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Correct Timing of *dnaA* Transcription and Initiation of DNA Replication Requires *trans* Translation▽†

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The *trans* translation pathway for protein tagging and ribosome release has been found in all bacteria and is required for proliferation and differentiation in many systems. *Caulobacter crescentus* mutants that lack the *trans* translation pathway have a defect in the cell cycle and do not initiate DNA replication at the correct time. To determine the molecular basis for this phenotype, effects on events known to be important for initiation of DNA replication were investigated. In the absence of *trans* translation, transcription from the *dnaA* promoter and an origin-proximal promoter involved in replication initiation is delayed. Characterization of the *dnaA* promoter revealed two *cis*-acting elements that have dramatic effects on *dnaA* gene expression. A 5′ leader sequence in *dnaA* mRNA represses gene expression by >15-fold but does not affect the timing of *dnaA* expression. The second *cis*-acting element, a sequence upstream of the −35 region, affects both the amount of *dnaA* transcription and the timing of transcription in response to *trans* translation. Mutations in this promoter element eliminate the transcription delay and partially suppress the DNA replication phenotype in mutants lacking *trans* translation activity. These results suggest that the *trans* translation capacity of the cell is sensed through the *dnaA* promoter to control the timing of DNA replication initiation.

The *trans* translation pathway for protein tagging and ribosome rescue is found in all bacteria and is required for normal physiology, development, or virulence in many species (14). During *trans* translation, a ribonucleoprotein complex consisting of tmRNA (transfer-messenger RNA) and SmpB enters substrate ribosomes that have a peptidyl-tRNA in the P site. The termini of tmRNA fold into a structure that mimics alanyl-tRNA, and tmRNA is charged with alanine. tmRNA-SmpB acts first like a tRNA, accepting the nascent polypeptide in a normal transpeptidation reaction. A specialized open reading frame within tmRNA is then decoded by the ribosome, producing a protein that has the tmRNA-encoded peptide at its C terminus and releasing the ribosome after termination at a stop codon. The tmRNA peptide is recognized by several intracellular proteases and targets the protein for rapid degradation. This *trans* translation reaction is important for both translational quality control and regulation of genetic circuits in many species (14, 25).

trans translation is particularly important for developmental processes in bacteria. tmRNA is required for cellular differentiation during sporulation in *Bacillus subtilis* (1), symbiosis in *Bradyrhizobium japonicum* (6), pathogenesis in *Salmonella enterica* (13) and *Yersinia pseudotuberculosis* (27), and the cell cycle of *Caulobacter crescentus* (17). For systems in which the *trans* translation phenotype has been investigated in molecular detail, developmental defects are due to misregulation of a key signaling molecule. For example, *B. subtilis* sporulation is disrupted in strains lacking tmRNA due to decreased expression of the SpoIVCA recombinase, an enzyme that is required for production of σ^K during the developmental sigma factor cascade (1). Likewise, pathogenesis by *Y. pseudotuberculosis* is impaired in the absence of *trans* translation because of misregulation of the VirF transcriptional regulator (27). *trans* translation is also clearly required for development in *C. crescentus*, but the molecular basis for this requirement has not previously been determined.

C. crescentus cells that lack *trans* translation activity because of a deletion in *smpB* or *ssrA*, the gene that encodes tmRNA, grow slower than wild-type cells due to a delay in the initiation of DNA replication (17). Newly divided *C. crescentus* cells in G_1 phase can be easily isolated, and these cells will pass synchronously through the cell cycle, allowing detailed studies of cell cycle-related processes (29). Unlike those of *Escherichia coli*, *C. crescentus* cells initiate DNA replication only once per cell division (22). Work with synchronized cultures has shown that initiation of DNA replication is controlled by several factors at the origin of replication. The essential response regulator CtrA binds to five sites in the origin and represses inappropriate initiation events (28). CtrA is degraded during the G_1–S-phase transition to allow replication to initiate (5). Another essential factor, DnaA, binds to the origin of replication and is assumed to act in the same manner as *E. coli* DnaA, unwinding the DNA and recruiting DNA polymerase to initiate replication (21). Expression of *dnaA* is cell cycle regulated, with maximum new protein synthesis immediately before DNA replication initiates (4, 34), and delaying production of new DnaA protein using an inducible promoter delays replication initiation, suggesting that new DnaA synthesis is required for replication initiation (11). In addition to these essential proteins, other factors also play a role in regulation of DNA replication. A strong promoter adjacent to the origin, P_s, is active during initiation and may help unwind DNA. P_s is required for replication in a plasmid model system but is dispensable in the chromosomal context (11, 20). IHF also binds

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TABLE 1. Plasmids and strains used in this study

Plasmid, strain, or genotype	Description	Reference
pRKlac290	RK2 vector for making *lacZ* transcriptional fusions	9
P_s-*lacZ*	*Ps* promoter in pRKlac290 (pGM976)	23
P_{dnaA}-*lacZ*	*dnaA* promoter from −369 to −1 in pRKlac290	This study
dnaA′-lacZ	*dnaA* gene from −369 to +163 in pRKlac290	This study
pLC17	*dnaA′-lacZ* with DnaA box TTATCCAAG changed to TGAGCGCAG	This study
P_{xylX}-*dnaA′-lacZ*	Xylose-inducible promoter followed by *dnaA* gene from +1 to +140 in pRKlac290	This study
P_{xylX}-*lacZ*	Xylose-inducible promoter in pRKlac290	This study
pLC43	*E. coli dnaA* gene from −190 to +151 in pRKlac290	This study
pLC44	*E. coli dnaA* gene from −190 to −1 in pRKlac290	This study
P_{dnaA}-*dnaA*	P_{dnaA}-*lacZ* with *dnaA* coding sequence replacing *lacZ*	This study
CB15N	Wild-type synchronizable *C. crescentus*	8
KCK116	CB15N Δ*ssrA* (in-frame deletion)	17
LC202	MG1655 Δ*lacX74* Δ*ssrA*::*nptII*	This study

within the origin and may play a role in replication control (31).

In Δ*ssrA* and Δ*smpB* cells, CtrA is degraded at the same time as in the wild type, but replication does not initiate for 30 to 45 min (17). In addition, the abundance of tmRNA and SmpB increases just before the initiation of DNA replication, and they are depleted in early S phase, consistent with a role for *trans* translation in replication initiation (16). In this article, the effects of *trans* translation on expression of *dnaA* and transcription from P_s were investigated to determine why the correct timing of replication initiation requires tmRNA and SmpB.

MATERIALS AND METHODS

Strains and plasmids. Plasmids and strains are listed in Table 1. The P_{dnaA}-*lacZ* reporter was constructed by using PCR to amplify the *dnaA* promoter from −369 to −1 with respect to the transcriptional start site and cloning the product into pRKlac290 (9), resulting in a *lacZ* gene containing its own translation initiation sequences under the control of the *dnaA* promoter. Deletions in the leader sequence were made in a similar fashion. Mutations in the *dnaA* promoter from −57 to −77 and in the putative DnaA box were constructed using the QuikChange mutagenesis kit (Stratagene). Note that although some pBBR1-derived plasmids will not replicate in Δ*ssrA* cells, RK2-derived plasmids are maintained in all strains used in this study.

C. crescentus strains were grown in PYE or M2 medium (7) with 0.2% glucose (M2G) or 0.3% xylose at 30°C, with 5 mg/ml kanamycin, 1 mg/ml chloramphenicol, or 1 mg/ml oxytetracycline as necessary, and growth was monitored by measuring the optical density at 660 nm (OD_{660}). Synchronized cultures were obtained by Ludox density gradient centrifugation as described previously (8), and all synchronized cultures were grown in M2G. The timing of DNA replication initiation was determined at each time point by incubating an aliquot with 15 μg/ml rifampin (rifampicin) at 30°C for 3 h, fixing cells by addition of ethanol to 70%, staining with Syto 13 (Invitrogen), and measuring DNA content by flow cytometry as described previously (32). *E. coli* strains were grown in LB broth at 37°C, and growth was monitored by measuring the OD_{660} (30).

Quantitative RT-PCR. Cells were harvested from synchronized cultures, and RNA was prepared using the RNeasy mini kit (Qiagen), including two incubations with RNase-free DNase I (Qiagen) on the column according to the manufacturer's instructions. RNA samples were diluted to 200 ng/μl and tested by PCR to ensure that there was no genomic DNA contamination before use in real-time PCR (RT-PCR). Reverse transcription was performed using the High Capacity RT kit (ABI), and cDNA was added to a PCR containing TaqMan 2× universal mix and amplified with the following protocol: 50°C for 2 min, 95°C for 10 min, and 40 repeats of 95°C for 15 s and 60°C for 1 min. The primers for *dnaA* gene were as follows: forward primer, 5′-GAGTTCGCGCACGCTGTAG-3′ (corresponding to bases 493 to 511 of the *dnaA* coding sequence); and reverse primer, 5′-CGTACGGGCCGTGGAA-3′ (complementary to bases 558 to 574 of the *dnaA* coding sequence). The TaqMan probe for *dnaA* was 5′-CGGACGGT CACTTCAATCCTGTGCT-3′. The 16S rRNA gene was used as a control with the following primers: 5′-GGGTTAAGTCCCGCAACGA-3′ and reverse primer 5′-ATGATTAGAGTGCCCAGCCAAA-3′. The TaqMan probe for 16S rRNA was 5′-CGCAACCCTCGTGATTAGTTGCCATC-3′. Both TaqMan probes were synthesized by Applied Biosystems, with 6-carboxyfluorescein at the 5′ end as the reporter and 6-carboxytetramethylrhodamine at the 3′ end as the quencher. The ABI 7300 sequence detection system was used to record data, and data were analyzed by the comparative cycle threshold (C_T) method for relative quantification according to the manufacturer's instructions (Applied Biosystems). The C_T was defined as the fraction of the cycle when the fluorescent intensity reached the threshold. For each time point, the average *dnaA* C_T was compared to the average C_T of 16S rRNA to get the ΔC_T value, and the ΔC_T value of this time point was then compared to the ΔC_T value of time zero min to get the $\Delta\Delta C_T$ value. The relative amount of *dnaA* mRNA was calculated by using the formula $2^{-\Delta\Delta C_T}$.

Pulse-labeling, pulse-chase, and immunoblotting. Pulse-labeling experiments were performed as described previously (16). Briefly, *C. crescentus* strains were grown in M2G medium to an OD_{660} of 0.3 to 0.4 and synchronized. At each time point, 1 ml culture was sampled and incubated with 1 μl [^{35}S]methionine (10 to 15 μCi) at 30°C for 5 min. The reaction was stopped by adding 950 μl labeled culture to 50 μl trichloroacetic acid, and labeled protein was recovered by centrifugation. Protein was resuspended in 50 μl IP-SDS buffer (10 mM Tris-HCl [pH 8], 1% SDS, 1 mM EDTA), and 750 μl RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) was added. Radioactivity was quantified by scintillation counting, and equal counts for each sample were added to anti-β-galactosidase or anti-DnaA antibody in 500 μl RIPA buffer with 15 μl protein A conjugated with Sepharose beads. After incubation at 4°C overnight, samples were washed twice with 900 μl RIPA buffer, resuspended in 2× Laemmli loading buffer, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Dried gels were exposed to phosphor screens, scanned using a Typhoon scanner, and analyzed using Imagequant software (Molecular Dynamics).

Pulse-chase experiments were performed as described for pulse-labeling experiments, except that after incubation with [^{35}S]methionine for 5 min, cold methionine was added to a 2% final concentration at time zero. Protein was isolated and analyzed by immunoprecipitation as described above.

For Western blots, cells were harvested by centrifugation and resuspended in a volume of 2× Laemmli loading buffer normalized to maintain a constant OD_{660} per sample. Electrophoresis, blotting, and development with anti-DnaA antibody were performed as described previously (30).

β-Galactosidase activity assays. Exponentially growing *C. crescentus* strains harboring a *lacZ* reporter were sampled, and β-galactosidase activity was measured using a modified Miller assay (15). Briefly, at each time point, 1 ml culture was taken and the OD_{660} was measured. Fifty μl chloroform was added to 50 μl culture and mixed with 750 μl Z buffer (composition), and *o*-nitrophenyl-β-D-galactopyranoside was added to 1 mg/ml. The reaction was incubated at 30°C until the color changed to yellow and stopped by addition of 500 μl Na_2CO_3, and the OD_{420} was measured. The increase in β-galactosidase activity was calculated by plotting OD_{420}/reaction time versus OD_{660}. Assays with *E. coli* were performed using the same procedure except that the OD was measured at 600 nm.

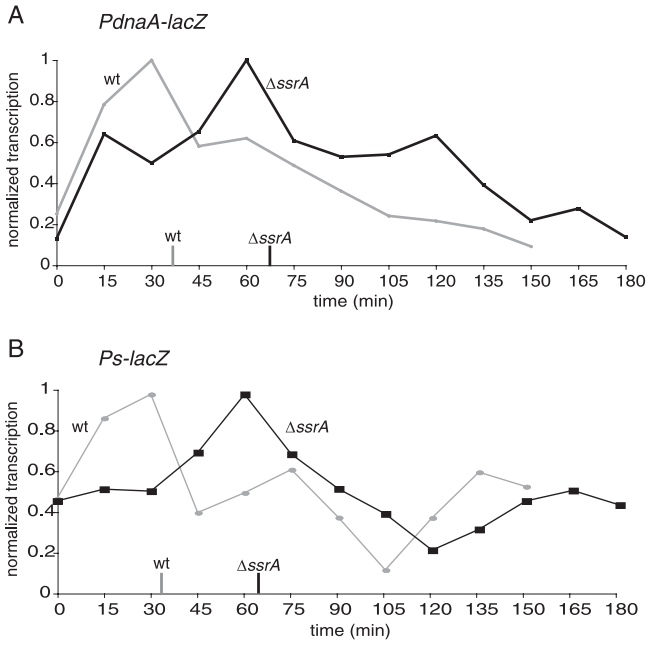


FIG. 1. tmRNA is required for correct timing of transcription from the *dnaA* promoter and origin-proximal promoter P_s. Cells containing the P_{dnaA}-*lacZ* reporter (A) or the P_s-*lacZ* reporter (B) were synchronized in wild-type (gray) or Δ*ssrA* (black) cells. The amount of promoter activity at each time point was measured by pulse-labeling followed by immunoprecipitation of β-galactosidase. The amount of radiolabeled β-galactosidase was normalized to the highest value for each strain. Vertical lines indicate the time at which half of the cells had initiated DNA replication as determined by flow cytometry assays (see Fig. S1 in the supplemental material). Representative curves from >5 repeats are shown.

RESULTS

Transcription of *dnaA* and P_s is delayed in Δ*ssrA* cells. To determine if *trans* translation is required for the correct timing of the earliest events in initiation of DNA replication, transcription from the *dnaA* and P_s promoters was measured throughout the cell cycle using *lacZ* transcriptional reporters. A *lacZ* gene containing its own translation initiation sequences was fused to the transcriptional start site of each promoter on a low-copy-number plasmid, and production of β-galactosidase was measured in synchronized cultures by pulse-labeling with [^{35}S]methionine followed by immunoprecipitation of β-galactosidase (Fig. 1). In these assays, the amount of radiolabeled β-galactosidase produced at each time point indicates the relative promoter activity. The initiation of DNA replication was monitored at each time point using flow cytometry assays (see Fig. S1 in the supplemental material). In wild-type cells, transcription from the *dnaA* promoter peaked at 30 min, 5 min before the initiation of DNA replication (Fig. 1A). This result is consistent with previously published observations (4, 34). In the Δ*ssrA* strain, peak transcription from the *dnaA* promoter occurred at 60 min, again just before the initiation of DNA replication (Fig. 1A). Transcription from the P_s promoter was also delayed in the Δ*ssrA* strain (Fig. 1B). These data are consistent with a role for *trans* translation in control of *dnaA* and P_s transcription or at an earlier step that is required for both processes. Because a delay in *dnaA* transcription can delay replication initiation (11), the role of *trans* translation in DnaA production was investigated as a possible mediator of the cell cycle delay phenotype.

***dnaA* expression is delayed in absence of *trans* translation.** To confirm that the delay in *dnaA* transcription observed in Δ*ssrA* cells results in altered cell cycle regulation of DnaA expression, the levels of *dnaA* mRNA, the timing of DnaA protein synthesis, and the accumulation of DnaA protein were assayed in synchronized cultures. *dnaA* mRNA levels were measured using quantitative RT-PCR and DNA microarrays (Fig. 2A; also data not shown). Both techniques showed that the level of *dnaA* mRNA in Δ*ssrA* cells changed during the cell cycle in a pattern similar to that for wild-type cells but delayed by 30 to 45 min. Likewise, pulse-labeling assays showed that the pattern of new synthesis of DnaA protein in Δ*ssrA* cells was delayed compared to that for the wild type (Fig. 2B). Western blotting for total DnaA protein levels showed that the peak accumulation of DnaA in the Δ*ssrA* strain was also delayed (Fig. 2C). These results show that the delay in transcribing *dnaA* does result in delayed DnaA protein production.

Because the primary effect of *trans* translation is to target substrate proteins for proteolysis and DnaA protein is degraded during the cell cycle, the effects of tmRNA on the degradation of DnaA were measured in exponentially growing cultures. Pulse-chase assays showed that the half-life of the DnaA protein was 30 to 35 min in both wild-type and Δ*ssrA* cells, indicating that deletion of *ssrA* does not have a significant effect on the overall stability of the DnaA protein (Fig. 2D). Therefore, *trans* translation is required for correct timing of *dnaA* gene expression but does not affect posttranscriptional control of DnaA levels.

***dnaA* expression is repressed by its 5′ leader sequence.** The *dnaA* gene has a 155-base leader sequence between the transcription start site and the ATG start codon (34). The amount of β-galactosidase activity produced from the P_{dnaA}-*lacZ* reporter, which does not contain the *dnaA* leader sequence, was significantly higher than those for previously described *dnaA′-lacZ* reporters in which the *lacZ* gene was fused after base 9 of the *dnaA* open reading frame (34). This observation suggested that the *dnaA* leader sequence is important for control of *dnaA* expression. To quantify the effects of the leader sequence on gene expression, β-galactosidase activity assays were performed with wild-type and Δ*ssrA* cells (Fig. 3). Reporters without the leader sequence had >15-fold-higher β-galactosidase activity in both strains. To more precisely map which sequences are important for decreasing gene expression, a series of deletions in the leader sequence was constructed and assayed (Fig. 3). Reporters containing ≥87 bases of the leader sequence had expression levels within twofold of that of the reporter containing the full leader, suggesting that the 3′ portion of the leader sequence is not important for regulating gene expression. Conversely, when only the first 67 bases of the leader sequence were present, expression levels were similar to those when the leader sequence was completely removed, suggesting that some or all of the sequence between bases 68 and 86 is required for regulation.

A putative DnaA binding site in the leader region has been proposed to be involved in autoregulation of *dnaA* expression (34). To determine if this site affects regulation by the *dnaA* leader sequence, four point mutations were engineered to

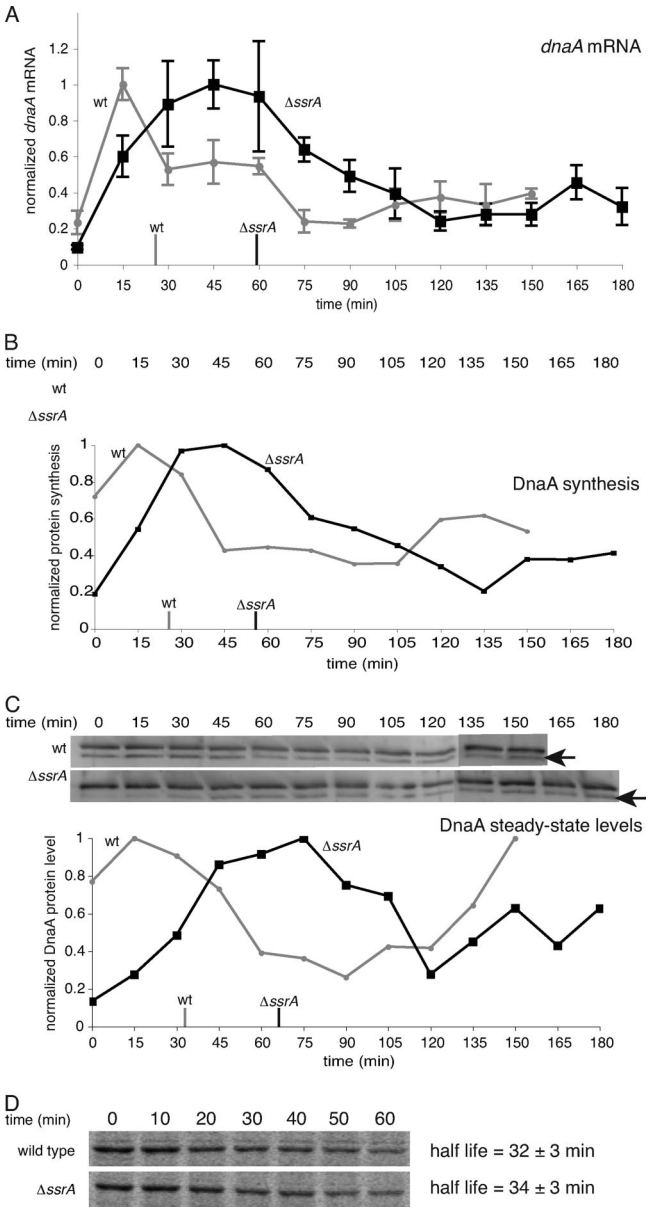


FIG. 2. Expression of DnaA is delayed in cells lacking *ssrA*. (A) Quantitative RT-PCR was used to measure the amount of *dnaA* mRNA in synchronized cultures of wild-type (gray) or Δ*ssrA* (black) cells. Values were normalized to the highest level for each strain, with error bars indicating the standard deviation after assays with three synchronized cultures. Vertical lines in panels A, B, and C indicate the time at which half of the cells had initiated DNA replication as determined by flow cytometry assays. (B) The amount of newly synthesized DnaA protein was determined in synchronized cultures by labeling cells with [^{35}S]methionine at each time point and immunoprecipitating DnaA. Representative gels and quantifications from three repeats are shown. (C) The amount of total DnaA protein was determined in synchronized cultures by Western blotting. Representative gels from five repeats are shown. Arrows indicate the band corresponding to DnaA (the higher band is GroEL). Protein levels were quantified and normalized to the highest level for each strain. (D) Pulse-chase experiments to measure the half-life of DnaA protein in exponentially growing cultures show that the half life was not affected by *ssrA*. Representative gels from three repeats are shown.

change the sequence from TTATCCAAG to TGAGCGCAG in the P_{dnaA}-*lacZ* reporter. β-Galactosidase production was increased by <2-fold compared to that with the unmutated P_{dnaA}-*lacZ* reporter in both wild-type and Δ*ssrA* cells (Fig. 3), indicating that this site plays at most a minor role in regulation by the leader sequence.

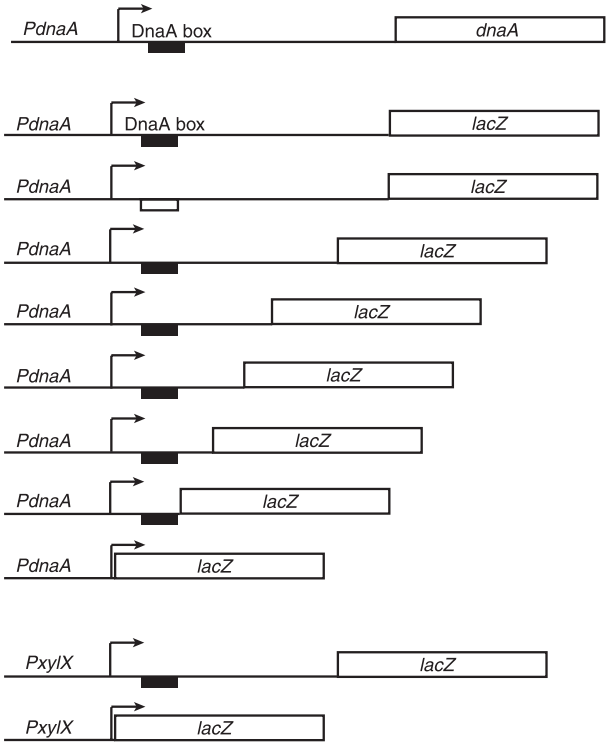
To test whether the leader sequence acts only in the context of the *dnaA* promoter or if it is a general repressor of gene expression, the effects of the leader on the xylose-inducible promoter P_{xylX} (24) were tested. When bases 1 to 140 of the *dnaA* leader sequence were inserted before *lacZ* in a P_{xylX}-*lacZ* reporter, β-galactosidase expression was decreased >50-fold in both wild-type and Δ*ssrA* cells (Fig. 3). These results indicate that the *dnaA* leader sequence is capable of decreasing gene expression independently of the *dnaA* promoter.

The similarities between cell cycle regulation patterns of transcription from P_{dnaA}-*lacZ* reporters that do not include the leader sequence (Fig. 1), results in previous studies using *dnaA′-lacZ* reporters that do include the leader sequence (4, 34), and expression of *dnaA* mRNA that includes the leader sequence (Fig. 2A) suggest that the leader sequence does not affect the timing of *dnaA* transcription. To confirm this hypothesis, production of β-galactosidase from the *dnaA′-lacZ* reporter was monitored in synchronized cultures of wild-type and Δ*ssrA* cells. In both strains, the patterns of β-galactosidase production were the same using *dnaA′-lacZ* and P_{dnaA}-*lacZ* (not shown), indicating that the *dnaA* leader is important for the amount of *dnaA* expression but not for timing of *dnaA* transcription with respect to the cell cycle.

Because the *E. coli dnaA* gene also has a long untranslated leader sequence (153 nucleotides) (26), transcriptional fusions of *lacZ* to the start codon and the transcriptional start site of the *E. coli* gene were engineered and tested with *E. coli* MG1655 and an isogenic strain deleted for *ssrA*. Removal of the leader sequence increased gene expression by a factor of 5.3 ± 1.3 in MG1655 and by a factor of 6.6 ± 0.6 in Δ*ssrA E. coli*. The *dnaA* leaders from *C. crescentus* and *E. coli* do not have any sequence homology or common RNA structure, but both leaders repress gene expression.

Timing of *dnaA* transcription is controlled through a promoter element in a *trans*-translation-dependent manner. It was previously reported that a mutation of base C(−71) to T in the *dnaA′-lacZ* reporter decreased transcription, suggesting the presence of a regulatory element at this site (3). To determine if this promoter element is involved in tmRNA-dependent regulation of *dnaA* expression, the effects of mutations at −71 and surrounding bases were examined in wild-type and Δ*ssrA* cells (Fig. 4). Consistent with previous results, in wild-type cells the C(−71)T mutation decreased *lacZ* expression by >80%. Likewise, mutations at positions −73 through −69, −67, and −64 through −60 decreased expression by >50%. Similar effects were observed in the Δ*ssrA* strain. These data suggest that the GTCAANANNAATAT sequence is required for full promoter activity. One possible explanation for these results is that a transcriptional activator binds to this site to promote transcription from P_{dnaA}.

To determine if the cell cycle regulation of *dnaA* transcription is affected by the GTCAANANNAATAT sequence element, expression of *lacZ* from the $P_{dnaA}C(-71)T$ promoter was assayed in synchronous cultures. In wild-type cells, peak



	relative activity	
dnaA leader	wild type	Δ*ssrA*
1-164	1.0 ± 0.1	0.6 ± 0.1
no DnaA box	1.6 ± 0.1	1.1 ± 0.2
1-140	1.9 ± 0.3	1.6 ± 0.4
1-87	1.2 ± 0.1	1.3 ± 0.6
1-67	16.4 ± 1.2	22.4 ± 5.1
1-47	13.4 ± 0.9	14.5 ± 1.2
1-27	12.2 ± 1.8	14.1 ± 1.0
none	18.3 ± 1.7	23.0 ± 3.7
1-140	1.0 ± 0.4	0.8 ± 0.3
none	57.5 ± 16.4	57.7 ± 22.8

FIG. 3. The *dnaA* leader sequence represses gene expression. Schematic diagrams show the wild-type *dnaA* locus and *lacZ* reporter constructs used to measure gene expression. β-Galactosidase assays were used to determine the relative expression from each promoter in wild-type and Δ*ssrA* cells. For *dnaA* promoter reporters, rates were normalized to the reporter with the entire leader sequence assayed in wild-type cells. For *xylX* promoter reporters, rates were normalized to the reporter with the *dnaA* 1–140 leader sequence assayed in wild-type cells. The construct with no DnaA box has four point mutations in the putative DnaA binding site.

transcription from the $P_{dnaA}C(-71)T$ promoter occurred at 15 min, just before the initiation of DNA replication at 30 min (Fig. 4B). However, in the Δ*ssrA* strain, transcription from the $P_{dnaA}C(-71)T$ promoter peaked at 15 min, even though DNA replication did not initiate until 57 min. Moreover, transcription from $P_{dnaA}C(-71)T$ in Δ*ssrA* cells was indistinguishable from that from P_{dnaA} or $P_{dnaA}C(-71)T$ in wild-type cells. Because mutation of C(−71) restores the wild-type transcription pattern in Δ*ssrA* cells, it is likely that the GTCAANANNA ATAT element is responsible for the delay in P_{dnaA} transcription in the absence of *trans* translation. It is not yet clear whether *trans* translation acts directly on a transcription factor that binds to this site or whether the mechanism is less direct.

Position −71 overlaps with a GANTC methylation site for CcrM, a cell-cycle-regulated DNA methyltransferase. However, this methylation site does not appear to be important for tmRNA-dependent regulation of *dnaA* transcription. A(−74) would be critical for methylation at this site, but mutation of A(−74) to T did not significantly affect transcription activity in wild-type or Δ*ssrA* cells (Fig. 4A). The A(−74)T mutation also had no effect on the timing of transcription from the P_{dnaA}-*lacZ* reporter in synchronized cultures (not shown). Therefore, position −71 and methylation of the GAGTC site by CcrM are not important for regulation of *dnaA* transcription by *trans* translation.

Expression of *dnaA* from a mutant promoter partially suppresses *ssrA* phenotype. Based on the data presented above, it is possible that the DNA replication delay in Δ*ssrA* cells is caused by misregulation at the GTCAANANNAATAT promoter element, which disrupts the timing of *dnaA* transcription. This hypothesis predicts that if *dnaA* transcription was uncoupled from the GTCAANANNAATAT promoter element, replication would initiate at the correct time in Δ*ssrA* cells. To test this prediction, the *dnaA* coding sequence was cloned under the control of the $P_{dnaA}C(-71)T$ promoter. Because the C(−71)T mutation decreases transcription, the *dnaA* leader sequence was not included in the $P_{dnaA}C(-71)T$-*dnaA* construct to increase *dnaA* expression levels. The combined effects of the C(−71)T mutation and no leader sequence were tested using *lacZ* reporters, and the $P_{dnaA}C(-71)T$-*lacZ* reporter had ~4-fold-higher activity than the *dnaA′-lacZ* reporter in exponentially growing cultures (not shown). A low-copy-number plasmid with $P_{dnaA}C(-71)T$-*dnaA* was introduced into wild-type and Δ*ssrA* cells, and the growth rate and timing of DNA replication initiation were measured (Table 2). Δ*ssrA* cells with $P_{dnaA}C(-71)T$-*dnaA* grew in exponential phase with a doubling time of 135 ± 9 min, significantly faster than Δ*ssrA* cells with $P_{dnaA}C(-71)T$-*lacZ* (Student's *t* test, $P = 0.05$) or with no plasmid (Student's *t* test, $P = 0.02$). $P_{dnaA}C(-71)T$-*dnaA* did not increase the growth rate of wild-type cells, indicating that the increased growth rate in the Δ*ssrA* strain was due to specific suppression of the *ssrA* phenotype and not an unrelated increase in the growth rate caused by the plasmid.

Measurements of DNA replication initiation in synchronized cultures showed that $P_{dnaA}C(-71)T$-*dnaA* did not affect wild-type cells but caused Δ*ssrA* cells to initiate replication earlier (Table 2). Wild-type cells containing $P_{dnaA}C(-71)T$-

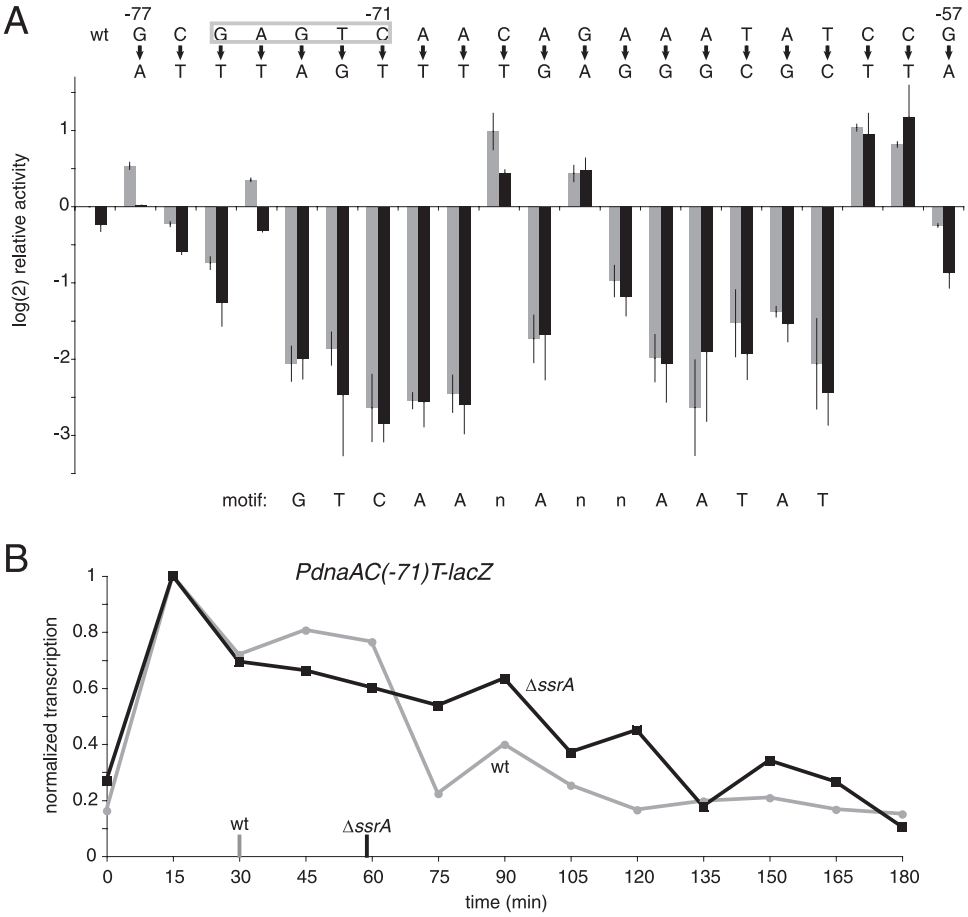


FIG. 4. A *dnaA* promoter element is responsible for delayed transcription in the absence of *ssrA*. (A) Point mutations were introduced from −57 to −77 in the *dnaA* promoter fused to *lacZ*, and β-galactosidase assays were used to measure the effects on promoter activity in wild-type (gray bars) or Δ*ssrA* (black bars) cells. The wild-type promoter sequence is shown on top, with the point mutation indicated below. The GANTC site proposed to be methylated by CcrM is boxed in gray. Data are plotted as $\log_2$ of the activity relative to that of the wild-type promoter in wild-type cells, so negative numbers indicate a decrease in expression. The promoter motif that includes all changes of >2-fold is indicated. (B) Transcription from the C(−71)T mutant promoter in wild-type and Δ*ssrA* cells was determined as described in the legend for Fig. 1A.

dnaA or $P_{dnaA}C(-71)T$-*lacZ* initiated replication at the same time, indicating that expression of *dnaA* from this promoter does not alter the timing of initiation. Δ*ssrA* cells with $P_{dnaA}C(-71)T$-*dnaA* initiated replication 20 min earlier than isogenic cells with $P_{dnaA}C(-71)T$-*lacZ*, suggesting that earlier transcription of *dnaA* partially suppresses the replication delay, even in the presence of a wild-type copy of the *dnaA* locus. However, the possibility that suppression was due to the amount of *dnaA* expression from $P_{dnaA}C(-71)T$, instead of the timing of expression, could not be excluded. Providing a plasmid-borne copy of *dnaA* expressed from the wild-type gene (including the leader sequence) had no effect on the growth rate of Δ*ssrA* cells, and expressing *dnaA* from P_{dnaA} with no leader sequence caused slower growth (not shown), but these promoters have fourfold-lower activity than $P_{dnaA}C(-71)T$ and sixfold-higher activity than $P_{dnaA}C(-71)T$, respectively. Nevertheless, the observations that the C(−71)T mutation relieves the P_{dnaA} transcription delay in Δ*ssrA* cells and expression of *dnaA* from the mutant promoter partially suppresses the replication delay are consistent with a model in which much of the DNA replication delay observed in the absence of *trans* translation is due to misregulation of *dnaA* expression through the GTCAANANNAATAT promoter element.

TABLE 2. Suppression of growth phenotypes by C(−71)T mutant

Description of strain[a]	Doubling time (min)	Time of replication initiation (min)
wt + vector	136 ± 11	26 ± 1
Δ*ssrA* + vector	158 ± 8	55 ± 3
wt + $P_{dnaA}C(-71)T$-*lacZ*	136 ± 6	35 ± 4
Δ*ssrA* + $P_{dnaA}C(-71)T$-*lacZ*	151 ± 9	62 ± 2
wt + $P_{dnaA}C(-71)T$-*dnaA*	133 ± 8	32 ± 3
Δ*ssrA* + $P_{dnaA}C(-71)T$-*dnaA*	135 ± 9	44 ± 2

[a] wt, wild type.

DISCUSSION

The results presented here reveal two mechanisms for regulating expression of *dnaA*: a promoter element that affects transcription and an untranslated leader sequence that re-

presses expression. Previously described mechanisms for regulating DnaA include putative transcriptional repression (15), regulated proteolysis (12), and regulatory inactivation by hydrolysis of bound ATP (21). Why are so many control mechanisms required, particularly when constitutive expression of *dnaA* does not have severe consequences during growth in culture (11)? A likely explanation is that these mechanisms provide the opportunity for input from multiple pathways that impact the ability of the cell to successfully complete DNA replication under different growth conditions. Possible regulatory roles for the *dnaA* leader sequence and promoter motif are discussed below.

The first 87 nucleotides of the *dnaA* leader sequence decreased expression from both the *dnaA* promoter and the unrelated xylose-inducible promoter >15-fold, suggesting that the leader sequence acts after transcription initiation. This leader sequence might act through a transcription attenuation mechanism or by destabilizing the mRNA. Most attenuators contain transcriptional termination sequences which are controlled by changes in RNA structure (10), but these features are not evident in the *dnaA* leader sequence. There is no predicted hairpin structure or a run of uridines characteristic of an intrinsic transcriptional terminator. Secondary structure predictions using the software program MFold (33) did not reveal alternative structures that would be predicted to block transcription elongation or translation initiation. Nevertheless, it is possible that this sequence uses a factor-dependent transcriptional terminator that has not been characterized. Alternatively, the leader sequence could target the mRNA for degradation, for example through binding of a small RNA. Searches of the *C. crescentus* genome showed no other sequences similar to the *dnaA* leader, so this repression mechanism may be dedicated to *dnaA* regulation. Although the leader sequence had profound effects on the magnitude of *dnaA* expression, it did not significantly alter the cell cycle timing of transcription. One possible use for this leader sequence would be to allow for a large burst in *dnaA* expression when DnaA protein concentrations are depleted. DnaA levels decrease dramatically under some starvation conditions (12), so a high rate of *dnaA* expression might facilitate restarting the cell cycle when nutrients become available. A temporary inactivation of the regulator that acts at the leader sequence would provide a large increase in *dnaA* expression without requiring a large increase in *dnaA* transcription.

How does the GTCAANANNAATAT element affect transcription? Mutations in the element decreased transcription, suggesting that this element is the binding site for a transcriptional activator. However, the C(−71)T mutation eliminates the delay in promoter activity in Δ*ssrA* cells, suggesting removal of a repressor binding site. One possibility is that two different transcriptional regulators bind to this sequence. Alternatively, a dual regulator may bind this sequence, repressing *dnaA* transcription in swarmer cells but switching transcription on just before the G_1–S-phase transition. There are several examples of dual regulators, including ArgP (also called IciA), a LysR-type transcription factor that binds a single promoter site to repress or activate transcription (18). Transcription is repressed when ArgP is bound to lysine but activated when it is bound to arginine. ArgP regulates *dnaA* transcription in *E. coli* (19), and *C. crescentus* contains several possible homologues. Identification and characterization of the proteins that bind to the GTCAANANNAATAT promoter element will clarify how this sequence regulates *dnaA* transcription and how the mechanism is affected by *trans* translation.

trans translation was previously shown to be required for correct timing of DNA replication initiation (17), and the data presented here show that *trans* translation is required for correct timing of transcription from the P_{dnaA} and P_s promoters, two of the earliest steps in DNA replication initiation. In Δ*ssrA* cells, mutation of the GTCAANANNAATAT element affects the timing of transcription from the *dnaA* promoter, and the growth rate and delay of replication initiation phenotypes are partially suppressed when there is a copy of *dnaA* expressed from the $P_{dnaA}C(-71)T$ mutant promoter. Why would *dnaA* expression and replication initiation be sensitive to *trans* translation activity? One possibility is that *trans* translation regulates a transcription factor that binds to the GTCAANANNA ATAT sequence, and in the absence of tmRNA the factor is misregulated and slows induction of *dnaA* transcription. An example of misregulation in the absence of *trans* translation has been observed for LacI in *E. coli* (2). LacI is tagged by *trans* translation as part of an autoregulatory circuit, and excess LacI accumulates in cells deleted for *ssrA*. This excess LacI delays transcription of the *lac* operon under inducing conditions (2). A similar defect in control of a regulator that binds to the GTCAANANNAATAT sequence could delay *dnaA* transcription, thereby delaying initiation of DNA replication. Alternatively, there may be a regulatory checkpoint that intentionally senses *trans* translation levels to ensure that there is sufficient translation capacity or that there is not a large amount of aberrant translation occurring before the cell commits to S phase. In this model, *trans* translation could directly affect the activity of a transcription factor or *trans* translation activity could be sensed indirectly, for example through the presence of stalled ribosomes. These models can be tested once factors that act at the GTCAANANNAATAT element have been identified.

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REFERENCES

1. **Abe, T., K. Sakaki, A. Fujihara, H. Ujiie, C. Ushida, H. Himeno, T. Sato, and A. Muto.** 2008. tmRNA-dependent trans-translation is required for sporulation in Bacillus subtilis. Mol. Microbiol. **69:**1491–1498.
2. **Abo, T., T. Inada, K. Ogawa, and H. Aiba.** 2000. SsrA-mediated tagging and proteolysis of LacI and its role in the regulation of *lac* operon. EMBO J. **19:**3762–3769.
3. **Collier, J., H. H. McAdams, and L. Shapiro.** 2007. A DNA methylation ratchet governs progression through a bacterial cell cycle. Proc. Natl. Acad. Sci. USA **104:**17111–17116.
4. **Collier, J., S. Murray, and L. Shapiro.** 2006. DnaA couples DNA replication and the expression of two cell cycle master regulators. EMBO J. **25:**346–356.
5. **Domian, I. J., K. C. Quon, and L. Shapiro.** 1997. Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1-to-S transition in a bacterial cell cycle. Cell **90:**415–424.
6. **Ebeling, S., C. Kundig, and H. Hennecke.** 1991. Discovery of a rhizobial RNA that is essential for symbiotic root nodule development. J. Bacteriol. **173:**6373–6382.
7. **Ely, B.** 1991. Genetics of *Caulobacter crescentus*. Methods Enzymol. **204:**372–384.
8. **Evinger, M., and N. Agabian.** 1977. Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. J. Bacteriol. **132:**294–301.
9. **Gober, J. W., and L. Shapiro.** 1992. A developmentally regulated *Cau-*

lobacter flagellar promoter is activated by 3′ enhancer and IHF binding elements. Mol. Biol. Cell **3:**913–926.
10. **Gollnick, P., and P. Babitzke.** 2002. Transcription attenuation. Biochim. Biophys. Acta **1577:**240–250.
11. **Gorbatyuk, B., and G. T. Marczynski.** 2001. Physiological consequences of blocked *Caulobacter crescentus dnaA* expression, an essential DNA replication gene. Mol. Microbiol. **40:**485–497.
12. **Gorbatyuk, B., and G. T. Marczynski.** 2005. Regulated degradation of chromosome replication proteins DnaA and CtrA in *Caulobacter crescentus*. Mol. Microbiol. **55:**1233–1245.
13. **Julio, S. M., D. M. Heithoff, and M. J. Mahan.** 2000. *ssrA* (tmRNA) plays a role in *Salmonella enterica* serovar Typhimurium pathogenesis. J. Bacteriol. **182:**1558–1563.
14. **Keiler, K. C.** 2008. Biology of *trans*-translation. Annu. Rev. Microbiol. **62:** 133–151.
15. **Keiler, K. C., and L. Shapiro.** 2001. Conserved promoter motif is required for cell cycle timing of *dnaX* transcription in *Caulobacter*. J. Bacteriol. **183:** 4860–4865.
16. **Keiler, K. C., and L. Shapiro.** 2003. tmRNA in *Caulobacter crescentus* is cell cycle regulated by temporally controlled transcription and RNA degradation. J. Bacteriol. **185:**1825–1830.
17. **Keiler, K. C., and L. Shapiro.** 2003. tmRNA is required for correct timing of DNA replication in *Caulobacter crescentus*. J. Bacteriol. **185:**573–580.
18. **Laishram, R., and J. Gowrishankar.** 2007. Environmental regulation operating at the promoter clearance step of bacterial transcription. Genes Dev. **15:**1258–1272.
19. **Lee, Y., H. Kim, and D. S. Hwang.** 1996. Transcriptional activation of the *dnaA* gene encoding the initiator for *oriC* replication by IciA protein, an inhibitor of in vitro *oriC* replication in *Escherichia coli*. Mol. Microbiol. **19:**389–396.
20. **Marczynski, G., A. Dingwall, and L. Shapiro.** 1990. Plasmid and chromosomal DNA replication and partitioning during the *Caulobacter crescentus* cell cycle. J. Mol. Biol. **212:**709–722.
21. **Marczynski, G., and L. Shapiro.** 2002. Control of chromosome replication in *Caulobacter crescentus*. Annu. Rev. Microbiol. **56:**625–656.
22. **Marczynski, G. T.** 1999. Chromosome methylation and measurement of faithful, once and only once per cell cycle chromosome replication in *Caulobacter crescentus*. J. Bacteriol. **181:**1984–1993.
23. **Marczynski, G. T., K. Lentine, and L. Shapiro.** 1995. A developmentally regulated chromosomal origin of replication uses essential transcription elements. Genes Dev. **9:**1543–1557.
24. **Meisenzahl, A. C., L. Shapiro, and U. Jenal.** 1997. Isolation and characterization of a xylose-dependent promoter from *Caulobacter crescentus*. J. Bacteriol. **179:**592–600.
25. **Moore, S. D., and R. T. Sauer.** 2007. The tmRNA system for translational surveillance and ribosome rescue. Annu. Rev. Biochem. **76:**101–124.
26. **Ohmori, H., M. Kimura, T. Nagata, and Y. Sakakibara.** 1984. Structural analysis of the *dnaA* and *dnaN* genes of *Escherichia coli*. Gene **28:**159–170.
27. **Okan, N. A., J. B. Bliska, and A. W. Karzai.** 2006. A role for the SmpB-SsrA system in *Yersinia pseudotuberculosis* pathogenesis. PLoS Pathog. **2:**e6.
28. **Quon, K. C., B. Yang, I. J. Domian, L. Shapiro, and G. T. Marczynski.** 1998. Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. Proc. Natl. Acad. Sci. USA **95:**120–125.
29. **Ryan, K. R., and L. Shapiro.** 2003. Temporal and spatial regulation in prokaryotic cell cycle progression and development. Annu. Rev. Biochem. **72:**367–394.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
31. **Siam, R., A. Brassinga, and G. Marczynski.** 2003. A dual binding site for integration host factor and the response regulator CtrA inside the *Caulobacter crescentus* replication origin. J. Bacteriol. **185:**5563–5572.
32. **Winzeler, E., and L. Shapiro.** 1995. Use of flow cytometry to identify a *Caulobacter* 4.5 S RNA temperature-sensitive mutant defective in the cell cycle. J. Mol. Biol. **251:**346–365.
33. **Zuker, M.** 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. **31:**3406–3415.
34. **Zweiger, G., and L. Shapiro.** 1994. Expression of *Caulobacter dnaA* as a function of the cell cycle. J. Bacteriol. **176:**401–408.