). Unlike the genomic arrangement in *E. coli* (20, 36, 38) and many other bacteria species (15), *dnaN*, encoding the β subunit of DNA polymerase III, is not directly downstream of *dnaA*. *C. crescentus* homologs of *dnaN*, *recF*, and *gyrB* have been cloned and identified by sequence comparison with the respective *E. coli* genes, and their map positions are shown in Fig. 1A (37). As in *E. coli*, they form a gene cluster, but they lie approximately 150 kb from the *dnaA* gene. The *dnaKJ* operon (18) resides 1.0 kb downstream of the *dnaA* translation stop codon.

Identification of the transcription start site. Primer extension from two different primers revealed a single transcription start site (Fig. 4). The first nucleotide transcribed is 151 nucleotides upstream of the proposed translation start site. A sequence upstream at an appropriate distance from the transcription start resembles the consensus sequence of *C. crescentus* sigma 70 promoters (26). Two 9-bp sequences, one 18 to 27 bp downstream of the transcription start site (Fig. 4) and the other located just over halfway through the coding sequence of *dnaA* (Fig. 2), match 8 of 9 bp of the consensus sequence for an *E. coli* DnaA binding site (a DnaA box). Other DnaA boxes have been found at the *C. crescentus* origin of replication and have been shown to be necessary for replication (29).

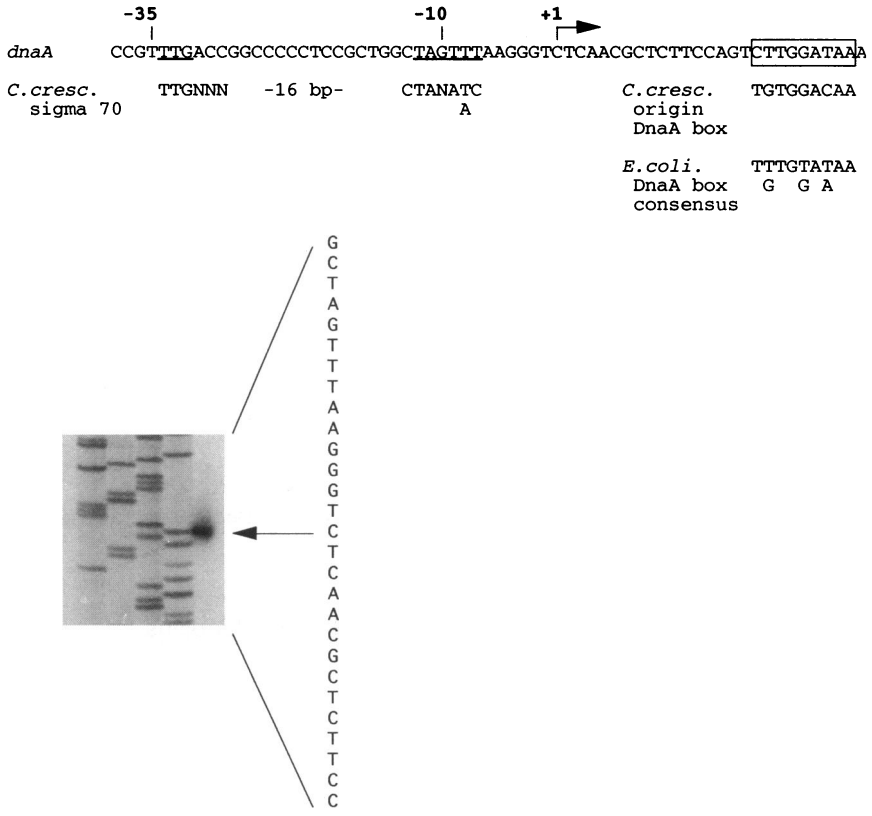

FIG. 4. Transcriptional start site of *dnaA*. An autoradiogram of a primer extension experiment beside the products of a sequencing reaction reveals the position of the first transcribed nucleotide. The sequence upstream of the *dnaA* coding sequence includes a putative sigma 70-type promoter, (underlined) and a putative DnaA box (boxed). The consensus sequence for several *C. crescentus* sigma 70-type promoters (26, 45a), the sequence of an essential DnaA box from the *C. crescentus* origin of replication (29), and the consensus *E. coli* DnaA box sequence (39) are shown under the *dnaA* sequence. An arrow shows the transcriptional start site and the direction of transcription.

Expression of the *dnaA* gene during the cell cycle. Expression of the *dnaA* gene was assayed in a strain (LS771) containing a chromosomal insertion of a portion of the *dnaA* gene fused to a β-lactamase reporter gene (Fig. 5A). Strain LS771 has an endogenous *dnaA* promoter and three-fourths of the *dnaA* coding sequence fused in frame to the *bla* gene in addition to an intact *dnaA* gene with its endogenous promoter. Swarmer cells of LS771 were isolated and allowed to proceed through the cell cycle. At intervals, culture samples were labeled with [^{35}S]methionine and cell extracts were immunoprecipitated with antibody to β-lactamase (Fig. 5B). A portion of each sample was immunoprecipitated with antibody to flagellins as an internal control of the synchrony. The DnaA′–β-lactamase fusion protein was detected throughout the cell cycle. Peak expression (twice the lowest level) occurred at 10 to 20 min into the cell cycle (Fig. 5C), just prior to replication initiation in nascent stalked cells (Fig. 5D).

The function of *dnaA* in *C. crescentus*. To help determine whether *dnaA* is limiting for the initiation of DNA replication, we introduced a plasmid (present in two to five copies per cell [28]), expressing *dnaA*, into wild-type strain NA1000. The DNA content of cells of strain NA1000, bearing either plasmid pGZ13 (LS770), carrying the *dnaA* gene under control of the strong P_{tac} promoter, or pGZ14 (LS769), an isogenic control plasmid containing only the amino-terminal 60 codons of the *dnaA* gene (Fig. 6A), was examined by flow cytometry. A *lacZ* reporter gene, transcriptionally fused to the full or truncated *dnaA* gene on the plasmids, indicated that both fusions were expressed in the presence of an intact chromosome copy of *dnaA*.

DNA replication in strains NA1000, LS770, and LS769 was allowed to go to completion, but not to reinitiate, by the addition of chloramphenicol. We measured the DNA content (in the presence of the fluorescent dye chromomycin A3) of individual cells by flow cytometry (49) using FACStar Plus (Fig. 6B). In each panel, the first peak represents cells with one chromosome and the second peak represents cells with two chromosomes. Cells with a DNA content equivalent to more than two chromosomes were not observed in the truncated *dnaA* transcript producer strain LS769 (Fig. 6B), or in the isogenic NA1000 strain. However, in cultures of strain LS770, we consistently observed cells with an amount of DNA equivalent to more than two chromosomes but less than three chromosomes. This increased DNA content may be due to additional DNA replication initiations.

No differences between cells of LS769 and LS770 and the parental strain NA1000 were observed by light microscopy. Strains LS769 and LS770 had identical generation times. Swarmer cells derived from LS769 and LS770 cultures appeared to proceed normally through the cell cycle. Thus, the expression of additional *dnaA* in strain LS770 had no noticeable effect on cell morphology and growth rate.

To determine whether *dnaA* is an essential gene, we attempted to disrupt it. Introduction of a nonreplicating plasmid, pGZ16, with 673 bp of the terminus of the coding region of *dnaA* into *C. crescentus* NA1000 yielded hundreds of viable

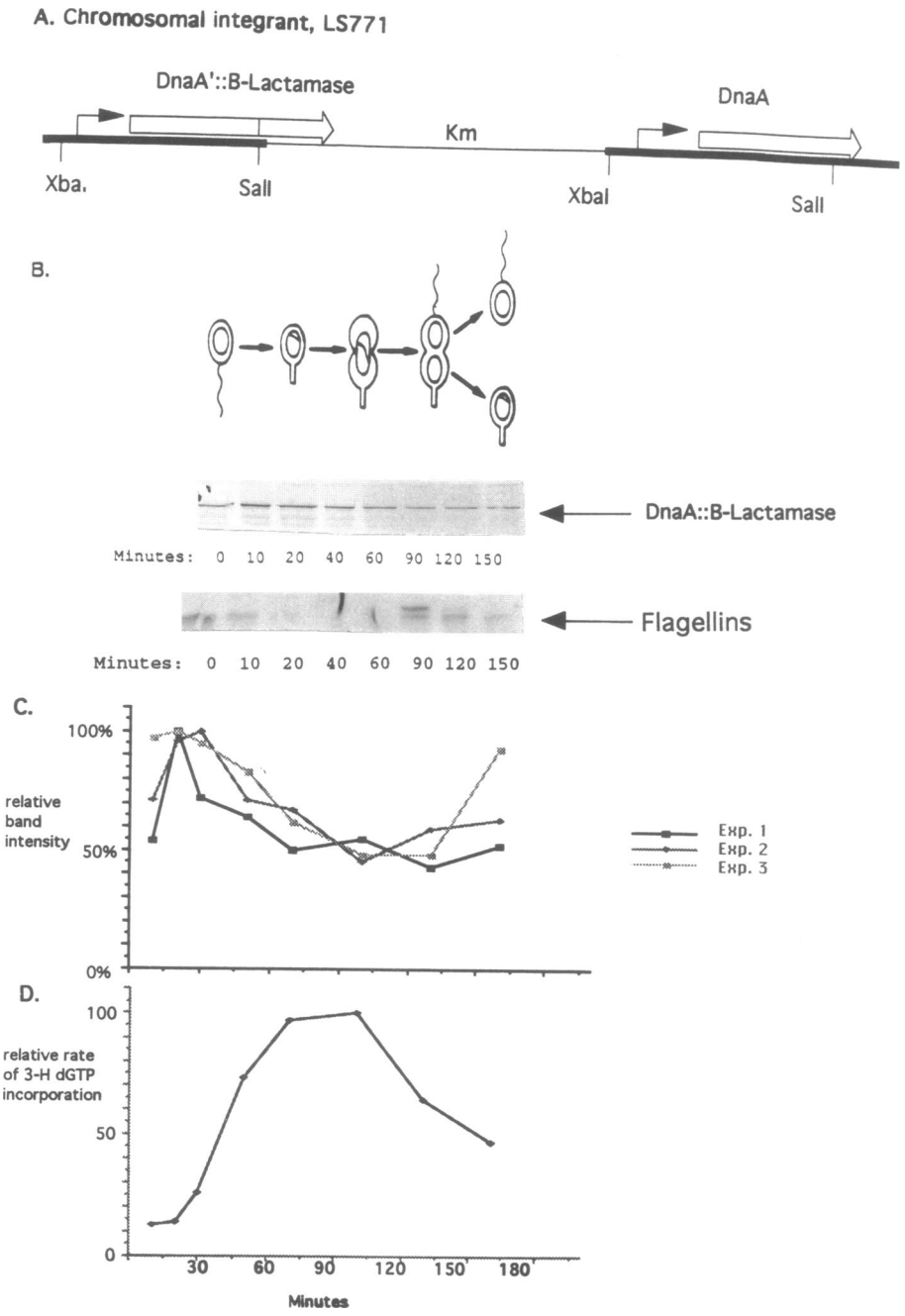

FIG. 5. Expression of a DnaA′–β-lactamase fusion protein as a function of the cell cycle. (A) Restriction map of the plasmid integration site at the *dnaA* gene of *C. crescentus* LS771. The thick line represents *C. crescentus* DNA, and the thin line represents vector DNA. DnaA′::β-lactamase and DnaA, shown with open arrows, are each expressed from copies of the native *dnaA* promoter. (B) Under a diagram of the *C. crescentus* cell cycle is an autoradiogram of the immunoprecipitation of ^{35}S-labeled DnaA′::β-lactamase from strain LS771 at the cell cycle time points indicated. The same samples were also immunoprecipitated with antiflagellin antibody as a control for the cell synchrony. An autoradiogram of ^{35}S-labeled flagellins is also shown. (C) The intensities of the autoradiogram patterns from three independent experiments of DnaA′::β-lactamase expressed during the cell cycle were measured by Molecular Dynamics software and used to generate the graph. (D) Incorporation of [^{3}H]dGTP into DNA was used to monitor the rate of DNA replication in a synchronized population of cells. The relative amount of incorporation is shown on the vertical axis. The 100% value represents 4.7×10^3 cpm.

integrants per microgram of transformed DNA. This finding indicates that interruption of sequences downstream of *dnaA* has no effect on viability in rich medium at normal growth temperatures. The introduction of a nonreplicative plasmid, pGZ15, with 785 bp of *dnaA* sequence internal to the coding region into *C. crescentus* NA1000 yielded only three colonies per microgram of transformed DNA. Integration by homologous recombination would have disrupted the *dnaA* gene, but Southern blot analysis of DNA from these three colonies indicated that the *dnaA* gene was intact. Therefore, acquisition

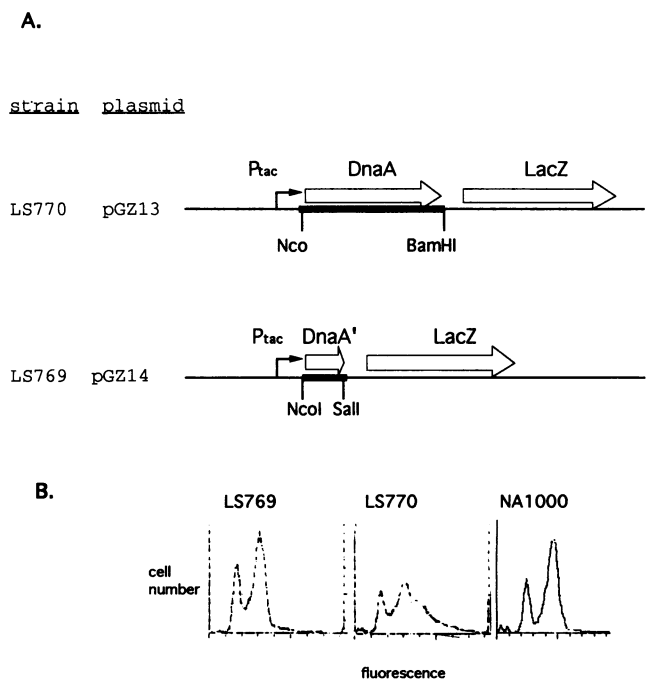

FIG. 6. FACS analysis of cells containing *dnaA*-expressing plasmids. (A) Partial maps of plasmids pGZ13 and pGZ14. *C. crescentus*-derived sequences are represented by thick lines; vector-derived sequences are represented by thin lines. The P_{tac} promoter is indicated by a thin arrow; DnaA, LacZ, and DnaA′ proteins are represented by open arrows. (B) *C. crescentus* LS770 (carrying plasmid pGZ13 containing the *dnaA* gene under control of the strong P_{tac} promoter), LS769 (carrying an isogenic control plasmid, pGZ14, deleted for the amino-terminal four-fifths of the *dnaA* gene [Fig. 6A]), and the wild-type strain NA1000 were examined by flow cytometry as described in Materials and Methods. The vertical axis indicates relative cell number. The horizontal axis indicates the relative amount of fluorescence.

of the selectable marker by these colonies was most likely the result of an illegitimate recombination event. The failure to obtain disruptions of *dnaA* suggests that such events may be lethal.

DISCUSSION

dnaA has been cloned from at least nine bacterial species to date. There is a growing body of evidence that the protein's sequence and replication functions have been conserved in an evolutionarily diverse group of bacteria (14, 16). A difference between the predicted *C. crescentus* DnaA protein and all others is that there are 21 amino-terminal amino acids that are found only in the *C. crescentus* protein. The regions of the bacterial DnaA protein that interact with DnaA boxes, the site of origin unwinding, membrane components, and other proteins are not known. Analysis of the growing array of wild-type and mutated DnaA proteins is being used to correlate domains with functions (3, 10, 45).

In *E. coli*, overexpression of *dnaA* can lead to additional DNA replication initiations that are often not completed (1a, 6, 33). The excess DNA content per cell that was found in *C. crescentus* cells in which the *dnaA* gene was expressed both from plasmid pGZ13 and from the chromosomal gene suggests that as in *E. coli*, excess DnaA in *C. crescentus* may induce additional initiations of chromosomal replication.

We found that expression of a DnaA fusion protein expressed from the *dnaA* promoter varies by twofold during the cell cycle, with peak expression occurring just prior to the stalked-cell phase. A sequence upstream of the start of *dnaA* transcription has homology to sigma 70-type *C. crescentus* promoters. The *C. crescentus dnaA* gene also has two potential DnaA boxes, one located 18 bp downstream of the transcription start site, but before the coding sequence, and the other located halfway through the coding sequence. In *E. coli*, there are DnaA boxes before and within the coding region that have been found to modulate *dnaA* expression (1a, 4, 35). It is possible that in *C. crescentus*, the DnaA protein also modulates its own expression, perhaps in a cell cycle-dependent manner.

Although the level of expression of a DnaA fusion protein appears to vary during the cell cycle, it is present in all cell types. DnaA is clearly available throughout the cell cycle, as IncP1 plasmids that require DnaA for replication were found to replicate throughout the cell cycle (28). However, the rate of plasmid replication is significantly increased in the stalked cell relative to the swarmer cell, perhaps indicative of limiting amounts of one or more replication factors. In contrast, chromosome replication is strictly limited to stalked and predivisional cells (7, 28). It may be that the level of the limiting replication factors is below the threshold required for chromosome replication. Ohta et al. have suggested that DNA synthetic enzymes may be selectively increased in stalked cells (31). They found that the rate of expression of a gene required for DNA elongation, referred to as *dnaC*, but not a homolog of the *E. coli dnaC* gene, is higher in stalked cells than in swarmer cells (31). We found that just prior to the initiation of replication in the stalked cell, the rate of expression of the initiator protein DnaA increased. The modulation in *dnaA* expression may contribute to the cell cycle regulation of the initiation of DNA replication. Although *dnaA* expression is clearly not limited to stalked cells, an increase in DnaA production in swarmer cells as they make the transition to stalked cells might actuate a critical concentration of DnaA in the nascent stalked cells, such that chromosomal replication is triggered. Alternatively, factors that have been found to activate *E. coli* DnaA in vitro, such as acidic phospholipids (41), ATP (40), or DnaK (23), may specifically activate DnaA in stalked cells. One *C. crescentus* mutant, with genotype *fatB*, cannot initiate replication without a fatty acid supplement, such as oleic acid (22). Since both *dnaK* expression (18) and phospholipid synthesis (27, 32) have been shown to be cell cycle regulated in *C. crescentus*, it will be interesting to determine whether cell cycle changes in DnaK or phospholipids activate DnaA. Perhaps activation of DnaA already present in the cell acts as a cell cycle-specific step that contributes to the regulated initiation of chromosome replication.

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