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# Transcription of Genes Encoding DNA Replication Proteins Is Coincident with Cell Cycle Control of DNA Replication in *Caulobacter crescentus*

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**DNA replication in the dimorphic bacterium *Caulobacter crescentus* is tightly linked to its developmental cell cycle. The initiation of chromosomal replication occurs concomitantly with the transition of the motile swarmer cell to the sessile stalked cell. To identify the signals responsible for the cell cycle control of DNA replication initiation, we have characterized a region of the *C. crescentus* chromosome containing genes that are all involved in DNA replication or recombination, including *dnaN*, *recF*, and *gyrB*. The essential *dnaN* gene encodes a homolog of the *Escherichia coli* β subunit of DNA polymerase III. It is transcribed from three promoters; one is heat inducible, and the other two are induced at the transition from swarmer to stalked cell, coincident with the initiation of DNA replication. The single *gyrB* promoter is induced at the same time point in the cell cycle. These promoters, as well as those for several other genes encoding DNA replication proteins that are induced at the same time in the cell cycle, share two sequence motifs, suggesting that they represent a family whose transcription is coordinately regulated.**

The bacterium *Caulobacter crescentus* proceeds through two main developmental intermediates during each cell cycle: a motile swarmer cell incapable of DNA replication and a sessile stalked cell that is replication proficient (reviewed in references 8 and 16). Regions of the *C. crescentus* chromosome containing homologs of *dnaA*, *dnaX*, *dnaN*, *recF*, and *gyrB* have been identified: the *dnaA* gene is linked to the origin of replication (31, 66), but the putative homologs of the genes downstream of *dnaA*, including *dnaN*, *recF*, and *gyrB* (which in most other bacteria are located immediately downstream of *dnaA*), were found clustered at least 115 kb away (47). The heat-inducible operon encoding *hrcA* and *grpE* is located immediately upstream of *dnaN* (48). We report here an analysis of the transcription and patterns of cell cycle regulation of the genes in the *dnaN* region of the chromosome.

The *recF* gene, which is shown to play a role in DNA repair, appears to be transcribed constitutively during the cell cycle (47). The RecF protein in both *Escherichia coli* and *Bacillus subtilis*, along with the *recA*, *recO*, and *recR* gene products, functions in the α or RecF pathway of DNA repair and recombination (2, 20; reviewed in reference 25). The *C. crescentus dnaN* gene is a homolog of the *E. coli* gene that encodes the β subunit of the *E. coli* DNA polymerase III holoenzyme, which in dimeric form acts as a sliding clamp to impart processivity to this polymerase (24; reviewed in reference 27). The β subunit is found as part of the holoenzyme at the site of concerted leading- and lagging-strand replication (58) and appears to be left behind upon completion of an Okazaki fragment, with the polymerase rapidly reassociating with a new β clamp (36, 59). Functional homologs of this protein have been identified in both higher eukaryotes (proliferating cell nuclear antigen [PCNA] [26]) and bacteriophage T4 (gp45 [22, 45]). In these systems, it appears that the sliding clamp functions in events beyond DNA replication. The gp45 sliding clamps may serve to activate transcription of the late class of phage T4 promoters in response to the process of DNA replication (19, 61). Eukaryotic PCNA subunits serve as a link between the detection of DNA damage and the consequent inhibition of DNA synthesis (15, 56, 63).

The essential *C. crescentus dnaN* gene is transcribed from three promoters, two of which share a pattern of temporal transcription with the *gyrB* gene and other genes encoding replication proteins; their transcription is stimulated several-fold just after the transition from swarmer to stalked cell is made. Analysis of the promoter sequences for *dnaN* and the linked *gyrB* gene, encoding a homolog of the β subunit of the type II topoisomerase DNA gyrase (reviewed in references 28 and 53), revealed two motifs, and 8-mer and a 13-mer, with strong sequence conservation among these promoters and the promoters of other DNA replication genes. This class of temporally controlled genes provides valuable access to the regulatory signals that mediates the swarmer-to-stalked cell transition.

## MATERIALS AND METHODS

**Materials.** Oligonucleotides were obtained from Operon Technologies. DNA-modifying enzymes, including restriction endonucleases, T4 DNA polymerase, T4 polynucleotide kinase, T4 DNA ligase, and Klenow enzyme, were obtained from Boehringer Mannheim or New England Biolabs and used according to the manufacturer's specifications. [$^{35}$S]methionine Trans-label was obtained from ICN, and [α-$^{35}$S]dATP, [α-$^{32}$P]dCTP, [$^{3}$H]dGTP, and [γ-$^{32}$P]ATP were obtained from Amersham. Sequencing was performed using a Taquence kit from U.S. Biochemicals. Other reagents were obtained from Sigma Chemical Co.

**Strains, growth conditions, and plasmids.** Bacterial strains used are listed in Table 1. *E. coli* cells were cultured in LB broth at 37°C (51), and *C. crescentus* cells were grown in PYE medium (42) or M2G minimal medium (13) at 30°C. For *C. crescentus*, ampicillin and nalidixic acid were used at a concentration of 20 μg/ml, kanamycin was used at 5 μg/ml, and tetracycline was used at 2 μg/ml. For *E. coli*, ampicillin and kanamycin were used at 50 μg/ml and tetracycline was used at 10 μg/ml.

Plasmids used in this study are also listed in Table 1. Sequencing of the region from *dnaN* to the 5′ end of *gyrB*, determined entirely on both strands, was based on subclones generated from the cosmid pGF-1 (47), using Taquence DNA polymerase and following the Sanger dideoxynucleotide chain termination method with single-strand templates (51). All of the sequences obtained were aligned and analyzed to identify putative open reading frames and potential

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TABLE 1. Strains and plasmids used

| Strain | Relevant genotype or characteristics | Reference or source |
|---|---|---|
| *E. coli* | | |
| TG1 | Δ*lac-pro* (F′ *lac*I$^q$ *proA*$^+$*B*$^+$ *lacZ*ΔM15) | 9 |
| S17-1 | F$^-$ *recA*, integrated RP4-2 Tc::Mu, Km::Tn7 | 54 |
| *C. crescentus* | | |
| NA1000 | Synchronizable derivative of wild type (also known as CB15N) | 14 |
| LS107 | NA1000 Δ*bla* | M. R. K. Alley |
| LS102 | NA1000 *rec-526* | 39 |
| LS1917 | NA1000 *flgI*::pDAG302 | 35 |
| LS2132 | LS107 Δ*dnaN::nptI* (with pRR304 in *trans*) | This work |
| LS2133 | LS107 Δ*dnaN::nptI* (with pRR304ΔH in *trans*) | This work |
| LS2290 | LS107 Δ*dnaN::nptI* wild-type *dnaN::xyl* | This work |
| LS2290 | LS107 Δ*dnaN::nptI dnaN*Δ$P_{hs}$::xyl | This work |
| LS2288 | NA1000 Δ*dnaN-recF* region, Tet$^r$ in one orientation | This work |
| LS2289 | NA1000 Δ*dnaN-recF* region, Tet$^r$ in other orientation | This work |
| LS2395 | NA1000 Δ*recF* | |
| Plasmids | | |
| pUC4K | Source of *nptI* cassette | 62 |
| pUC7 | Cloning vector | 62 |
| pBluescript SK(+) | Cloning vector | Stratagene |
| pAL4000 | Transcriptional reporter vector, source of Tet$^r$ | 18 |
| pRKlac290 | Cloning vector for transcriptional fusions to *lacZ* | 1 |
| pBGST18 | pBGS18 (57) with RK2 *oriT* inserted | M. R. K. Alley |
| pNPTS138 | pLitmus 38 vector with added *nptI*, *sacB*, and RK2 *ori*T sequences, deleted *bla* gene | M. R. K. Alley |
| pIC20R-*sacB* | Source of *sacB* gene | M. R. K. Alley |
| pMR20 | Mini-RK2 cloning vector; carries RK2 replication and stabilization functions | R. Roberts and C. Mohr |
| pRR158 | Source of RK2 Tet$^r$ region in pUC7 | This work |
| pRR244-1 | *Sma*I fragment carrying the *dnaN* promoter region cloned into the *Sma*I site of pBluescript SK(+) | This work |
| pRR261 | pMR20 carrying the *dnaN* gene from *Nco*I to *Nde*I | This work |
| pRR266 | pBluescript SK(+) carrying *sacB* and *dnaN*::*nptI* | This work |
| pRR304 | pMR20 carrying wild-type *dnaN* gene from *Stu*I to *Nde*I | This work |
| pRR304ΔH | pMR20 carrying Δ$P_{hs}$ *dnaN* gene from *Stu*I to *Nde*I | This work |
| pRR310-1 | pNPTS138 carrying *dnaN-recF* intergenic region disrupted by RK2 Tet$^r$ in one orientation | This work |
| pRR310-2 | pNPTS138 carrying *dnaN-recF* intergenic region disrupted by RK2 Tet$^r$ in opposite orientation | This work |
| pRR318 | pBGST18 with an internal fragment of the *recF* gene | This work |
| pRR319 | pMR20 carrying wild-type *recF* gene as a *Pst*I fragment | This work |

homologous genes from other organisms (in PIR and SWISSPROT databases), using the Genetics Computer Group sequence analysis program package (11).

The regions upstream of *dnaN* or *gyrB* were analyzed for promoter activity by creating deletions of these regions first in pBluescript SK(+), using the restriction sites diagrammed in Fig. 4 for *dnaN* and Fig. 8A for *gyrB*. These deletions were transferred to the reporter vector pRKlac290 to create *lacZ* transcriptional fusions oriented such that transcriptional activity toward *dnaN* or *gyrB* was assayed.

Transcriptional regulatory activity downstream of *dnaN* was assessed by generating transcriptional fusions of deletions with or without the downstream region to *lacZ* in the transcriptional reporter vector pRKlac290. The deletions used are diagrammed in Fig. 7B; the restriction sites used are indicated. The terminator region was subcloned by PCR amplification with oligonucleotides introducing an *Asp*718 site within *dnaN* (using the oligonucleotide 5′-CGTCTG CGGTACCATCGCCGGTCC-3′) and an *Xba*I site on either side of the putative terminator sequence (using either 5′-GATGACCCGGCTCTAGATCAGACC C-3′ or 5′-GCCCGGCGAATCTAGAAAGCAAAAACG-3′). These PCR-amplified products were then introduced in the proper reading frame downstream of the *dnaN* promoter region (*Nco*I to *Hin*dIII) in pRKlac290.

To disrupt the chromosomal *dnaN* gene, *dnaN* with flanking sequence both upstream and downstream was subcloned to pBluescript SK(+) with approximately 2 and 1 kb of flanking upstream and downstream sequences, respectively. The *nptI* cassette from pUC4K was inserted internally to replace 507 bp of the 5′ half of *dnaN*, between the two *Hin*dIII sites (see Fig. 4). The *sacB* gene of *B. subtilis* was then inserted into the polylinker of this plasmid as an *Xba*I fragment from pIC20R-*sacB* to create the plasmid pRR266. Disruption of the region between the *dnaN* and *recF* genes was accomplished by subcloning a fragment containing this region plus flanking sequence on either side into pNPTS138, a vector which carries *nptI* and *sacB* for counterselection of plasmid loss. A 640-bp segment of the *dnaN-recF* intergenic region between *Nde*I and the most distal *Stu*I site was deleted and replaced with the tetracycline resistance (Tet$^r$) locus in either orientation from plasmid pRR158. The resulting disruption plasmids were named pRR310-1 and pRR310-2, to reflect the two orientations of the inserted Tet$^r$ locus. pRR158 carries the 2.3-kb *tetA-tetR* region from plasmid RK2 cloned as a blunted *Eco*RI fragment from plasmid pAL4000 into a single *Hin*cII site of pUC7, oriented with the *Pst*I site of the polylinker adjacent to *tetA*. Inactivation of *recF* was accomplished by a single homologous recombination using a ~550-bp internal fragment of the *recF* gene, from *Xho*I to *Bsm*I (see Fig. 8A), inserted in the polylinker of pBGST18. This plasmid was called pRR318.

Complementation of the *dnaN* or *recF* disruption strains was tested by using plasmids carrying these genes in *trans*. Such plasmids included the *dnaN* region from *Nco*I to *Nde*I or the *recF* region as a 2.4-kb *Bam*HI fragment, cloned into the polylinker of the mini-RK2 broad-host-range replicon pMR20 (carrying replication and plasmid stabilization functions as well as a polylinker, *oriT*, and Tet$^r$) to create pRR261 (*dnaN*) or pRR319 (*recF*). Further pMR20-based vectors were also generated to supply *dnaN* in *trans*, carrying this gene from the *Stu*I site upstream to the downstream *Nde*I site either with a wild-type promoter region (pRR304) or with the heat shock promoter ($P_{hs}$) inactivated (pRR304ΔH).

**Disruption of genes in the *dnaN* region of the *C. crescentus* chromosome.** The *dnaN* gene and the region between *dnaN* and *recF* were independently disrupted by double homologous recombination, using sucrose resistance as selection of the second recombination event, as described elsewhere (7). For *dnaN* inactivation, plasmid pRR266, carrying *dnaN* disrupted with the *nptI* cassette encoding kanamycin resistance, was integrated into the chromosome of *C. crescentus* LS107 by homologous recombination, to generate strain LS2036 by selecting for acquisition of resistance to both kanamycin and ampicillin (plasmid vector encoded). This strain was diploid for the targeted genes, with one copy wild type and the other disrupted. Such a strain, with or without an added plasmid in *trans* to supply the gene product of the targeted disruption, was plated on medium containing 3% sucrose. Cells able to grow had either inactivated the integrated

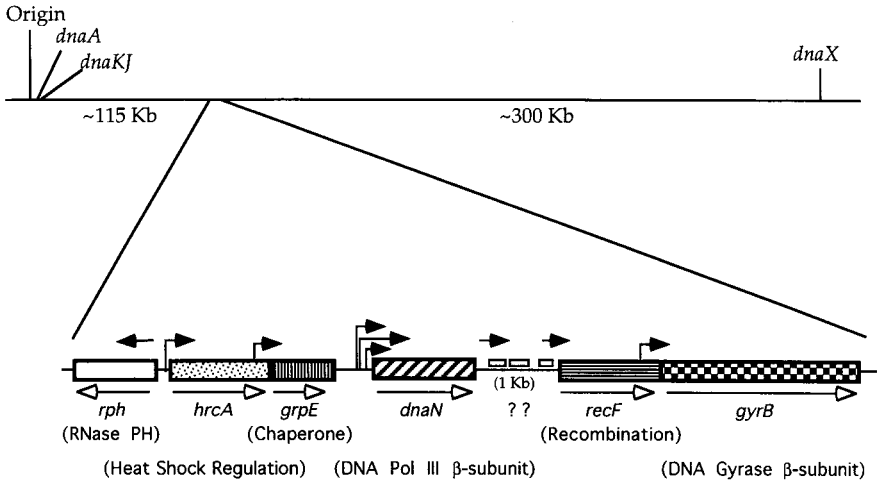


FIG. 1. Genetic region surrounding the *C. crescentus dnaN* gene. The genes flanking *dnaN* are indicated, with direction of transcription indicated by the open arrows below the line. The genetic structure and function of the ~1-kb sequence between *dnaN* and *recF* are unknown, as indicated by the question marks; small open boxes above the region indicate the approximate positions of the three putative open reading frames. Closed arrows above the line indicate transcriptional activity, bent arrows indicate promoters with known start sites (by primer extension), and straight arrows indicate promoter activity approximately located by transcriptional fusions. The proposed functions for each of these genes are shown below the gene names. This region is located >100 kb from the clustered origin of replication, *dnaA* and *dnaKJ* genes (47), and ~300 kb from the *dnaX* gene (64), as indicated by the line diagram above the *dnaN* genetic region. Pol, polymerase.

*sacB* gene (~10% of the colonies) or excised the integrated plasmid by a second homologous recombination (~90% of the colonies), thus leaving behind either the native or the disrupted gene. Determination of antibiotic resistance profiles allowed these events to be distinguished. For disruption of the region between *dnaN* and *recF*, the same strategy was used, integrating either pRR310-1 or pRR310-2 and then using *sacB* counterselection to eliminate the wild-type region to generate strains LS2288 and LS2289. Chromosomal disruption of these regions was confirmed by Southern blot analysis.

*recF* was disrupted by introducing plasmid pRR318 (carrying an internal fragment of the *recF* gene) into NA1000 by conjugal mating and then selecting for integration by acquisition of kanamycin resistance. Such an integrant, called LS2395, has two incomplete halves of *recF*, thus disrupting its function. Proper integration was confirmed by Southern blot analysis.

**Quantitation of recombination proficiency.** Strains disrupted for either the *recF* gene (LS2395) or the region between *dnaN* and *recF* (LS2288 and LS2289) were assayed for susceptibility to UV irradiation as described previously for analysis of the *C. crescentus rec-526* mutation in LS102 (39). Recombination proficiency was also monitored by determining the capacity of these strains to integrate exogenous DNA by generalized transduction. Strain LS1917 carries a *flgI* gene disrupted with the Ω cassette (35). A φCR30 transducing lysate was prepared from this strain and used for transduction into wild-type strain NA1000 or strain LS102, LS2288, LS2289, or LS2395. Successful transduction was determined by acquisition of the proper antibiotic resistances (spectinomycin, streptomycin, and gentamicin) and disruption of motility.

**Site-directed promoter mutagenesis.** Site-directed mutagenesis was used for the inactivation of the three putative *dnaN* promoters by the protocol of Kunkel (as described in reference 51). Single-stranded DNA was generated from pRR244-1 [pBluescript SK(+) carrying a *Sma*I fragment encoding the *dnaN* promoter region], and then mutagenic oligonucleotides were used to prime synthesis of the complementary strand. Transformation into the mutagenic *E. coli* BMH 71-18 *mutS* allowed subsequent mutant identification by restriction digestion and sequencing. The oligonucleotides used were 5′-GAACCATTCTC AACCCCGGGCATCTCGATG-3′ for $P_{hs}$ inactivation, 5′-CTTGCGGTGCGG GCCGCGGGCTCCATTTA-3′ for $P_{distal}$ inactivation, and 5′-GGGACCGCCG ACCGGTGCGAAAAGACGC-3′ for $P_{proximal}$ inactivation. For mutagenesis of the 8-mer boxes upstream of *dnaN* $P_{proximal}$, the oligonucleotides used were 5′-GGAAGAGGCCGGGGCCCTTGCGGTGC-3′ and 5′-CGACATTAGCGC TAAGACGCGCGC-3′, targeting the upstream and downstream boxes, respectively (underlined bases differ from the wild-type sequence).

**Primer extension.** For primer extension reactions, oligonucleotide primers with sequences complementary to the predicted RNA sequences at the 5′ end of the *dnaN* and *gyrB* genes (5′-GCTTCATAGTCCGGTCCAGATGTC-3′ and 5′-CAGCGACTCGTCCGTGCCG-3′, respectively) were first radioactively labeled by using T4 polynucleotide kinase and [γ-$^{32}$P]ATP as described elsewhere (51). Approximately 0.5 pmol of labeled primer was then annealed to 40 μg of yeast tRNA (included as a control) or to 40 μg of *C. crescentus* cellular RNA that was isolated via standard protocol (51) from cells grown at 30 or 42°C for 5, 10, 15, or 20 min. These oligonucleotides were then used as primers for the enzyme reverse transcriptase. The reaction products were separated on a 6.5% polyacrylamide sequencing gel and autoradiographed, using DNA sequencing products obtained with these same primers as standards to identify the positions of the transcriptional start sites (51).

**Promoter expression in synchronous cultures.** Transcription of the *dnaN* promoters was examined to determine their pattern of expression during the cell cycle. Cultures of *C. crescentus*, harboring the plasmid-borne *dnaN* transcriptional fusions to *lacZ*, were synchronized as described in reference 14. At various times during synchronous progression through the cell cycle, 1-ml aliquots of cells were incubated for 5 min with 1 μCi of [$^{35}$S]methionine Trans-label to label newly synthesized proteins and then centrifuged to collect the cells, and these cells were lysed and immunoprecipitated as described previously (21). Equivalent counts per sample were simultaneously incubated with anti-β-galactosidase and antiflagellin antibodies. The flagellins were monitored as an internal control and indicator of the quality of cell synchrony.

For the mock synchronization to test whether *dnaN* and *gyrB* promoter induction was the result of this process, *C. crescentus* cells carrying the reporter fusions were synchronized to separate swarmer cells from stalk and predivisional cells, and then these two populations were remixed. The cells were treated as usual for the remainder of the protocol, including the labeling and processing of the [$^{35}$S]methionine samples.

**Measurement of DNA synthesis rates.** The rate of cellular DNA synthesis was monitored to assess either the effect of the deletion of *dnaN* $P_{hs}$ or of a disruption in the chromosomal region between *dnaN* and *recF*, or alternately to determine the time of DNA replication initiation in synchronous cultures. The synthesis rate was determined in duplicate as described previously (31), using a short (2-min) pulse of [$^{3}$H]dGTP to label only DNA and then both rapidly lysing the cells and precipitating the nucleic acids by dilution in 10% trichloroacetic acid. The labeled DNA was recovered and counted to estimate total incorporation over the pulse period.

**Nucleotide sequence accession number.** The DNA sequence data described in this work have been deposited in GenBank with accession number U37793.

## RESULTS

**Analysis of the genes and open reading frames in the *dnaN* region.** In previous work, Rizzo et al. identified the *C. crescentus* homolog of the *gyrB* gene by complementation of novobiocin-resistant mutants and by sequence analysis of a short region of the predicted amino terminus (47). As in other organisms, the *gyrB* homolog resides downstream of *recF* and *dnaN* homologs (Fig. 1). The complete DNA sequence of this region, extending approximately 7 kb upstream of the *gyrB* gene, was determined. In most bacteria, the *dnaA* gene and the chromosomal origin of replication are located directly upstream of *dnaN*. However, in *C. crescentus*, while *dnaA* is linked to the origin of replication (31, 66), the *dnaN* gene cluster is at least 115 kb away (47), while a heat shock operon

and an RNase gene are located directly upstream of *dnaN* (48). Analysis of partial sequence information from the region downstream of *gyrB* (data not shown) demonstrated that the *gyrA* gene must be located elsewhere, as is generally the case in gram-negative bacteria.

The predicted amino acid sequence for the *C. crescentus* DnaN showed substantial similarity to DnaN homologs from other organisms (Fig. 2A), with identity ranging from 19.8% with the *Borrelia burgdorferi* homolog to 40.1% with the *Pseudomonas putida* homolog. The *dnaN* translational start site that best aligns with a consensus *C. crescentus* Shine-Dalgarno sequence is located just prior to the first *Hin*dIII restriction site (see Fig. 6), with no other potential upstream start codons in this reading frame. The in vivo use of this start site was confirmed by constructing fusions to *lacZ* at this *Hin*dIII site in a translational fusion vector series (which contain the *lacZ* reporter devoid of any transcriptional or translational start signals preceded by a polylinker): only the predicted *dnaN* reading frame produced a functional fusion in *C. crescentus* (575 Miller units [34] of activity versus 12 U for the two out-of-frame fusions), thus confirming assignment of the start of DnaN translation.

Downstream of *dnaN* there is a 996-bp gap between the end of *dnaN* and the start codon for *recF* (Fig. 1). Three short open reading frames in this region showed no significant similarity to proteins in PIR or SWISSPROT database, and *Caulobacter*-specific codon preference for all of these potential open reading frames was quite poor. The presence of a gap between *dnaN* and *recF* coding sequences is not novel; such gaps are also seen between the homologous genes in *Staphylococcus aureus* (32), *Mycobacterium genitalium* (6), and *B. subtilis* (37). A 640-bp *Nde*I-to-*Stu*I segment of the chromosomal region between *dnaN* and *recF*, comprising the first two of the open reading frames but not the proposed promoter for *recF* or the third open reading frames was replaced with a cassette encoding the RK2 Tet$^r$ gene inserted in either orientation. Such strains showed no discernible defect in the rate of growth or DNA synthesis at 30 or 42°C, response to heat shock, sensitivity to UV irradiation, or generalized transduction, a test of recombination proficiency (results not shown). These results suggest that this region does not have a major role in cellular processes under laboratory conditions.

The predicted RecF amino acid sequence showed a lower degree of conservation with other bacterial RecF proteins, with identities ranging from 24.5 to 33.7% (Fig. 2B). The last codon of the *recF* homolog overlapped the proposed start codon for *gyrB*, suggesting the possibility of translational coupling (addressed below). Finally, the N-terminal region of *C. crescentus* GyrB showed substantial similarity with other GyrB and ParE topoisomerase genes (not shown), with identities as high as 73.1% with *E. coli gyrB* homolog (65).

**(i) The *C. crescentus dnaN* homolog is an essential gene.** The β subunit of DNA polymerase III is an essential gene in *E. coli* (49). To determine if *dnaN* is essential in *C. crescentus*, an attempt was made to replace the chromosomal gene with a disrupted copy of *dnaN* by using homologous recombination. While a single crossover resulting in the duplication of *dnaN* (one copy wild type and the other defective) was easily obtained, a second recombination resulted exclusively in loss of the defective copy; in 1,357 colonies tested that had undergone a second homologous recombination, every one had deleted the disrupted copy of *dnaN*. This finding suggests that the *C. crescentus dnaN* gene is essential. This was confirmed by demonstrating that if a functional *dnaN* gene was supplied in *trans* from plasmid pRR261, the chromosomal *dnaN* gene could be disrupted readily (135 colonies disrupted out of 199 colonies tested). Subsequent attempts to displace pRR261 from the strain lacking a chromosomal *dnaN* were unsuccessful, while this plasmid could readily be displaced from the parental NA1000 strain (results not shown), indicating that pRR261 is essential in the *dnaN* disruption strain.

**(ii) The *C. crescentus recF* homolog has a role in recombination.** A plasmid carrying an internal fragment of the *recF* gene was integrated into the *C. crescentus* chromosome by homologous recombination to disrupt *recF* (by creating two defective chromosomal copies, one lacking the 5′ end of the gene and the other lacking the 3′ end). This strain showed increased susceptibility to UV irradiation (Fig. 3), suggesting that, as in *E. coli* (20) and *B. subtilis* (2), RecF plays a significant role in recombination, most notably in DNA damage repair. The increased susceptibility to UV irradiation was complemented only by providing an intact copy of the *C. crescentus recF* gene in *trans* from plasmid pRR319 (Fig. 3). The UV sensitivity was similar in degree to the disruption of another *C. crescentus* recombination gene (*rec-526* [39]). However, disruption of *recF* produced no discernible reduction in φCR30-mediated generalized transduction, whereas the *rec-526* mutation dramatically reduced transduction efficiency (results not shown), confirming that *rec-526* lies in a different gene that also functions in recombination, perhaps *recA*.

**Transcriptional regulation of the *dnaN* gene. (i) Promoter localization.** The promoters responsible for the transcription of *dnaN* were first localized by fusing upstream DNA fragments of *dnaN* to the promoterless *lacZ* gene (Fig. 4B and C). Analysis of β-galactosidase synthesis from the resulting nested deletions, proceeding in both 5′ and 3′ directions, indicated that a 200-bp fragment between *Bbs*I and *Hin*dIII has the majority of the transcriptional activity. The decreases in transcriptional activity observed as deletions were constructed proceeding toward *dnaN* (Fig. 4B) are likely due to alteration in positioning of the fragment within the reporter vector. The low level of activity present in the *Hin*dIII-*Hin*dIII fragment may indicate that weak transcriptional activity initiates from within the *dnaN* gene. Further subdivision of *Bbs*I to *Hin*dIII fragment (Fig. 4D) revealed two distinct promoter activities: a stronger activity proximal to *dnaN*, between *Bss*HII and *Hin*dIII (~2,000 Miller units), and a weaker activity distal to *dnaN*, between *Bbs*I and *Bss*HII (~600 Miller units).

These promoters, labeled $P_{proximal}$ and $P_{distal}$ (Fig. 4A), were precisely localized by determining their transcriptional start sites using primer extension (Fig. 5). At physiological growth temperature (30°C), we observed two major transcriptional start sites, located proximal and distal to *dnaN* and separated by the *Bss*HII restriction site, as expected from the activity of the *lacZ* transcriptional fusions shown in Fig. 4. The identities of these start sites were confirmed by S1 nuclease protection assays (not shown). It was noted that the *Bss*HII site was located between the $P_{proximal}$ −35 and −10 regions; bisection here would be expected to inactivate $P_{proximal}$. In looking at the sequence of the *Bss*HII fusion made in the vector polylinker, we noted that the new sequence placed adjacent to the remaining $P_{proximal}$ −10 region bears significant similarity (five-of-six match) to the native $P_{proximal}$ −35 region and thus is likely to functionally substitute for it.

In addition to these promoters, examination of sequences upstream of *dnaN* revealed a $\sigma^{32}$ heat shock recognition sequence (Fig. 6A) with significant identity to known *C. crescentus* heat shock promoters (4, 17, 46, 48). Examination of transcription of the *dnaN* region at elevated temperatures showed that transcription from this putative heat shock promoter could be detected after a shift to 42°C (Fig. 5), confirming that this promoter (labeled $P_{hs}$) is used in vivo and is indeed heat

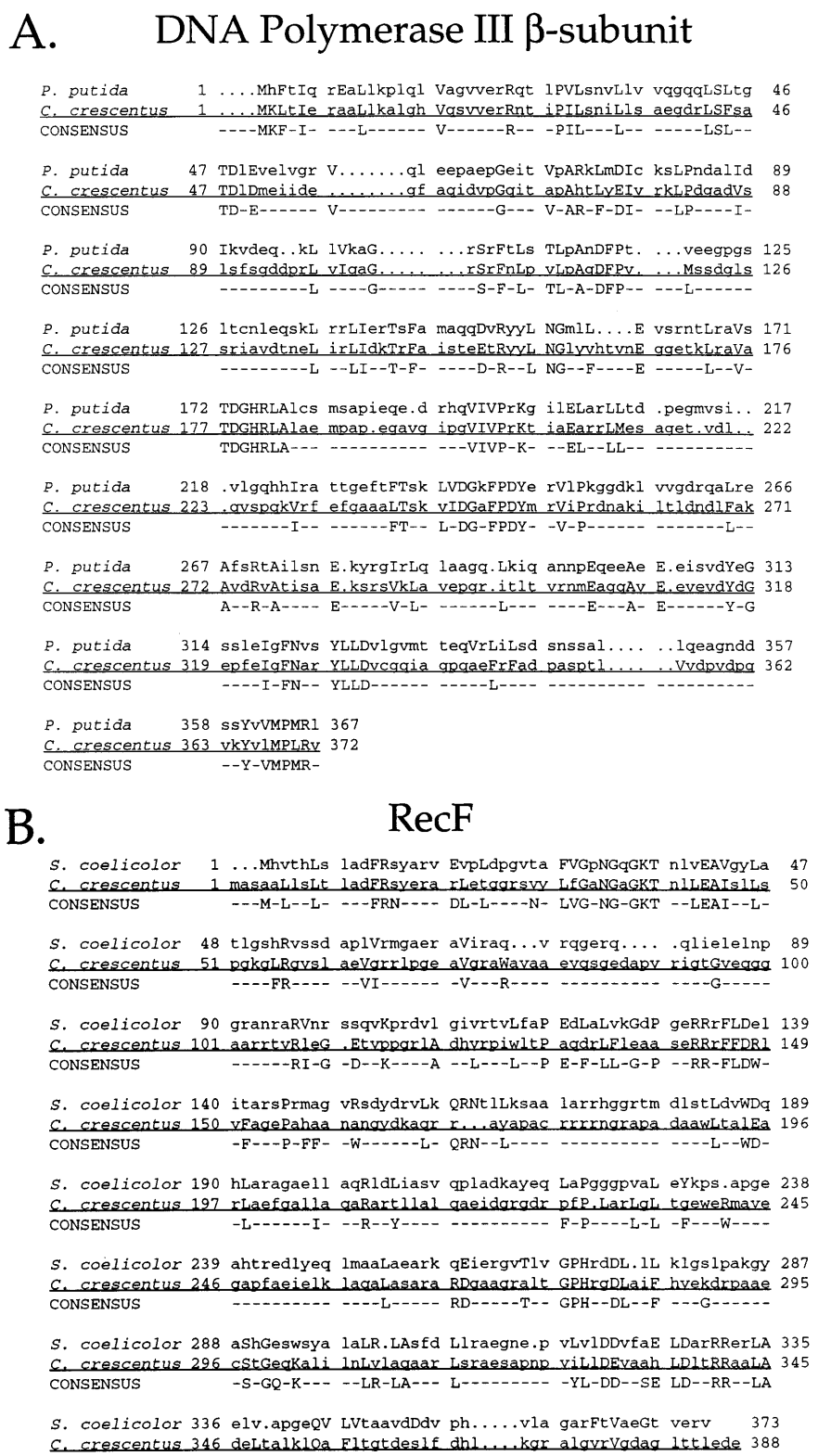


FIG. 2. Alignment of *C. crescentus* DnaN and RecF homologs. The predicted amino acid sequences for *dnaN* and *recF* were aligned with those for homologous genes from PIR and SWISSPROT databases. (A) Alignment of DnaN with the DnaN protein of the most highly conserved homolog, from *P. putida* (accession number P13455; 40.1% overall identity). The consensus sequence indicated was derived from alignment of eight full-length DnaN proteins; conserved residues (either identical or chemically similar) present in six of the eight homologs are indicated by capital letters. The additional DnaN genes used to calculate the consensus were from *Proteus mirabilis* (accession number P22838; 40.3% identity with *C. crescentus dnaN*), *E. coli* (accession number P00583; 38.0% identity), *B. subtilis* (accession number P05649; 31.9% identity), *Buchnera aphidicola* (accession number P29439; 27.9% identity), *S. coelicolor* (accession number P27903; 27.7% identity), and *Borrelia burgdorferi* (accession number S34928; 19.8% identity). (B) Alignment of *C. crescentus* RecF with the homolog from *S. coelicolor* (accession number P336176; 33.7% identity). The consensus sequence indicated was derived from alignment of eight full-length RecF proteins; conserved residues (either identical or chemically similar) present in six of the eight homologs are indicated by capital letters. The additional RecF genes used to calculate the consensus were from *P. putida* (accession number X62504; 27.5% identity with the *C. crescentus* RecF), *E. coli* (accession number A03547; 25.9% identity), *Salmonella typhimurium* (accession number X62505; 25.6% identity), *B. subtilis* (accession number P05651; 25.4% identity), an *Actinomyces* sp. (accession number P24718; 25.1% identity), and *P. mirabilis* (accession number M58352; 24.5% identity).

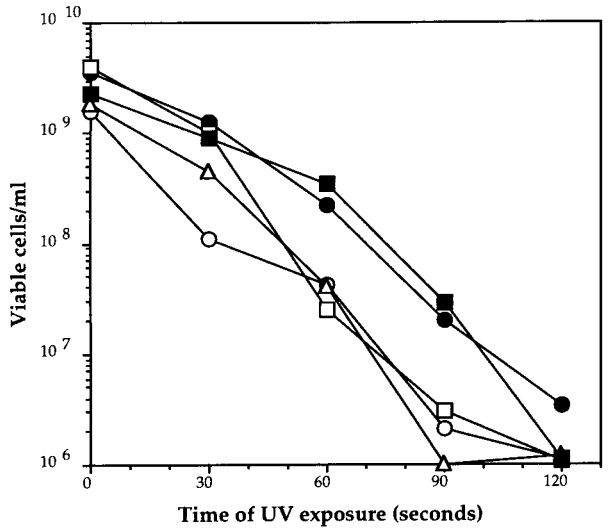


FIG. 3. UV light susceptibility of *recF*-disrupted *C. crescentus*. Cells with the indicated genotypes (all derivatives of NA1000) in late log phase were exposed to a germicidal UV lamp for the indicated times, at which aliquots were removed to determine the number of viable cells remaining (plotted). Closed circles, NA1000; open circles, LS102 (39); open squares, LS2395; open triangles, LS2395 carrying the vector pMR20; closed squares, LS2395 carrying pRR319, which encodes an intact copy of the *C. crescentus recF* gene.

inducible. The diminishing of $P_{distal}$ and $P_{proximal}$ transcripts and concomitant appearance of a number of apparently intermediate-size RNAs upon heat shock is likely related to a heat-induced shutoff of these promoters and breakdown of their transcriptional products.

**(ii) Functional confirmation by site-directed mutagenesis.** To confirm the assignment of these promoter elements and to examine their use in vivo, we made site-directed mutations, targeting A/T-rich sequences in the −10 regions (Fig. 6A). The mutated −10 regions, in the context of the whole promoter region, were fused to *lacZ* in pRKlac290 (Fig. 6B). Transcriptional analysis of these mutated promoters by primer extension, using a pRKlac290-specific oligonucleotide, demonstrated proper inactivation of each targeted promoter (not shown). The β-galactosidase activity of each of these transcriptional fusions (Fig. 6B) showed that inactivation of $P_{hs}$ had little effect on overall transcriptional activity at 30°C; activity from the mutated promoter was also undetectable at elevated temperatures when assayed by primer extension (not shown). Inactivation of $P_{distal}$ resulted in a 22% decrease in overall activity, proportional to what one would predict based on the relative strengths of $P_{distal}$ and $P_{proximal}$ demonstrated by the *lacZ* fusions in Fig. 4D. Four base pair changes in the $P_{proximal}$ −10 region abolished all transcription from this region, suggesting that $P_{proximal}$ is the major promoter for *dnaN* expression. Absence of detectable expression from $P_{distal}$ in the $P_{proximal}$ mutant may indicate that this particular mutation had a general adverse effect on transcription or translation of *dnaN*, possibly by introducing secondary structure which affects either the processing of the $P_{distal}$ mRNA or its translation.

**(iii) Role of the heat-inducible promoter transcribing *dnaN*.** In general, bacterial DNA replication genes are not a part of the heat shock response, and it was therefore surprising to detect a *dnaN* promoter, $P_{hs}$, that conformed to a $\sigma^{32}$ consensus sequence and was activated upon shift to 42°C. The strategy chosen to address the significance of $P_{hs}$ transcription of *dnaN* was to disrupt the chromosomal copy of this promoter and assay the phenotype of the resulting mutant strain. This approach was complicated, however, by heat-inducible transcription from the upstream *hrcA-grpE* operon that continued into *dnaN* (48). To ensure inactivation of heat-inducible *dnaN* expression, both readthrough from the *hrcA-grpE* operon and transcription from $P_{hs}$ had to be eliminated. This was accomplished by first relocating *dnaN* to a plasmid vector, either with a wild-type promoter region (pRR304) or with $P_{hs}$ disrupted by site-directed mutagenesis at the −10 region (pRR304ΔH). When the native chromosomal *dnaN* gene was disrupted, leaving only the relocated gene to provide the β subunit of DNA polymerase, no difference could be detected between the non-heat-inducible *dnaN* and its isogenic parent (results not shown). Parameters examined included growth rates at 30, 37, and 42°C, survival at elevated temperatures, establishment of a thermotolerant state, and rates of DNA synthesis at 30 and 42°C. In a separate experiment, *dnaN* with or without a functional $P_{hs}$ was moved by homologous recombination to the *xyl* locus on the *C. crescentus* chromosome (33), under the control of its own promoters (and not the *xyl* promoter). Disruption of the native *dnaN* genes in these strains produced the same results as seen for the plasmid-borne *dnaN* studies: no detectable phenotypic difference with or without $P_{hs}$ (not shown). While this finding does not prove that $P_{hs}$ transcription plays no role in the cell, it does suggest that such a role must be somewhat limited in scope or significance.

**(iv) Transcription termination.** Initial examination of the sequence following the stop codon for *dnaN* demonstrated the presence of an inverted repeat followed by a relatively T-rich region (Fig. 7A), much like ρ-independent transcriptional terminator sequences in *E. coli* (41). To determine whether this element acts as a transcriptional terminator in *C. crescentus*, deletions from this region were generated and transcriptionally fused to *lacZ* in pRKlac290 (Fig. 7B). Analysis of the transcriptional activity from fusions with the *dnaN* promoter region with or without the inverted repeat motif demonstrated that this element does function as a weak transcriptional terminator, reducing transcriptional readthrough by more than 60%. When the *dnaN* promoter region was deleted, transcriptional activity could be detected if 60 bp downstream of the terminator sequence was present (Fig. 7B), demonstrating the presence of another promoter immediately downstream of *dnaN*. This promoter is most likely the same as the weak, cell cycle-regulated promoter previously identified (47).

**Transcriptional and translational start site determination for the *gyrB* gene.** Since the products of both the *dnaN* and *gyrB* genes are involved in the process of DNA replication, a closer examination of the *gyrB* gene was initiated. Initial experiments have mapped the *gyrB* promoter to a 700-bp fragment (47). To more precisely determine the location and sequence of the *gyrB* promoter(s), subclones of the region upstream of the coding sequence for *gyrB* (all within the *recF* coding sequence) were fused to *lacZ* and transcriptional activity was examined (Fig. 8). The most significant activity was localized to a 180-bp region between *Nar*I and *Stu*I restriction sites; the low level of expression from the *Stu*I-*Sal*I fragment may represent a weak secondary promoter. One 9-bp sequence within this region was particularly A/T rich and also encoded quite rare codons within *recF*, both clues that it might form a part of the *gyrB* promoter element. Primer extension reactions confirmed the location of the transcriptional start site (Fig. 8B), aligning with the A/T-rich region at approximately −10.

The proposed translational start site for *gyrB* (47) overlaps the identified stop codon for *recF*. However, another potential start codon is present 48 bp downstream; use of this second start codon would eliminate most of the N-terminal region of

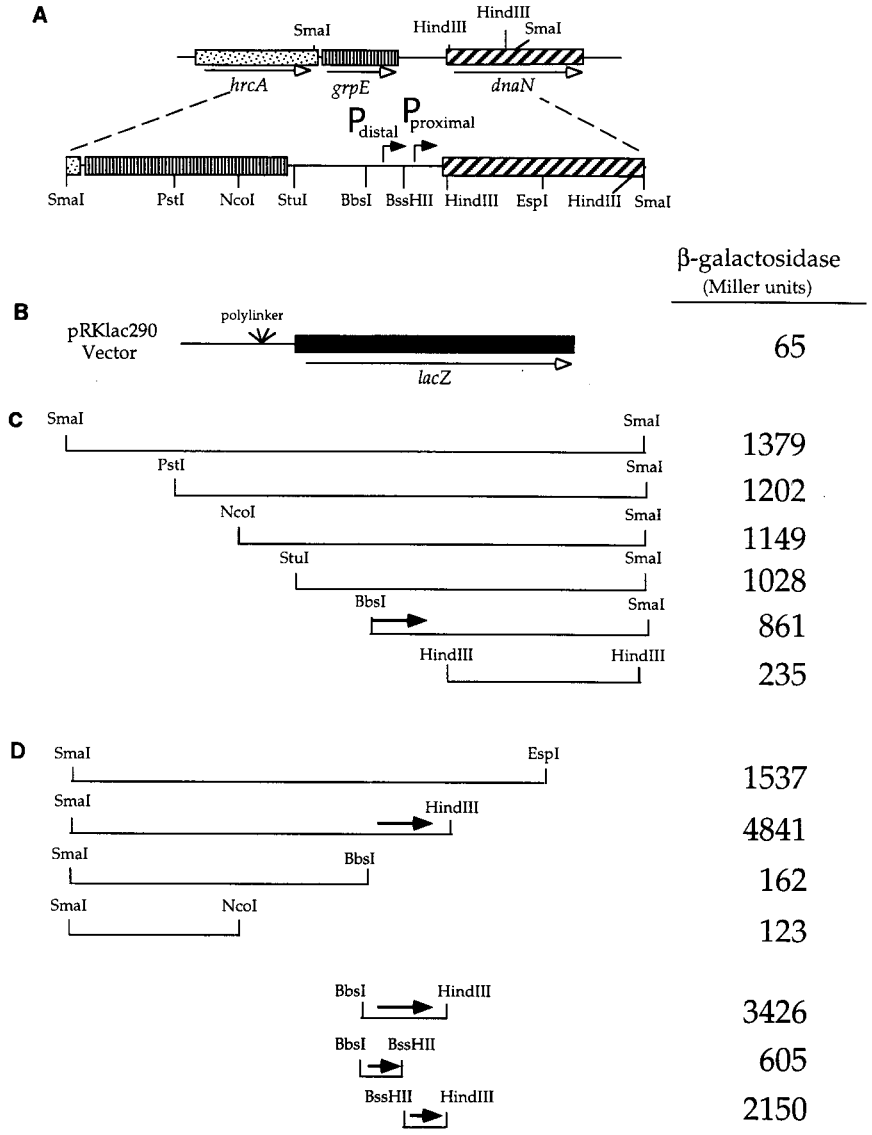


FIG. 4. Genetic localization of promoters transcribing *dnaN*. (A) Restriction fragment subclones of the region upstream of *dnaN* were tested for transcriptional activity by fusing them to a promoterless *lacZ* reporter gene in the vector pRKlac290 (1). The fragments tested are indicated below the genetic map, and the resulting β-galactosidase activities averaged from triplicate assays are reported as Miller units (34). (B) Deletions proceeding from upstream of *dnaN*. (C) Deletions proceeding from downstream. (D) Defined minimal fragments. Solid arrows indicate the general location of transcriptional activity as defined by these data. Note that the order of restriction sites has been corrected from that given in reference 47.

*C. crescentus* GyrB that does not align with other family members (47). (It should be noted that the 16 codons between the two potential start sites follow typical *C. crescentus* codon usage [not shown].) To determine which of these start codons are used in vivo, fusions to *lacZ* in a translational fusion vector series were generated at a *Sal*I site located between these two putative start sites. A fusion at this site should synthesize the reporter β-galactosidase only if the upstream translational start site is used in the proper reading frame. When assayed, a high level of expression was indeed observed (2,566 Miller units), specific to the *gyrB* reading frame (fusions made out of frame for *gyrB* generated only 9 to 12 U of activity). This finding confirms that the 5′ start codon is used and reinforces the possibility of translational coupling between these two genes.

**Expression of the *dnaN* and *gyrB* genes as a function of the cell cycle.** Replication of the *C. crescentus* chromosome occurs specifically during the stalked-cell phase of the cell cycle (10, 12, 31). It has been reported that transcription of several genes involved in the process of replication, including *dnaA* (66), *gyrB* (47), an uncharacterized gene referred to as *dnaC* (38), and more recently *dnaX* (64), is increased to various degrees at the start of the stalked-cell phase of the cell cycle. The temporal pattern of expression of *dnaN* (transcribed from the wild-type promoter region encompassing all three identified promoters) during the cell cycle was determined by transcriptionally fusing this region, from the *Sma*I site within *grpE* to the *Hin*dIII site at the N terminus of *dnaN*, to the reporter gene *lacZ* in pRKlac290. Independently, the more precisely localized *gyrB* promoter was also fused to *lacZ* in this vector.

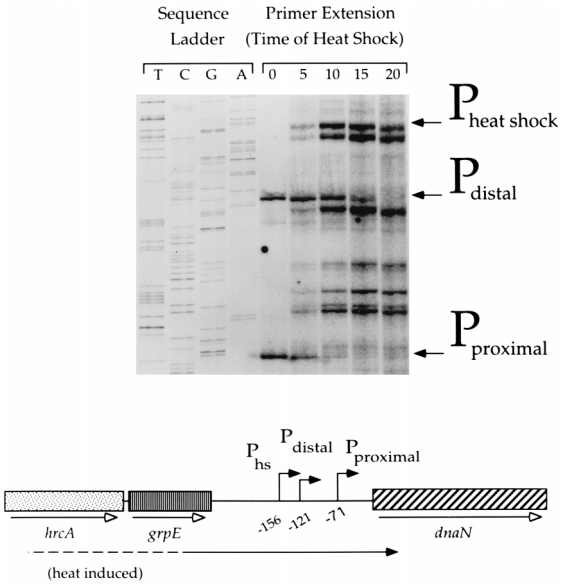


FIG. 5. Precise locations of *dnaN* transcriptional start sites. Primer extension analysis using an oligonucleotide near the translational start site of *dnaN* (Fig. 6) is shown adjacent to a DNA sequencing ladder generated by using the same oligonucleotide. Arrows indicate the locations of transcriptional start sites identified here and also confirmed by S1 nuclease mapping. Below is shown a diagram of the location of the major start sites in the context of the genetic region and the distance from the start of *dnaN* translation, shown in Fig. 6A, showing the heat-induced transcriptional readthrough from the upstream *hrcA-grpE* operon.

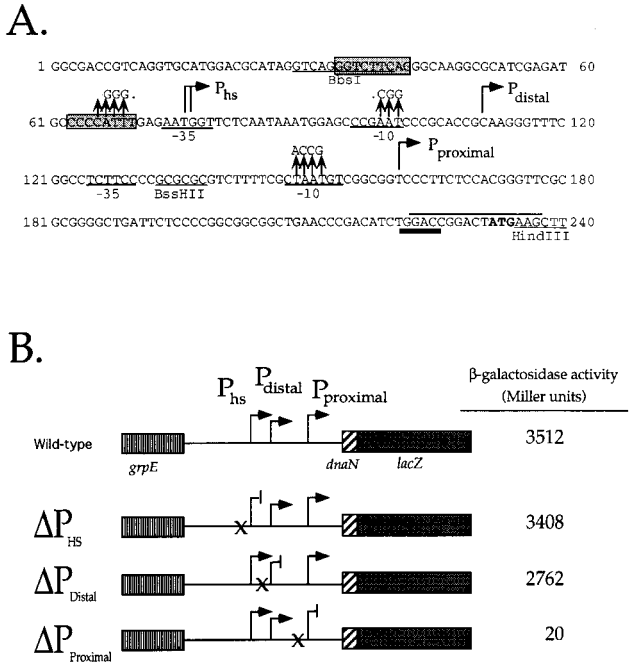


FIG. 6. Site-directed mutations in *dnaN* promoter sequences. (A) The 240-nucleotide *dnaN* promoter sequence shown lies in the region between *grpE* and *dnaN*, with the stop codon of *grpE* located 146 bp upstream; the *dnaN* ATG start codon is boxed. Relevant restriction sites are underlined and identified below the sequence. Transcriptional start sites identified by primer extension and S1 nuclease mapping are indicated by open boxed bases and bent arrows. $P_{hs}$ shows conservation with the *C. crescentus* $\sigma^{32}$ consensus and is indicated by the shaded boxes. The region whose complement was used as the primer for the primer extension reaction shown in Fig. 5 is indicated by a line above the sequence. The changes made by site-directed mutagenesis of the promoter −10 regions (underlined) are indicated with vertical arrows above the sequence. The region with homology to the consensus *C. crescentus* Shine-Dalgarno sequence is underlined with a black bar. (B) Transcriptional activity of the *dnaN*::*lacZ* fusions, each carrying the base substitutions indicated in panel A. A bent arrow indicates an intact promoter element, and a blocked arrow with an associated "X" indicates a mutated promoter. The average β-galactosidase activities for triplicate assays are shown.

Transcriptional activities of such constructs were then measured by pulse-labeling total protein at different times during synchronous growth and recovering the reporter β-galactosidase via immunoprecipitation (Fig. 9). Both the *dnaN* and *gyrB* promoter regions showed induced transcription just prior to or at the time of the swarmer-to-stalked-cell transition (as judged by microscopic observation), lasting through the stalked-cell phase and then decreasing in the predivisional cell (Fig. 9). Induction of transcription of these genes just preceded the onset of DNA replication, as measured by [$^3$H]dGTP incorporation (Fig. 9B). It should be noted that no decrease in the rate of DNA synthesis was observed in the predivisional stage; this is due to the short duration of the cell cycle after initiation of replication (~105 min) compared to the time required to replicate the chromosome (~100 min). When the two major *dnaN* promoters, $P_{distal}$ from a subcloned fusion with $P_{proximal}$ truncated downstream of the *Bss*HII site and $P_{proximal}$ from a construct with site-directed mutations at the −10 regions for both $P_{hs}$ and $P_{distal}$, were tested independently, they showed essentially identical patterns of cell cycle expression, demonstrating the difference between these promoters is not one of timing of expression (not shown).

Because the induction of *dnaN* and *gyrB* transcription occurred shortly after the isolated swarmer cells resumed growth following their synchronization, it was possible that the induction observed represented an artifact of some aspect of the synchronization process. Induction of transcription from the *dnaN* promoters was assayed in mock-synchronized populations. While an increase in transcription was noted upon completion of the synchronization protocol, when the mixed culture was returned from suspension in Ludox at 4°C to growth medium at 30°C, the degree of induction (~2-fold) was substantially lower than the average induction in synchronous cultures, and the duration of induction (~40 min) was much shorter than that seen in synchronized cultures (data not shown). This evidence confirms that the transcriptional response of *dnaN* seen at the swarmer-to-stalked-cell transition is genuine.

## DISCUSSION

The expression of several *C. crescentus* genes encoding proteins that are involved in the replication and repair of DNA, including *dnaX* (64), *dnaA* (66), *dnaC* (38), and *dnaKJ* (5, 17), has been shown to be cell cycle controlled, with maximal expression coinciding with the period of chromosomal replication. Here we have identified and examined the expression patterns of a group of contiguous genes encoding homologs of *dnaN*, *recF*, and *gyrB*. Although *recF* is expressed constitutively, both *dnaN* and, as described previously (47), *gyrB* are under strict temporal control.

The *C. crescentus recF* gene was shown genetically to play a role in repair of UV damage. This finding correlates well with the results found for *E. coli* or *B. subtilis*, where mutations in the *recF* homolog mildly affect susceptibility to DNA-damaging treatments (2, 20). Examination of the alignment of the putative *C. crescentus* RecF protein demonstrated only weak sequence similarity to other RecF proteins; its closest homolog was RecF from the gram-positive bacterium *Streptomyces coeli-*

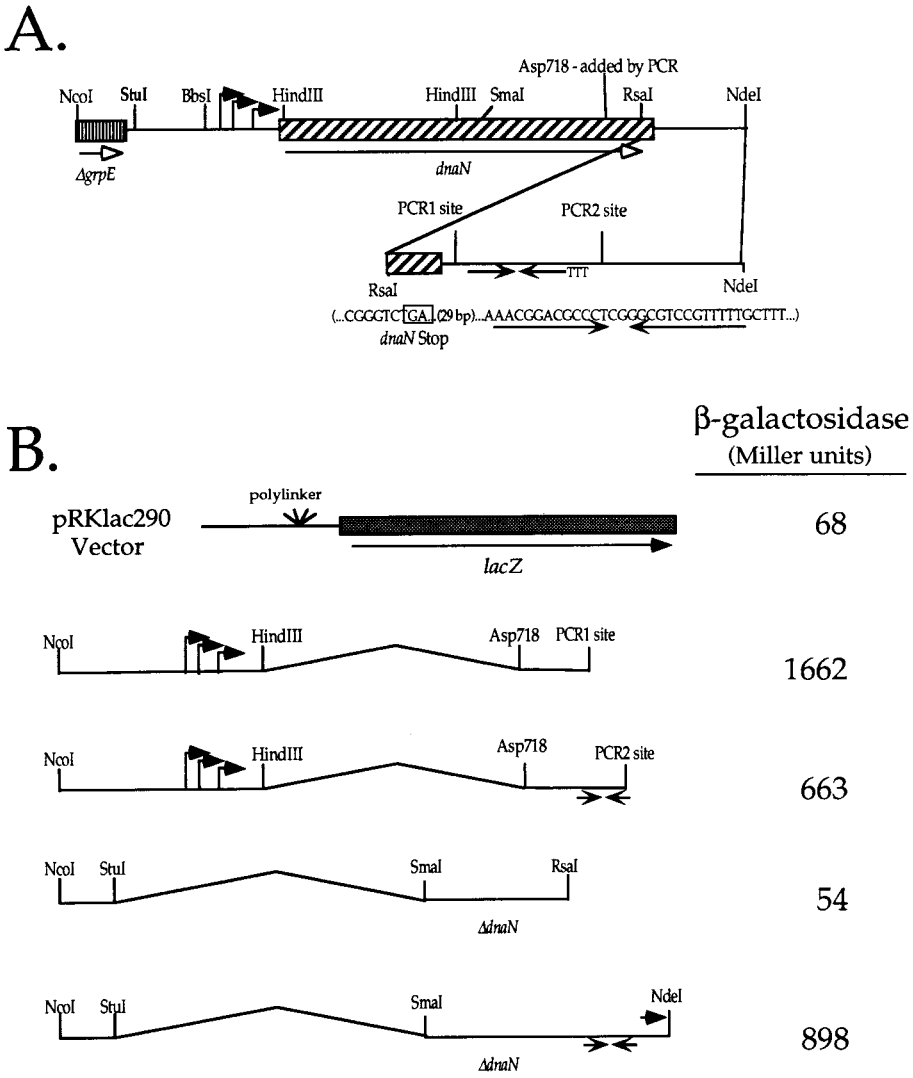


FIG. 7. Transcriptional terminator activity downstream of *dnaN*. (A) Genetic map of the *dnaN* region, with vertical bars representing a portion of the *grpE* gene and diagonal stripes representing *dnaN*. The sequence immediately downstream is expanded below to show the identified inverted repeat. (B) Horizontal lines show the different regions fused to *lacZ* in the transcriptional reporter vector pRKlac290 (regions deleted are represented by bridged lines), either with the *dnaN* promoter region indicated by the three bent arrows or lacking this region. Opposed small arrows indicate the putative terminator stem-loop, and the solid straight arrows represent the general location of the detected transcriptional activity. The average β-galactosidase activities for triplicate assays of these fusions are shown; the activity for the indicated pRKlac290 vector alone was not subtracted from these values.

*color*. The flanking *dnaN* and *gyrB* genes, however, show greatest similarity to the phylogenetically more closely related gram-negative *E. coli* or *P. putida*. However, key regions of the *C. crescentus* RecF protein, including the ATP-binding P-loop at the N-terminal end (52) and other highly conserved residues scattered throughout the protein sequence, were conserved. While the weak promoter directly upstream of the *C. crescentus recF* gene is constitutively expressed (47, 47b), several promoters further upstream are temporally expressed and may possibly impart temporal transcription to *recF* as well, a possibility not addressed by the promoter fusions used in these studies.

The only sequence within the *dnaN-recF-gyrB* region for which no function has been demonstrated is the ~1-kb span between *dnaN* and *recF*. Although a weak cell cycle-regulated promoter was detected downstream of the *dnaN* gene, no convincing open reading frames were discerned within this region. Furthermore, deletion of a 640-bp portion of this sequence in the chromosome and replacement with a $Tet^r$ determinant showed no detectible phenotype in growth, DNA replication, sensitivity to UV damage, or recombination. While these studies do not definitively show that this region is unimportant, the function of this region and/or its transcribed products in the *C. crescentus* cell remains elusive.

The genetic organization of three *C. crescentus* genes involved in DNA replication and recombination, *dnaN*, *recF*, and *gyrB*, is very similar to that seen in other bacteria, although the genes upstream of *dnaN* are not usually found in that position. In *E. coli*, the *dnaA* and *dnaN* genes are transcribed as an operon (50), although *dnaN* does have two of its own, apparently constitutive promoters, found in the coding region of *dnaA* (3). In *C. crescentus*, *dnaN*, while separated from *dnaA*, was also found to be under the control of a complex promoter region. Most surprising was the presence of a heat shock promoter. In *E. coli*, at least two mechanisms, either SOS response dependent (for *dnaA*, *dnaN*, and *dnaQ* [43, 60]) or SOS independent (*dnaB* [23]), appear to exist to induce expression of DNA replication genes upon detection of DNA damage. In-

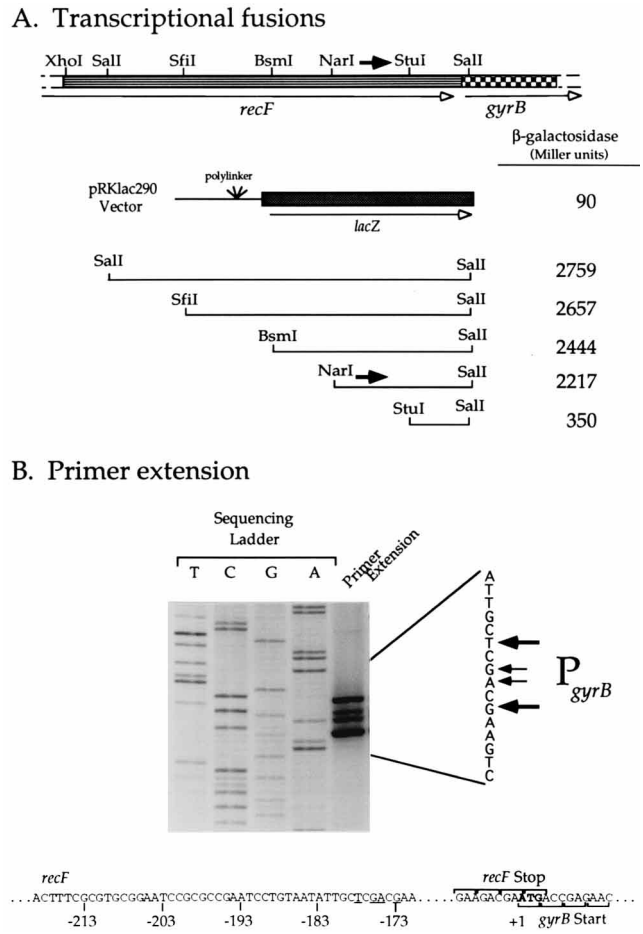


FIG. 8. Locations of *gyrB* transcriptional start sites. (A) Diagram of the *recF-gyrB* region and the restriction fragment subclones of this region tested for transcriptional activity by fusing them to *lacZ*. The β-galactosidase activities averaged from triplicate assays are shown; the background from the pRKlac290 vector alone was not subtracted. Solid arrows indicate the general locations of transcriptional activity as defined by these data. (B) Primer extension analysis using an oligonucleotide near the beginning of *gyrB*, adjacent to a DNA sequencing ladder generated by using the same oligonucleotide. Bases corresponding to potential start sites are underlined. The large arrows indicate bases corresponding to major bands in the start region, and small arrows indicate bases corresponding to minor bands. Below is shown the sequence of the promoter region, with numbering relative to the first nucleotide of the *gyrB* translational start codon.

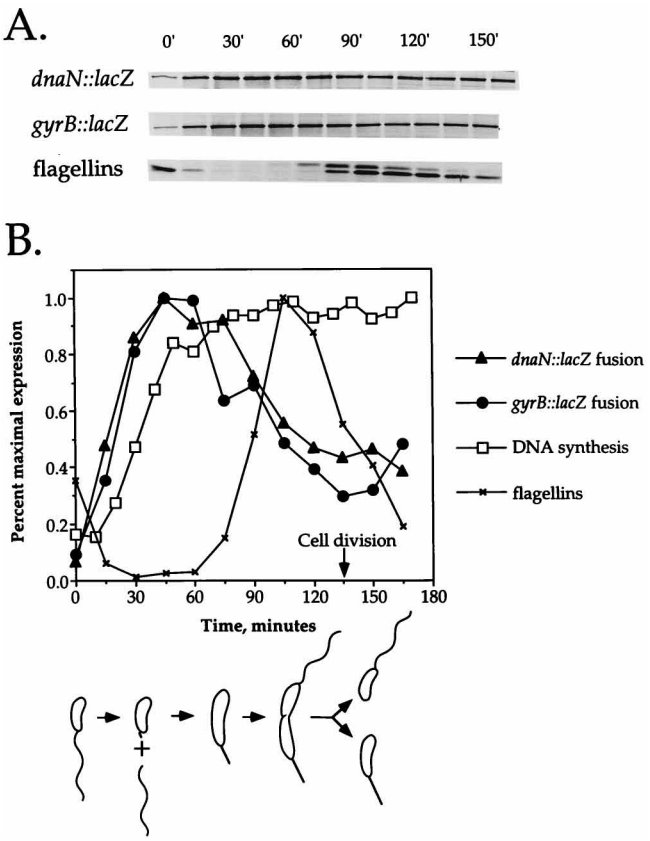


FIG. 9. Patterns of *dnaN* and *gyrB* transcription during the cell cycle. Levels of expression of *lacZ* fusions to the complete promoter regions upstream of either *dnaN* or *gyrB* in the transcriptional reporter vector pRKlac290 were monitored in synchronous *C. crescentus* cells as they progressed through the cell cycle. (A) Results of immunoprecipitation of $^{35}$S-labeled β-galactosidase from strains carrying either *dnaN*::*lacZ* (with all three identified promoters present) or *gyrB*::*lacZ* fusions and of $^{35}$S-labeled flagellar filament proteins (recovered from the same cells carrying the *dnaN*::*lacZ* fusion), followed by electrophoresis on a 12.5% polyacrylamide gel and autoradiography. Numbers above the images indicate the time elapsed, in minutes, from the beginning of synchronous growth. (B) Quantification of these data by using a Molecular Dynamics PhosphorImager, reported as the percentage of maximal expression for each protein. Closed triangles represent expression of the *dnaN*::*lacZ* fusion, closed circles represent expression of the *gyrB*::*lacZ* fusion, and crosses represent flagellin expression. The open boxes represent the DNA synthesis rates of these strains (plotted as the percentage of maximal synthesis), as determined by quantitating the amount of [$^3$H]dGTP label incorporated during 2-min pulses performed during the course of the synchrony. The time of cell division, determined by microscopic observation, is also indicated in the graph. A diagram of the *C. crescentus* cell cycle is keyed to the synchrony shown in the graph.

deed, the *E. coli dnaN* gene was found to encode a shorter polypeptide (β*) whose expression is specifically induced upon UV treatment of the cells (40, 55). This alternate form of the sliding clamp imparts resistance to the UV treatment in an undefined manner. However, neither *dnaN* nor any other bacterial DNA polymerase subunit genes have been shown to be expressed from a heat-inducible promoter. Biochemical start site analysis of the putative $\sigma^{32}$ promoter region of the *C. crescentus dnaN* gene demonstrated heat inducibility of transcription from this promoter, as well as heat-inducible transcription of *dnaN* by readthrough from the upstream *hrcA-grpE* heat shock operon (48). However, when heat-inducible transcription of *dnaN* was inactivated, no phenotype could be detected. The *dnaN* heat shock promoter may be a remnant of the transposition event that introduced the *hrcA-grpE* operon immediately upstream of *dnaN*; in most other organisms, the heat shock operon *dnaKJ* lies directly downstream of the *grpE* gene.

*dnaN* is transcribed at physiological temperatures from two promoters ($P_{distal}$ and $P_{proximal}$). These promoters share a common transcriptional expression pattern during the cell cycle, with low but discernible levels of expression in swarmer and predivisional cells and induction of ~4 to 5-fold upon differentiation of the swarmer cell into a stalked cell. Low-level expression in the swarmer cells was not unexpected, since previous studies have shown that while chromosomal replication is limited to stalked cells (10, 12, 30), plasmid replication does occur in swarmer cells, demonstrating the presence of a functional DNA replication machinery (31). The timing of induction of the *dnaN* promoters coincides with the initiation of chromosomal DNA replication; similar timing is also seen for several other *C. crescentus* genes whose products play a role in replication, including *dnaA* (66), *dnaK* (5), *dnaX* (64), and *gyrB* (47) and the uncharacterized *dnaC* gene (38).

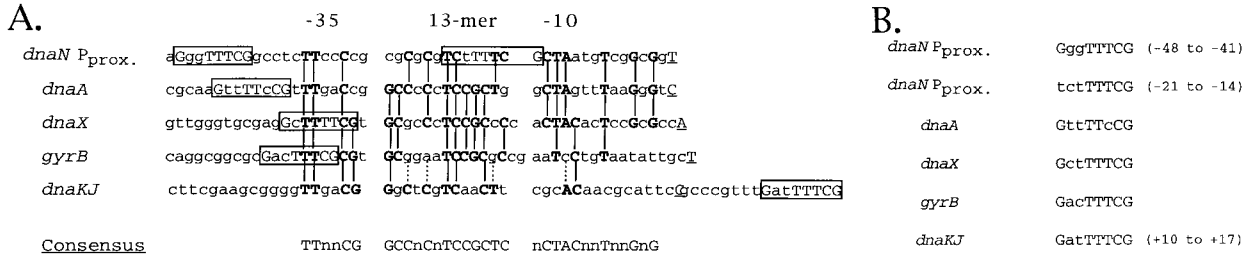


FIG. 10. Alignment of promoters for similarly timed DNA replication genes. (A) The *dnaX*, *dnaN* ($P_{proximal}$), *gyrB*, *dnaA*, and *dnaKJ* promoter sequences are shown, all of which are transcribed maximally near the time of swarmer-to-stalked-cell transcription (with $P_{dnaA}$ transcribed somewhat earlier than the others). Below is the consensus sequence for these promoters, as well as for *C. crescentus* $\sigma^{70}$ promoters (29). Transcriptional start sites are shown as underlined, capitalized bases, and the approximate −10 and −35 regions, as well as the position of the conserved 13-mer, are indicated above the alignment. The bases conserved among all of the strongly expressed promoters are shown in capitalized boldface letters with connecting lines. Open boxes in each sequence show the 8-mer repeat (consensus, GnnTTTCG) found in common for all the promoters, indicating the different relative positions of this repeat. (B) Alignment of the conserved 8-mer sequences for the promoters shown in panel A.

Recently, a *C. crescentus* response regulator protein, CtrA, has been shown to regulate the transcription of several genes that are under cell cycle control, including those involved in flagellar biogenesis, DNA methylation, and DNA replication initiation (44). The DNA replication genes under examination here were found not to be under the control of CtrA (47a), suggesting that expression of these genes is linked to the cell cycle by a distinct mechanism. Comparison of the sequences for the DNA replication gene promoters with similar temporal patterns of transcription revealed conserved sequence elements (Fig. 10). Both the −10 and −35 regions show conserved sequences, and similarities were also found between approximately −17 and −29 (labeled the 13-mer). As many as 10 of the 13-mer bases are conserved between *dnaA* and *dnaX* promoter sequences (with lesser degrees of conservation for the other promoters), and in the case of *dnaX*, base pair changes in this conserved sequence resulted in a fourfold increase in transcription, suggesting a possible repressor binding site (64). Additionally, an 8-mer sequence of GnTTTCG is found within all of these promoters, although its positioning is variable, from ~−45 for *dnaN* $P_{proximal}$ to +15 for $P_{dnaK}$ (Fig. 10). Further, another putative *C. crescentus* DNA repair gene, with similarity to the *E. coli alkB* gene that is involved in repair of DNA damage due to alkylating agents, also contains two sequences with substantial similarity to the 8-mer consensus (16a). The *dnaN* $P_{proximal}$ promoter has two 8-mer sequences, and significant conservation in the 13-mer region, but the weak *dnaN* $P_{distal}$ promoter did not show strong conservation to either the 8-mer or the 13-mer consensus.

The *E. coli dnaN*-encoded β subunit of DNA polymerase, functioning as a sliding clamp, forms an integral part of the replication machinery (27). In addition to this master function, sliding-clamp homologs in other systems have been shown to play other roles. These include serving as a link to regulate DNA replication in response to DNA damage (PCNA in eukaryotic systems [15, 56, 63]) and as a transcriptional activator of late genes in response to the process of DNA replication (gp45 in bacteriophage T4 [19, 61]). The DnaN homolog from *C. crescentus* may also play other roles in coordinating cell cycle events. Mutagenesis of this gene and examination of resulting phenotypes should provide insight into this possibility.

## ACKNOWLEDGMENTS

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