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Feedback Control of DnaA-Mediated Replication Initiation by Replisome-Associated HdaA Protein in *Caulobacter*▽

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Chromosome replication in *Caulobacter crescentus* is tightly regulated to ensure that initiation occurs at the right time and only once during the cell cycle. The timing of replication initiation is controlled by both CtrA and DnaA. CtrA binds to and silences the origin. Upon the clearance of CtrA from the cell, the DnaA protein accumulates and allows loading of the replisome at the origin. Here, we identify an additional layer of replication initiation control that is mediated by the HdaA protein. In *Escherichia coli*, the Hda protein inactivates DnaA after replication initiation. We show that the *Caulobacter* HdaA homologue is necessary to restrict the initiation of DNA replication to only once per cell cycle and that it dynamically colocalizes with the replisome throughout the cell cycle. Moreover, the transcription of *hdaA* is directly activated by DnaA, providing a robust feedback regulatory mechanism that adjusts the levels of HdaA to inactivate DnaA.

The events involved in the initiation of chromosomal replication are similar in eubacteria, eukaryotes, and archaea: replication starts with the binding of specific initiator protein(s) to chromosomal origins, resulting in the unwinding of the DNA duplex. Bacteria replicate their chromosome(s) from a single replication origin, and replication is initiated by DnaA, which binds to DnaA boxes within the chromosomal origin (34). In general, replication initiation takes place only once per cell cycle, although in rapidly dividing cells in some bacterial families, the next round of replication can start before the ongoing round has terminated (6, 9).

The gammaproteobacterium *Escherichia coli* has been a long-standing model system to study the regulation of DNA replication initiation. In this bacterium, at least three mechanisms prevent the reinitiation of DNA replication from the newly replicated origins (21, 34): (i) the inhibition of DnaA activity (22, 35), (ii) the titration of free DnaA molecules by the *datA* locus (23, 24), and (iii) the sequestration of the chromosomal origin by the SeqA protein (3, 42). When bound to ATP, DnaA is in its active form. ATP-DnaA initiates DNA replication, leading to the loading of the DNA polymerase III holoenzyme. The *r*egulated *i*nactivation of *D*na*A* (RIDA) occurs by the conversion of ATP-DnaA into ADP-DnaA (22, 35) that is stimulated by a protein complex composed of the β-clamp subunit of the DNA polymerase (DnaN) and the Hda protein. The requirement of the DNA-loaded clamp can ensure the timely inactivation of DnaA by Hda, only once DNA replication has initiated (45). The Hda protein is *h*omologous to the ATPase domain of *D*na*A*, and it directly interacts with DnaN through a β-clamp binding motif located at its N terminus (25, 44). *E. coli* strains for which the *hda* gene is deleted accumulate suppressor mutations, suggesting that the *hda* gene is essential for viability or that Hda deficiency leads to severe growth impairment in *E. coli* (12, 22, 39). The overproduction of DnaA in the absence of Hda leads to growth retardation or cell death, due to the overinitiation of DNA replication by ATP-DnaA (39).

In the aquatic alphaproteobacterium *Caulobacter crescentus*, the initiation of DNA replication takes place only once per cell cycle, and only at a specific time of the cell cycle (29). *Caulobacter* divides asymmetrically, yielding a swarmer cell and a stalked cell. The stalked cell immediately initiates DNA replication, while the swarmer cell is unable to initiate the replication of its chromosome until it has differentiated into a stalked cell. The DnaA protein is essential for the initiation of DNA replication in *Caulobacter* (14). Although *E. coli* DnaA is stable for more than 24 h (47), *Caulobacter* DnaA is actively degraded (15) so that levels of DnaA can rapidly change as a function of the cell cycle (5). The transcription of *dnaA* is also cell cycle regulated, peaking in swarmer cells prior to the initiation of DNA replication (27, 49). The transcription of *dnaA* is regulated by the methylation state of the *dnaA* promoter: the *dnaA* promoter is preferentially transcribed when it is in the fully methylated state prior to the initiation of replication (4). Chromosomal loci are fully methylated at the beginning of the cell cycle. The loci then become hemimethylated by the passage of the replication fork and are not fully methylated again until just before cell division, because the cell cycle-regulated DNA methylase CcrM accumulates only in late predivisional cells. Once DNA replication is initiated and the replication fork passes through *dnaA*, the two copies of *dnaA* are maintained in the hemimethylated state until the completion of DNA replication, resulting in decreased levels of transcription from the *dnaA* promoter. This DNA methylation-dependent regulation of *dnaA* transcription contributes to the transient high levels of DnaA observed at the swarmer-to-stalked cell transition.

In swarmer cells, replication initiation is inhibited by the

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TABLE 1. Strains and plasmids used in this study

Plasmid or strain	Relevant characteristics, construction, or genotype	Source or reference
Plasmids		
pXGFP4C1	Integrating plasmid	D. Alley, unpublished data
pX-DnaA	*dnaA* under the control of the *xylX* promoter in pXGFP4C1	This study
pNPTS138	Integrating plasmid containing the *sacB* gene	D. Alley, unpublished
pNPTS138-Δ*hda*A	The regions upstream and downstream of the *hdaA* coding sequence cloned into pNPTS138	This study
pMR20	Low-copy-number replicating plasmid	R. Roberts, unpublished data
pX-HdaA	*hdaA* under the control of the *xylX* promoter in pMR20	This study
pXGFP-HdaA	*gfp-hdaA* under the control of the *xylX* promoter in pXGFP4C1	This study
pCHYC-1	Integrating plasmid containing the *mcherry* gene	46
pDnaN-RFP	*dnaN-mcherry* under the control of the *dnaN* promoter once integrated into the chromosome	This study
pJS14	High-copy-number replicating plasmid	J. Skerker, unpublished data
pJSX-DnaA	*dnaA* under the control of the *xylX* promoter in pJS14	This study
p*lacZ*290	Low-copy-number plasmid to create transcriptional fusions with *lacZ*	13
p*lacZ*290-*hdaA*P(WT)	*lacZ* gene under the control of the wild-type *hdaA* promoter in p*lacZ*290	This study
p*lacZ*290-*hdaA*P(box3)	*lacZ* gene under the control of the mutant *hdaA* promoter (box3) in p*lacZ*290	This study
pET21a	Overexpression plasmid to clone histidine-tagged proteins	Novagen
pET21a-HdaA	*hdaA* cloned into pET21a to overexpress His6-HdaA	This study
Strains		
E. coli		
S17-1	294::RP4-2(Tc::Mu)(Km::Tn*7*)	41
Rosetta (DE3)/pLysS	Designed to enhance the expression of proteins that contain rare codons	Novagen
C. crescentus		
NA1000	Synchronizable derivative of wild-type strain CB15 (CB15N)	11
JC249	pX-DnaA integrated at *xylX* into NA1000	This study
JC353	NA1000 Δ*hdaA* pX-HdaA	This study
JC208	pXGFP-HdaA integrated at *xylX* into NA1000	This study
JC388	pDnaN-RFP integrated at *dnaN* into JC208	This study
LS1	P*lacZ*::*ccrM bla6 rsaA2*	48
JC362	NA1000 P*lacZ*::*ccrM*	This study
GM2471	NA1000 Δ*dnaA*::Ω P*xylX*::*dnaA*	14

CtrA~P response regulator that binds directly to five sites within the *Caulobacter* origin of replication (*Cori*) (37). At the swarmer-to-stalked cell transition, CtrA is degraded by the ClpXP protease (7, 18, 31), which releases the *Cori* for replication initiation by DnaA. Following the initiation of DNA replication, *ctrA* transcription is activated (7, 8). The accumulation of CtrA then contributes to the prevention of replication reinitiation. We asked if CtrA silencing of the origin of replication is sufficient to restrict the initiation of DNA replication to the swarmer-to-stalked cell stage of the cell cycle and to only once per cell cycle. To address this question, we identified the *Caulobacter* homologue of the *E. coli* Hda protein, named HdaA, and showed that it is required to confine the initiation of DNA replication to specific times in the cell cycle. Cells deficient in HdaA overinitiate DNA replication, and they exhibit severe growth defects. HdaA dynamically colocalizes with the β-clamp of DNA polymerase throughout the S phase, suggesting that HdaA is a component of the *Caulobacter* replisome. The stimulation of *hdaA* transcription by DnaA provides a feedback loop that fine-tunes the timing of DNA replication initiation. Thus, controlled *dnaA* transcription mediated by the differential methylation state of its promoter (4), the inhibition of DNA replication initiation by HdaA (this report), the temporally controlled proteolysis of DnaA (15), and the restriction of the CtrA silencer of DNA replication initiation to swarmer and predivisional cells (7) together provide robust restriction of DnaA function as an initiator of DNA replication to once per cell cycle.

MATERIALS AND METHODS

Bacterial strains, synchronization, and growth conditions. *Caulobacter crescentus* strains were grown in peptone yeast extract (PYE) complex medium or M2 minimal salts plus 0.2% glucose (M2G) minimal medium (10) at 28°C. The plasmids and strains used are listed in Table 1. The antibiotics used for the *Caulobacter* liquid cultures include rifampin (rifampicin) (15 μg/ml), chloramphenicol (1 μg/ml), kanamycin (5 μg/ml), and oxytetracycline (1 μg/ml). The antibiotics used for the *E. coli* liquid cultures include chloramphenicol (20 μg/ml), kanamycin (30 μg/ml), oxytetracycline (12 μg/ml), and ampicillin (100 μg/ml). Plasmids were mobilized from *E. coli* S17-1 (41) into *Caulobacter* strains by bacterial conjugation or introduced by transformation. Bacteriophage φCR30 was used for general transduction into *Caulobacter*. Synchronized cell cultures were obtained by centrifugation in a Ludox density gradient, followed by the isolation of swarmer cells (11). Swarmer cells were resuspended into M2G medium and allowed to proceed synchronously through their cell cycle.

Plasmid constructions. For the construction of the pX-DnaA plasmid, oligonucleotides 5′-CCCATATGACCATGAAGGGCGGGGTTGCC-3′ and 5′-CCGGATCCTTAGCCCCGCAGCTTGCGCGT-3′ were used to amplify the *dnaA* coding sequence by PCR. The corresponding PCR product was digested with NdeI and BamHI and cloned into NdeI-BamHI-digested pXGFP4C1 (with the *gfp* gene eliminated), giving the pX-DnaA plasmid.

For the construction of the pNPTS138-Δ*hdaA* plasmid, the *hdaA* downstream region was PCR amplified using primers 5′-CCGGATCCGAGGGGGATGAGGGGTAGGC-3′ and 5′-CCGGGCTAGCAGGCGTTGATGCGGGTCAGCT-3′. The 500-bp product was digested with NheI and BamHI and cloned into an NheI-BamHI-digested pNPTS138 plasmid, giving pNPTS138-*hdaA*down. The *hdaA* upstream region was PCR amplified using primers 5′-GGTAAGCTTAC

CGGAAGGCGAAATGCCACT-3′ and 5′-CCGGATCCTTTGAACTGGGTG GACAATCCT-3′. The 500-bp product was then digested with HindIII and BamHI and cloned into BamHI-HindIII-digested pNPTS138-*hdaA*down, giving the pNPTS138-Δ*hdaA* plasmid.

For the construction of the pX-HdaA plasmid, the *hdaA* coding sequence was PCR amplified using primers 5′-CCCCATATGTTGTCCACCCAGTTCAAAC TGCCGC-3′ and 5′-CCGGATTCCTACCCCTCATCCCCCTCGAAC-3′. The product was digested by NdeI and BamHI and cloned into an NdeI-BamHI-digested pXGFP4C1 plasmid (with the *gfp* gene eliminated). The resulting plasmid was used as a template to amplify a DNA region containing the *hdaA* gene under the control of the *xylX* promoter using primers 5′-CCTCTAGACTACC CCTCATCCCCCTCGAAC-3′ and 5′-AAGGTACCCAGCCGATCAGGCGG AACTGG-3′. This second product was digested by KpnI and XbaI and cloned into a KpnI-XbaI-digested pMR20 low-copy-number plasmid, giving the pX-HdaA plasmid.

For the construction of the pXGFP-HdaA plasmid, the *hdaA* coding sequence was PCR amplified using primers 5′-CCAAGCTTTGTCCACCCAG TTCAAACTGCC-3′ and 5′-CCGGATCCCTACCCCTCATCCCCCTCGAA C-3′. The product was digested by BamHI and HindIII and cloned into a BamHI-HindIII-digested pXGFP4C1 plasmid, giving pXGFP-HdaA.

For the construction of the pDnaN-RFP plasmid, the 3′ half of the *dnaN* coding sequence was PCR amplified using primers 5′-CCCATATGCCGAGGG CGCGGTCGGCATC-3′ and 5′-AAGGTACCGACCCGCAGCGGCATCAGC AC-3′. The product was digested by NdeI and KpnI and cloned into NdeI-KpnI-digested pCHYC-1.

For the construction of the pJSX-DnaA plasmid, the pX-DnaA plasmid was used to amplify a DNA region containing the *dnaA* gene under the control of the *xylX* promoter by using primers 5′-AAGGTACCCAGCCGATCAGGCG GAACTGG-3′ and 5′-ACGCGCAAGCTGCGGGGCTAAGGATCCGG-3′. The product was digested by KpnI and BamHI and cloned into a KpnI-BamHI-digested pJS14 plasmid, giving pJSX-DnaA.

For the construction of the p*lacZ*290-*hdaA*P(WT) and p*lacZ*290-*hdaA*P(box3) plasmids, the wild-type *hdaA* promoter region [*hdaA*P(WT)] was PCR amplified using primers 5′-CCGGATTCACCGGAAGGCGAAATGCCACTT-3′ and 5′-GGTAAGCTTGTTTGAACTGGGTGGACAATCCT-3′. The product was digested by HindIII and BamHI and cloned into a HindIII-BamHI-digested p*lacZ*290 plasmid, giving p*lacZ*290-*hdaA*P(WT). The targeted mutagenesis of the DnaA box 3 in the *hdaA* promoter [*hdaA*P(box3)] was generated by PCR using the mutagenic primers 5′-CGCGGCCTAACCCCCATCCGTGCCTCCT CCGCCCC-3′ and 5′-GGGGCGGAGGAGGCACGGATGGGGGTTAGGCC GCG-3′. The resulting product was also cloned into a HindIII-BamHI-digested p*lacZ*290 plasmid, giving p*lacZ*290-*hdaA*P(box3).

For the construction of the pET21a-HdaA plasmid, the *hdaA* coding sequence was amplified using primers 5′-CCCCATATGTTGTCCACCCAGT TCAAACTGCCGC-3′ and 5′-CCCTCGAGCCCCTCATCCCCCTCGAACC C-3′. The product was digested by NdeI and XhoI and cloned into an NdeI-XhoI-digested pET21a plasmid, giving pET21a-HdaA.

Strain constructions. For the construction of the JC249 strain, plasmid pX-DnaA was integrated into the *xylX* promoter (32) of strain NA1000 by a single integration event.

For the construction of the JC353 strain, plasmid pNPTS138-Δ*hda*A was introduced into strain NA1000 by conjugation, selecting for kanamycin-resistant colonies with the plasmid integrated at the *hdaA* locus by PCR. Plasmid pNPTS138-Δ*hda*A was integrated into the *Caulobacter* chromosome by single homologous recombination. Plasmid pX-HdaA was then introduced into the resulting strain by transformation. The resulting strain was grown to stationary phase in PYE medium lacking kanamycin. The cells were plated on PYE medium plus 3% sucrose and incubated at 28°C. Single colonies were picked and transferred in parallel onto plain PYE plates and PYE plates containing kanamycin. Kanamycin-sensitive clones, which had lost the integrated plasmid due to a second recombination event, were then tested for the presence of the mutated allele by colony PCR.

For the construction of the JC208 strain, plasmid pXGFP-HdaA was integrated at the *xylX* promoter of strain NA1000 by a single integration event.

For the construction of the JC388 strain, plasmid pDnaN-RFP was integrated at the *dnaN* locus of strain JC208 by a single integration event.

For the construction of the JC362 strain, transduction was used for introducing the P*lac*::*ccrM* construct from phage lysates grown on strain LS1 into NA1000.

HdaA antibody preparation. The HdaA protein tagged with hexahistidine was overproduced using pET21a-HdaA in the Rosetta *E. coli* strain. The tagged protein was purified by nickel affinity chromatography under standard nondenaturing conditions (Qiagen) and used to immunize rabbits for the production of polyclonal antibodies (Josman).

Immunoblot analysis. DnaA and CcrM proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (40). CtrA and HdaA proteins were resolved on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. The gels were electrotransferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Immunodetection was performed with polyclonal antibodies. Anti-DnaA sera, anti-CtrA sera, and donkey anti-rabbit sera conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) were diluted 1:10,000. Anti-CcrM serum was diluted 1:5,000. Anti-HdaA serum was diluted 1:2,000. A chemiluminescent reagent (PerkinElmer, Wellesley, MA) and Kodak (Rochester, NY) Bio-Max MR films were used. The images were processed with Photoshop (Adobe, Mountain View, CA), and the relative band intensities were determined by using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Flow cytometry analysis. Rifampin-treated cells were fixed and stained with DNA-binding Vybrant DyeCycle orange (Invitrogen), as previously described (28). Fixed cells were analyzed on a Becton Dickinson FACScan fluorescence-activated cell sorter. Flow cytometry data were analyzed using FlowJo software (Tree Star).

Analysis of the copy-number ratio of *Cori* to *ter* by Southern hybridization. Chromosomal DNA was prepared using the Puregene Yeast/Bact. kit B (Qiagen, Valencia, CA). Chromosomal DNA (1 to 3 μg) was digested by BamHI and analyzed by Southern hybridization using digoxigenin (DIG)-labeled probes obtained by PCR (Roche, Mannheim, Germany) with primers 5′-CTGAGGACACGACAGC GACCTC-3′ and 5′-CGCGGCGTAGCAGGGCATTTC-3′ for the *Cori* probe and primers 5′-CTCAACATGCTTGACCGCCAGAT-3′ and 5′-ACCCAGGTCCTCG CCAAAGCTG-3′ for the *ter* probe. DIG-labeled probes were detected using anti-DIG-AP antibodies and CSPD (disodium 3-[4-methoxyspiro{l,2-dioxetane-3,2′-[5′-chloro]tricyclo[3.3.1.1^{3,7}]decan}-4-yl]phenylphosphate) following the protocol recommended by the manufacturer (Roche, Mannheim, Germany), and Bio-Max MR films (Kodak, Rochester, NY). The images were processed with Photoshop (Adobe, Mountain View, CA), and the relative band intensities were determined using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Microscopy. Cells were immobilized using a thin layer of medium plus 1% agarose. Normarski differential interference contrast (DIC) and fluorescence microscopy images were taken with a 100× DIC objective on an E800 microscope (Nikon, East Rutherford, NJ) with a 5-MHz Micromax 5600 cooled charge-coupled-device camera controlled through Metamorph (Universal Imaging, Downingtown, PA). The images were processed using Adobe Photoshop and Metamorph version 4.5.

Promoter activity assays. The β-galactosidase activities of strains containing p*lacZ*290 derivatives were assayed in log-phase cultures in PYE medium, as previously described (33). β-Galactosidase activities represent the averages of the results from at least three independent assays.

RESULTS

Control of the timing of replication initiation is maintained in cells that overexpress *dnaA* throughout the cell cycle. *Caulobacter* permits detailed analysis of the cell cycle timing of chromosome replication because one can easily obtain synchronized cell populations by differential density centrifugation, without perturbing normal physiology. Immunoblot analysis of the relative levels of DnaA and CtrA as a function of the cell cycle revealed that there is a significant period of the cell cycle when the DnaA replication initiator is detectable, while the CtrA inhibitor of replication initiation drops to undetectable levels (5). This period lasts for about 40 min during the swarmer-to-stalked cell transition and in stalked cells, and it is far more than the time needed to initiate the replication of a bacterial chromosome. This observation suggested that CtrA action may not be sufficient to restrict the initiation of DNA replication to only once per cell cycle. It was also recently shown that DNA replication does not overinitiate when CtrA binding sites are eliminated from the *Cori* by targeted mutagenesis of the *Caulobacter* chromosome (2). The function of CtrA in replication control is more likely to prevent chromosome replication in swarmer cells.

We observed that the levels of DnaA decrease after the initi-

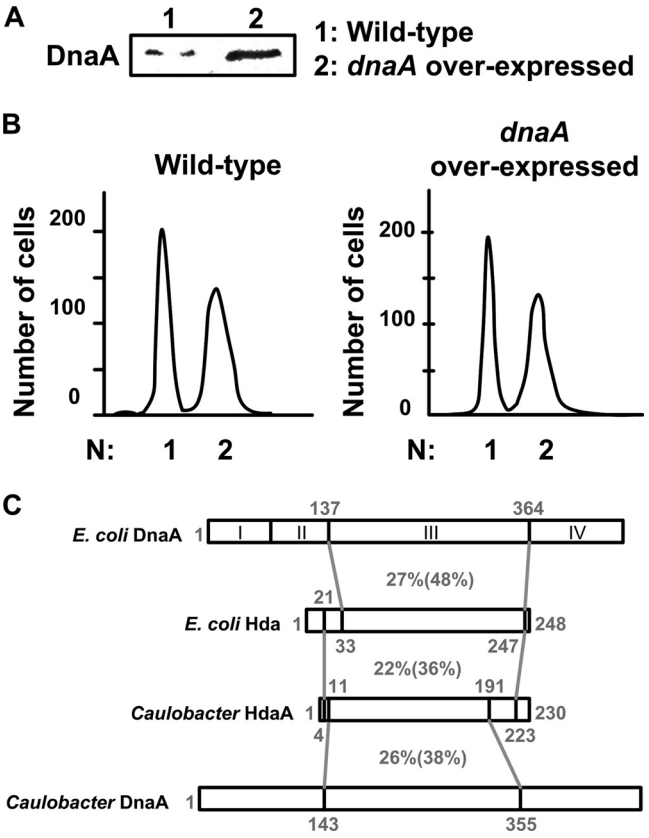


FIG. 1. The temporal control of *dnaA* transcription is not necessary for the correct timing of the initiation of DNA replication. (A) DnaA accumulates to high levels in cells bearing a chromosomal copy of *dnaA* that is transcribed from an inducible *xylX* promoter (*dnaA* overexpressed, strain JC249). Cells were grown for 4 h in PYE medium plus 0.2% glucose and 0.3% xylose (PYEGX medium), and then immunoblotting of the cell extracts was performed using antibodies to DnaA. (B) Cells from strain JC249 do not overinitiate DNA replication. Cells were grown for 4 h in PYEGX medium and then treated with rifampin for 3 h. Cells were fixed and stained with Vybrant DyeCycle orange, before analyzing their DNA content by flow cytometry. The horizontal axis indicates the number of complete chromosomes. (C) Regions of homology between the *E. coli* DnaA and Hda proteins and the *Caulobacter* DnaA and HdaA proteins are shown. The amino acid positions are shown in gray. Identities and similarities (in parentheses) between protein regions are indicated by percent values. DnaA domains I, II, III, and IV are indicated (44). DnaA domain III includes the AAA+ motifs for the ATPase activity of DnaA.

ation of DNA replication (5), even before the accumulation of CtrA in predivisional cells, suggesting that the levels of DnaA may become too limiting to allow the reinitiation of DNA replication. To test if the changes in the expression of *dnaA* over the cell cycle are necessary for the correct timing of the initiation of DNA replication, we constructed a mutant strain (JC249) that transcribes *dnaA* constitutively throughout the cell cycle and measured the DNA content in these cells by flow cytometry. The *dnaA* gene was expressed from the xylose-inducible *xylX* promoter so that the level of DnaA in JC249 cells grown in rich medium supplemented with xylose (PYEGX medium) for 4 h was significantly higher than that observed in wild-type cells (Fig. 1A). To examine the effect of high levels of DnaA on the initiation of chromosome replication, cultures of mutant and wild-type cells were examined by flow cytometry (Fig. 1B). JC249 cells and wild-type cells were treated with the RNA polymerase inhibitor rifampin for 3 h, which allows the completion of DNA replication but not cell division or the reinitiation of DNA replication (28). Flow cytometry analysis revealed that both wild-type cells and JC249 cells accumulate only one or two complete chromosomes, demonstrating that the control of replication initiation remains efficient when *dnaA* is overexpressed throughout the cell cycle. Thus, the temporal regulation of *dnaA* transcription is not solely responsible for restricting the initiation of DNA replication to once and only once during the cell cycle.

HdaA is required for normal cell growth, for cell division, and for restricting the initiation of DNA replication to once per cell cycle. The observation that the initiation of DNA replication still occurs only once per cell cycle in cells that express *dnaA* throughout the cell cycle (Fig. 1B), despite the absence of CtrA for about 40 min in stalked cells, suggested that there exists a second negative regulator of the initiation of DNA replication in *Caulobacter* cells. One possibility is that the activity of DnaA is temporally regulated by a mechanism similar to the RIDA mechanism found in *E. coli*. The inactivation of DnaA after the initiation of DNA replication is mediated by the Hda protein in *E. coli* (22). We identified the *Caulobacter* homologue of the *hda* gene, named the *hdaA* gene (CC_1711), encoding a putative protein that is similar to both the *E. coli* Hda protein and to the *Caulobacter* and the *E. coli* DnaA proteins (Fig. 1C). We were not able to obtain an *hdaA* deletion strain, unless we added a complementing low-copy-number plasmid that expressed *hdaA* under the control of the inducible *xylX* promoter. When the resulting strain (JC353) was grown in rich medium in the presence of glucose (PYEG medium) at all times, conditions that prevented the expression of *hdaA* from the *xylX* promoter, we observed that cells grew very slowly with a generation time of ~235 min, instead of ~90 min for a wild-type strain (data not shown). DIC microscopy showed that HdaA-depleted cells are filamentous (Fig. 2A), indicating that HdaA is required for normal cell growth. This phenotype is similar to that of mutant cells which overinitiate DNA replication when expressing a thermosensitive allele of *ctrA* (37), consistent with HdaA functioning as an inhibitor of replication initiation. When JC353 was grown in the presence of xylose (PYEX medium), the wild-type phenotype was restored (Fig. 2A).

To test if the initiation of DNA replication was deregulated in cells depleted for HdaA, we performed flow-cytometry experiments to observe the number of chromosomes in wild-type and JC353 cells grown for four generations in PYEG medium and treated with rifampin (Fig. 2B). DIC microscopy showed that cells were only slightly filamentous at the time when rifampin was added to the culture medium (Fig. 2B, bottom panels). We found that ~35% of the HdaA-depleted cells contained more than two chromosomes, suggesting that these cells had initiated a new round of DNA replication before the end of the previous round of replication, which happened only very rarely in wild-type *Caulobacter* cells (~2% of the cells). We also performed Southern hybridizations to examine the copy-number ratio of *Cori* to *ter* (the replication termination region) in JC353 cells grown in PYEX or PYEG medium. As shown in Fig. 2C, the *Cori* to *ter* ratio was 1.98 (±0.25)-fold

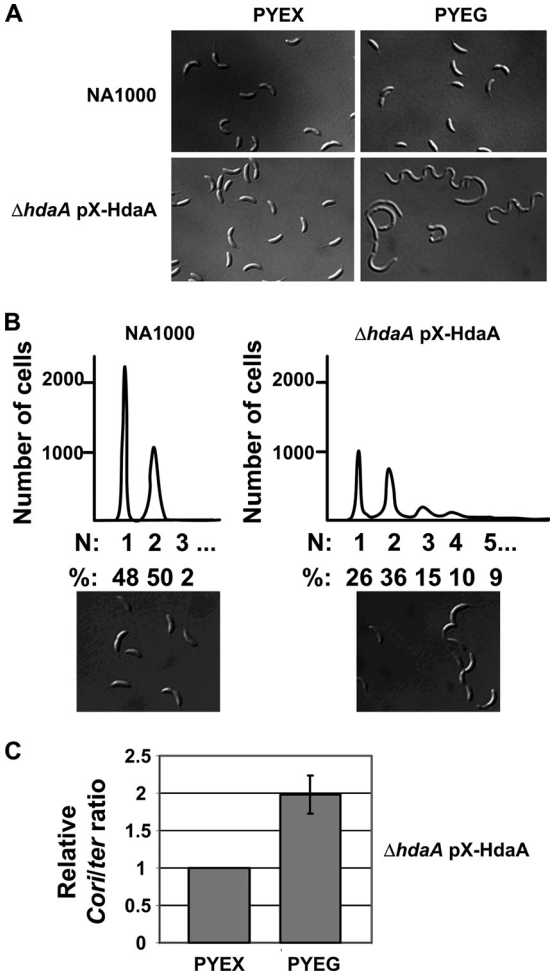


FIG. 2. The *Caulobacter hdaA* gene is required for cell division and for the temporal control of the initiation of DNA replication. (A) Nomarski DIC microscopy images of NA1000 (wild-type) and JC353 (Δ*hdaA* pX-HdaA) cells grown in PYE medium plus xylose (PYEX; *hdaA* expressed) or PYE medium plus glucose (PYEG; *hdaA* not expressed) at all times. (B) Flow cytometry analyses of the NA1000 and JC353 strains grown for four generations in PYEG medium. Cells were treated with rifampin for 3 h prior to fixing and staining with Vybrant DyeCycle orange. The horizontal axis indicates the number of complete chromosomes. The percentages correspond to the proportion of cells containing the indicated number of chromosomes per cell. Nomarski DIC microscopy images of NA1000 and JC353 cells at the time of rifampin addition are shown in the bottom panels. (C) The JC353 strain was grown in PYEX or PYEG medium at all times, and samples were harvested to quantify the copy numbers of the chromosomal *Cori* and *ter* sites in a Southern hybridization experiment using specific probes. The ratio of the *Cori* sites to *ter* sites in cells grown in PYEX medium is defined as 1.0, and the relative value for cells grown in PYEG medium is shown. Results are the averages of data from independently duplicated experiments. The error bar indicates the standard deviation.

higher in cells depleted of HdaA (PYEG medium) than in cells that contained HdaA (PYEX medium), indicating that HdaA-depleted cells often overinitiate DNA replication. Cumulatively, these results suggest that HdaA is a second inhibitor of the initiation of DNA replication in *Caulobacter*.

HdaA dynamically colocalizes with the replisome throughout the cell cycle. It has been shown in *E. coli* that Hda must interact with DnaN bound to DNA to inactivate DnaA. Thus, DnaA is inactivated by HdaA only once DNA replication has initiated (20, 22, 45). The *E. coli* Hda protein binds directly to DnaN in vitro (25, 44). In *Caulobacter*, DNA polymerase III is dynamically localized during the cell cycle (19). The replisome is assembled on the *Cori*, is positioned at the future stalked-cell pole during the swarmer-to-stalked cell transition, and then gradually moves to mid-cell as DNA replication progresses. Once DNA replication is complete in late predivisional cells, the replisome disassembles. To determine if the HdaA protein is in a complex with the *Caulobacter* replisome in vivo, we constructed a strain that would allow us to observe the subcellular localization of HdaA in live cells. We visualized HdaA in live cells by integrating an in-frame fusion between the N terminus of HdaA and the green fluorescent protein (GFP), under the control of the native *xylX* promoter. We isolated a pure population of swarmer cells from this strain (JC208) grown in minimal medium containing xylose (M2GX medium) for 3 h before synchronization. The intracellular location of GFP-HdaA was examined in individual cells by time-lapse fluorescence microscopy, as the cells progressed through their cell cycle. Figure 3A shows representative time-lapse images of cells bearing both wild-type HdaA and HdaA-GFP. At the 10-min time point, prior to the initiation of replication, we observed cells with fluorescence diffuse in the cytoplasm. Once a tight fluorescent focus was formed upon replication initiation, it proceeded to migrate toward mid-cell (between the 20-min and the 180-min time points), before disappearing in predivisional cells. During the migration process, we sometimes observed cells with two closely spaced GFP-HdaA foci. The pattern of HdaA cellular localization during the cell cycle is the same as that described for the localization of multiple replisome components in *Caulobacter* (19), suggesting that HdaA interacts with the replisome throughout the cell cycle.

To determine if HdaA colocalizes with DnaN in vivo, we observed a double-labeled strain by time-lapse fluorescence microscopy. In the strain containing the GFP-HdaA construct, we replaced the wild-type allele of DnaN by an in-frame fusion between the red fluorescent protein (RFP) and the C terminus of DnaN under the control of the endogenous *dnaN* promoter (strain JC388). The intracellular location of GFP-HdaA and DnaN-RFP was examined in individual JC388 cells as they progressed through the cell cycle (Fig. 3B). We observed that GFP-HdaA and DnaN-RFP colocalize throughout the whole replication cycle, suggesting that HdaA is a component of the replisome.

It was previously shown that ongoing replication is required for the formation of replisome foci in *Caulobacter* cells (19). The DNA gyrase inhibitor novobiocin blocks the elongation of DNA replication in *Caulobacter* cells (19). To determine if the dynamic localization of HdaA also requires active replication, we treated a population of replication-competent stalked JC388 cells with novobiocin and examined the cells for DnaN and HdaA foci (Fig. 3C, middle panels). Upon the inhibition of DNA replication, DnaN-RFP foci were not detected, but rather a diffuse fluorescent signal was observed, confirming that the presence of replisome foci requires ongoing replication. Under these conditions, GFP-HdaA also appeared diffuse. When the cells were washed and resuspended in fresh medium without novobiocin, both DnaN-RFP and GFP-HdaA

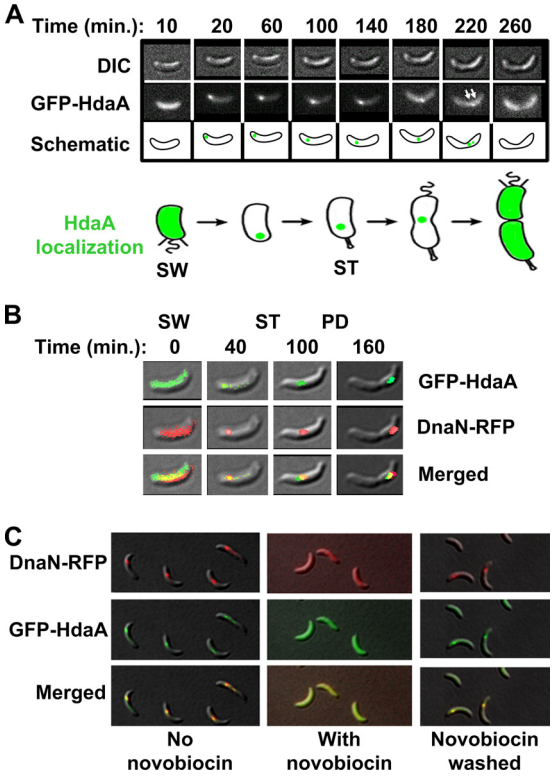


FIG. 3. HdaA dynamically colocalizes with the replisome. (A) Time-lapse fluorescence microscopy analysis of GFP-HdaA-expressing cells. Strain JC208 was cultivated in M2G medium plus 0.3% xylose (M2GX) for 3 h prior to synchronization of the culture. Swarmer cells were isolated and placed on a thin layer of agarose containing nutrients, and images of the same cells were acquired at the indicated time points as the cells progressed through their cell cycle. Cell division occurs at ~280 min under these growth conditions. DIC images, fluorescence images, and schematics of the same cells are shown. White arrows indicate cells with two closely spaced GFP-HdaA foci. The green color in the schematics indicates the intracellular position of GFP-HdaA. SW, swarmer cell; ST, stalked cell. (B) Time-lapse fluorescence microscopy analysis of GFP-HdaA and DnaN-RFP colocalization. Strain JC388 was cultivated in M2GX medium for 3 h prior to synchronization of the culture. Swarmer cells were isolated and placed on a thin layer of agarose containing nutrients, and images of the same cells were acquired at the indicated time points. Top panels, GFP-HdaA (green) over DIC image; middle panels, DnaN-RFP (red) over DIC image; bottom panels, GFP-HdaA and DnaN-RFP over DIC image to show colocalization of the fluorescence markers (yellow). SW, swarmer cell; ST, stalked cell; PD, predivisional cell. (C) Strain JC388 was synchronized, and novobiocin was added at the late stalked-cell stage (65 min into the cell cycle). Some cells were washed and resuspended in fresh M2GX medium 42 min later. Top row, DnaN-RFP (red) over DIC; middle row, GFP-HdaA (green) over DIC; bottom row, DnaN-RFP and GFP-HdaA (colocalization in yellow) over DIC; left column, untreated stalked cells 65 min into the cell cycle; middle column, cells 12 min after the addition of novobiocin; right column, cells treated with novobiocin for 42 min, washed, and incubated in fresh medium for 10 min.

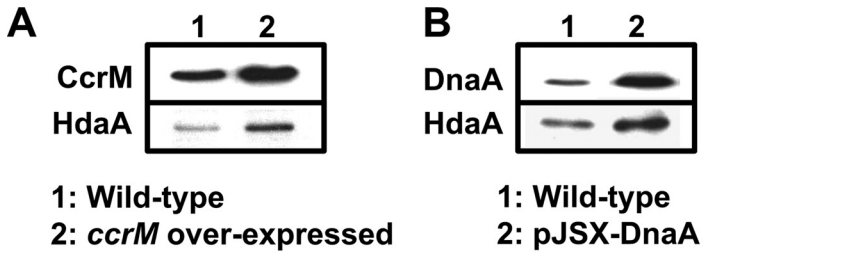
foci reappeared and colocalized, providing additional evidence that HdaA is a component of the *Caulobacter* replisome.

The levels of HdaA are adjusted to the needs of the cell to inhibit the initiation of DNA replication. We compared the levels of HdaA in the wild-type strain and in two mutant strains that accumulate excess DnaA. In the first mutant strain tested, the gene encoding the CcrM DNA methyltransferase is expressed constitutively throughout the cell cycle (data not shown). Because the *dnaA* gene is preferentially transcribed from a fully methylated promoter, high levels of DnaA are maintained throughout the cell cycle in such mutant strains (4). As for *hdaA* transcription, it is not directly controlled by CcrM, since the *hdaA* promoter region does not contain GANTC sequences that become methylated by CcrM in predivisional cells. Immunoblots of wild-type and mutant cell extracts, using antibodies raised against HdaA, showed a greater accumulation of HdaA in the mutant strains than in the isogenic wild type (Fig. 4A). To test whether it is the increase in DnaA levels that promotes the accumulation of HdaA, we constructed a second strain in which a high-copy-number plasmid expressed the *dnaA* gene under the control of the inducible *xylX* promoter (pJSX-*dnaA*). A strain carrying this plasmid and grown in PYEX medium for 4 h accumulated significantly more DnaA and HdaA than the isogenic wild-type strain (Fig. 4B). Thus, in *Caulobacter*, the levels of HdaA are adjusted to the levels of DnaA.

DnaA directly activates the transcription of *hdaA*. DnaA is a dual-function protein that acts both as an initiator of DNA replication and as a transcription factor by binding to DnaA boxes found in the origin of replication and in promoters of specific genes, respectively (14, 17). In *Caulobacter*, DnaA directly regulates the transcription of at least 40 genes, including the gene encoding the GcrA master regulator (5, 17).

Because we found that the cellular levels of HdaA increased upon an increase in the levels of DnaA (Fig. 4A and B), we tested the possibility that DnaA could directly activate the transcription of *hdaA*. An examination of the 350-base sequence upstream of the translational start site of *hdaA* revealed a surprisingly high number of DnaA boxes (Fig. 4C); six motifs matched a minimum of seven of the nine nucleotides of the consensus DnaA box from *Caulobacter* (17) and a minimum of five of the six nondegenerate nucleotides of the consensus. These motifs look as similar to the consensus DnaA box as the five putative DnaA boxes found in the *Cori* (Fig. 4D) (30).

To test if the transcription of *hdaA* is activated by DnaA, we constructed a transcriptional fusion of the *hdaA* promoter and the *lacZ* gene on a low-copy-number plasmid. The resulting plasmid p*lacZ*290-*hdaA*P(WT) was first introduced into strain GM2471, in which the sole copy of *dnaA* is under the control of the chromosomal *xylX* promoter. We measured β-galactosidase activity, in the presence or the absence of the xylose inducer, as an indication of *hdaA* promoter activity (Fig. 5A). Four hours after this strain was shifted from PYEX to PYEG medium to deplete DnaA, β-galactosidase activity decreased by ~25%. This change of activity is significant, since β-galactosidase is a very stable enzyme that takes a long time to disappear in cells when its synthesis is arrested. To confirm this result, we also introduced plasmid p*lacZ*290-*hdaA*P(WT) into an NA1000 strain containing the pJSXDnaA plasmid or an empty control vector. The activity of the *hdaA* promoter was 2.35-fold higher in the strain containing the pJSXDnaA plasmid than in the strain containing the control vector, when cells were grown in PYEX medium to overproduce DnaA from pJSXDnaA (Fig. 5C). We conclude that DnaA significantly activates transcription from the *hdaA* promoter.



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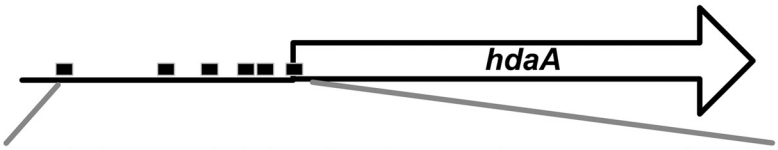

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D

Name	Sequence	Consensus Matches: Overall	Consensus Matches: Non-degenerate
Consensus	TGTGGATAA CCG	9	6
Cori Box 1	T**t**TGGACAA	8	5
Cori Box 2*	TGTGGATCA	9	6
Cori Box 3*	**a**GTGGATA**t**	7	5
Cori Box 4*	TGTGGA**gtc**	6	6
Cori Box 5*	TGTG**c**ATCA	8	5
*hdaA*P Box 1	TG**a**GGATC**t**	7	5
*hdaA*P Box 2*	TG**c**GGA**g**GA	7	5
*hdaA*P Box 3*	TGTGGATGG	9	6
Box3 mutant*	**cac**GGATGG	6	3
*hdaA*P Box 4*	TG**g**GGATAG	8	5
*hdaA*P Box 5*	TG**g**GGATGA	8	5
*hdaA*P Box 6	TG**a**GGA*T**t**G*	7	5

FIG. 4. DnaA promotes HdaA accumulation and the *hdaA* promoter region contains multiple putative DnaA boxes. (A) Strain JC362, with an extra chromosomal copy of *ccrM* under the control of the constitutive *lacZ* promoter (*ccrM* overexpressed) and the wild-type strain, with a single cell cycle-regulated copy of *ccrM*, were grown in PYE medium and used to perform immunoblotting using antibodies raised against CcrM and HdaA. (B) A wild-type strain, with or without pJSX-DnaA, was grown in PYE medium plus 0.2% glucose and 0.3% xylose (PYEGX) for 4 h and used to perform immunoblotting using antibodies raised against DnaA and HdaA. (C) The nucleotide sequence of the *hdaA* promoter region (345 nucleotides) is shown. The nucleotides corresponding to the six putative DnaA boxes are in bold. Italics indicate the beginning of the *hdaA* coding sequence. The black boxes in the schematic above indicate the DnaA boxes. (D) Consensus sequence for DnaA boxes in *Caulobacter* (17) and the putative DnaA boxes within the *Cori* (30) and the *hdaA* promoter. Reverse-complement sequences are presented for boxes marked with asterisks. Nucleotide positions differing from the consensus are denoted in lowercase bold lettering. The six nondegenerate nucleotides of the consensus DnaA box are highlighted in gray. The number of nucleotide matches to the overall and nondegenerate consensus DnaA box are shown for each sequence. The sequence of the mutated *hdaA*P(box3) is displayed, with lowercase bold letters indicating nucleotide changes compared to the wild type.

If DnaA activates the transcription of *hdaA* by directly binding to the *hdaA* promoter, then the elimination of one or more DnaA boxes in the *hdaA* promoter could affect the efficiency of transcription from that promoter. To test this hypothesis, we analyzed the transcription from a mutant *hdaA* promoter in which the DnaA box with a 9/9 base conservation (box 3 in Fig. 4C and D) was disrupted by targeted mutagenesis. This mutant promoter was fused to *lacZ*, yielding plasmid p*lacZ*290-*hdaA*P-(box3). We introduced this plasmid into wild-type cells and measured β-galactosidase activity. The activity of the mutant promoter was about twofold lower than the activity of the wild-type promoter (Fig. 5B), suggesting that DnaA directly activates the transcription of *hdaA*. We also introduced the p*lacZ*290-*hdaA*P(box3) plasmid into the GM2471 strain and

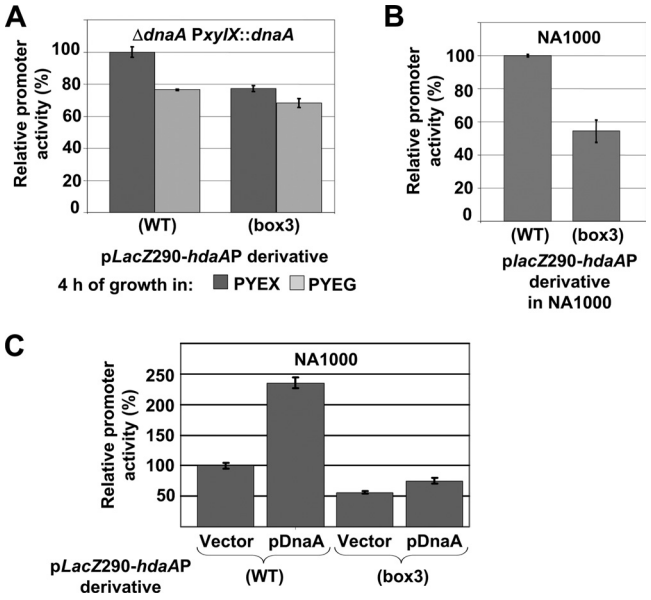

FIG. 5. DnaA directly activates the transcription of *hdaA*. (A) The graph shows the relative β-galactosidase activities from p*lacZ*290-*hdaA*P(WT) and p*lacZ*290-*hdaA*P(box3) in a GM2471 (Δ*dnaA* P*xylX*::*dnaA*) strain upon the depletion of DnaA 4 h after a shift from PYE medium plus xylose (PYEX) to PYE medium plus glucose (PYEG). (B) The graph shows the relative β-galactosidase activities from p*lacZ*290-*hdaA*P(WT) and p*lacZ*290-*hdaA*P(box3) in an NA1000 (WT) strain grown in PYE medium. Activities in Miller units were normalized so that the activity of *hdaA*P(WT) equals 100% in PYE (NA1000) or PYEX (GM2471) medium to facilitate comparison. (C) The graph shows the relative β-galactosidase activities from p*lacZ*290-*hdaA*P(WT) and p*lacZ*290-*hdaA*P(box3) in an NA1000 strain containing the DnaA-overexpressing plasmid pJSX-DnaA or the empty control vector pJS14 grown in PYEX medium for 4 h. Activities in Miller units were normalized so that the activity of *hdaA*P(WT) equals 100% in NA1000 containing pJS14 to facilitate comparison. Error bars indicate the standard deviations.

into the NA1000 strain containing the pJSXDnaA plasmid to test if the *hdaA*P(box3) mutant promoter is still sensitive to changing levels of DnaA in the cells. The β-galactosidase activity of the mutant promoter decreased by ~12% when the GM2471 strain was shifted from PYEX to PYEG medium for 4 h to deplete DnaA, compared to the 25% decrease observed for the wild-type *hdaA* promoter (Fig. 5A). The β-galactosidase activity of the mutant promoter was 1.34-fold higher in the NA1000 strain containing pJSXDnaA than in the NA1000 strain containing the pJS14 empty vector when the cells were grown in PYEX medium, compared to 2.35-fold for the wild-type *hdaA* promoter. The *hdaA*P(box3) mutant promoter is therefore less sensitive to changes in DnaA levels in the cell than the wild-type promoter (Fig. 5C).

Cumulatively, these results suggest that DnaA directly activates the transcription of *hdaA* by binding at minimum to DnaA box 3 within the *hdaA* promoter.

DISCUSSION

We have determined that the *Caulobacter* HdaA protein functions as a negative regulator of replication initiation, in concert with CtrA, to ensure that the initiation of DNA replication takes place only once per cell cycle. The HdaA protein is an apparent component of the *Caulobacter* replisome and it inhibits the initiation of DNA replication. The accumulation of HdaA is regulated by the activation of *hdaA* transcription by DnaA, providing an interesting feedback loop.

Model for the temporal regulation of DNA replication initiation in *Caulobacter*. As shown in Fig. 6A, we propose the following multistep control system that limits the initiation of DNA replication to only once per cell cycle: (i) the initiation process is inhibited in swarmer cells by CtrA bound to five sites within the origin region (37); (ii) CtrA is eliminated by targeted proteolysis (7) and DnaA accumulates during the swarmer-to-stalked cell transition (4), allowing the initiation of DNA replication; (iii) a second round of replication initiation is inhibited by an HdaA/DnaN complex bound to the chromosome once DNA replication has initiated in stalked cells (Fig. 2) and by the directed proteolysis of DnaA (15); and (iv) CtrA accumulates again to strengthen the inhibition of replication initiation in predivisional cells.

The regulated control of DnaA accumulation and activity is integrated in a wider regulatory network that controls the *Caulobacter* cell cycle (Fig. 6B) (4). The expression of about 550 genes is temporally regulated during the *Caulobacter* cell cycle (27). The two master transcriptional regulators CtrA and GcrA together control the expression of ~145 of these genes. The CtrA response regulator directly controls the transcription of 95 genes (26), while GcrA regulates the transcription of about 50 genes (16). CtrA and GcrA oscillate out of phase temporally and spatially. The DnaA protein is not only the initiator of DNA replication, but it is also a transcription factor that regulates the transcription of at least 40 genes in *Caulobacter* (17). One of these is the *gcrA* gene, which is directly activated by DnaA (5). The GcrA master regulator activates one of the two *ctrA* promoters, *ctrA* P1, when it is in the hemimethylated state, after the passage of the replication fork through the *ctrA* locus in late stalked cells (16, 38). CtrA activates its own transcription by binding to the *ctrA* P2 promoter (8), so that CtrA efficiently accumulates in predivisional cells to inhibit the initiation of DNA replication (37).

Feedback control of the activity of DnaA by HdaA. Evidence that DnaA activates the transcription of *hdaA* in vivo includes the observations that (i) the cellular levels of the HdaA protein increased in mutant strains that accumulate excess DnaA (Fig. 4A and B); (ii) the *hdaA* promoter contains multiple DnaA boxes, similar to those present in the *Cori* sequence (Fig. 4C and D); and (iii) *lacZ* transcribed from the *hdaA* promoter was activated by DnaA in vivo, and a mutation in a conserved 9/9 DnaA box in the *hdaA* promoter decreased activation by DnaA (Fig. 5). These results suggest that the synthesis of HdaA is proportional to the levels of active DnaA in the cell, providing an interesting feedback mechanism for the control of DnaA activity. We examined the level of HdaA protein as a function of the cell cycle and found that it did not change significantly (data not shown), suggesting that HdaA is a rather stable protein in *Caulobacter*. However, as is the case in *E. coli* (45), HdaA in *Caulobacter* is likely to only inactivate DnaA once it joins the replisome as it is assembled onto the DNA. Thus, we propose that the activity, and not the protein availability of HdaA, is cell cycle regulated so that it functions to inactivate DnaA only after the first round of DNA replication has initiated.

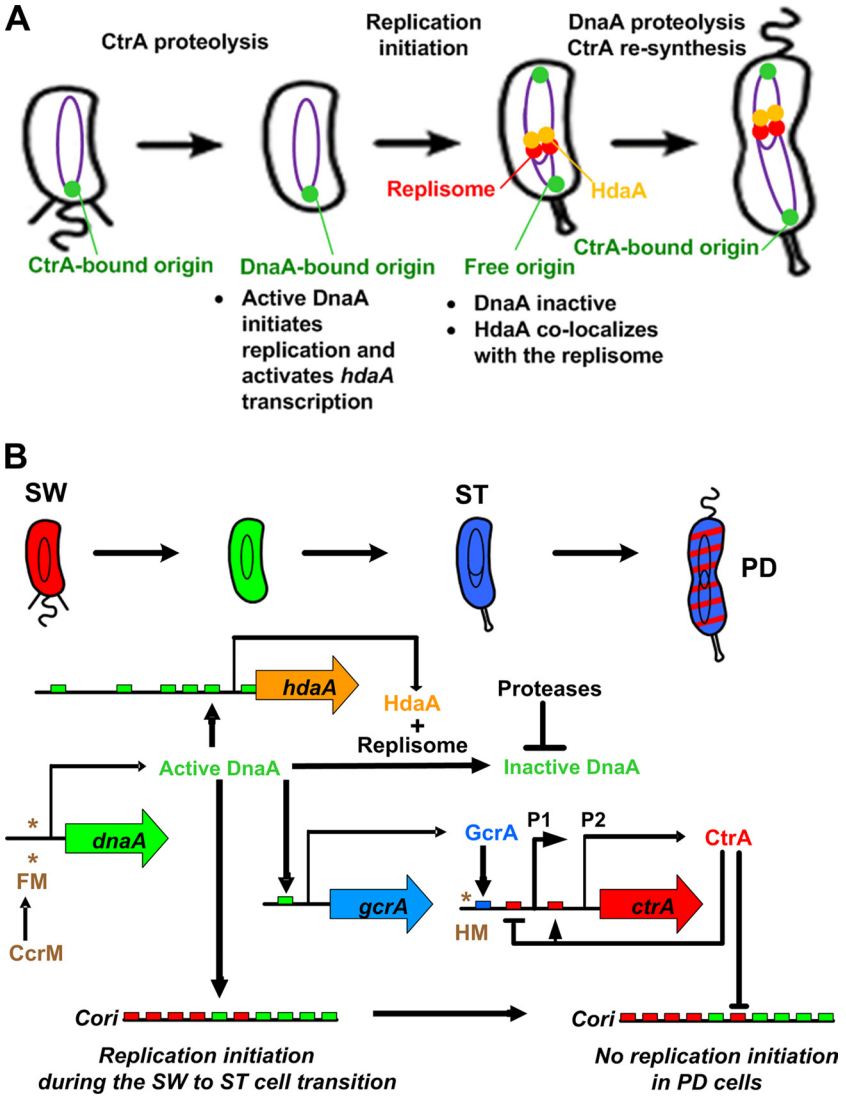

FIG. 6. Model for the temporal control of DNA replication initiation in *Caulobacter*. (A) A schematic of the beginning of the *Caulobacter* cell cycle is shown. Purple theta structures inside the cells indicate replicating DNA. The single origin of replication (green focus) in swarmer cells is bound to CtrA, which represses the initiation of DNA replication. During the swarmer-to-stalked cell transition, CtrA is rapidly degraded by the ClpXP protease, and active DnaA binds to the origin to initiate DNA replication. The replisome (red foci), associated with HdaA (orange foci), replicates the chromosome and inactivates DnaA once DNA replication is ongoing. CtrA reaccumulates in predivisional cells and binds to the origin to prevent more replication initiation events. (B) A schematic of the beginning of the *Caulobacter* cell cycle is shown. Red indicates CtrA accumulation, green indicates DnaA accumulation, and blue indicates GcrA accumulation. SW, swarmer cell; ST, stalked cell; PD, predivisional cell. DnaA is synthesized in swarmer cells, when the *dnaA* promoter is in the fully methylated state (FM, two asterisks). New molecules of DnaA initiate DNA replication and activate the transcription of *gcrA* and *hdaA* by directly binding to DnaA boxes (green boxes). Once the replisome is assembled, the replisome-HdaA complex inhibits the initiation of DNA replication, probably by a mechanism similar to the RIDA mechanism in *E. coli*, and the DnaA protein is degraded by a protease (15) to prevent more initiation events in stalked cells. Soon after the initiation of DNA replication, the *dnaA* and the *ctrA* genes are duplicated by the passage of the replication fork and therefore hemimethylated (HM, asterisk). Transcription from the hemimethylated *dnaA* gene is shut down, while transcription from the hemimethylated *ctrA* gene is turned on by the binding of GcrA to the *ctrA* P1 promoter (blue box). Accumulation of CtrA in early predivisional cells then contributes to the inactivation of replication initiation by directly binding to the CtrA sites in the *Cori* (red boxes), yielding a robust replication control system.

We believe that the main function of the feedback mechanism is not to control the accumulation of HdaA as a function of the cell cycle but to promote HdaA accumulation in cases when DnaA becomes too abundant in the cell and may cause overinitiation defects. This can happen, for example, when the transcription of *dnaA* or *ccrM* is mis-regulated (Fig. 4A and B). HdaA contributes to preventing the start of a second round of DNA replication in stalked cells, even when *dnaA* is transcribed constitutively throughout the cell cycle (Fig. 1A and B). We found previously that DnaA accumulates constitutively throughout the cell cycle when the *dnaA* locus is maintained in the fully methylated state by moving the position of the *dnaA* gene on the chromosome from its native location next to the *Cori* to a location next to the terminus of replication (4). Interestingly, this strain still initiates the replication of its chromosome only once per cell cycle, likely due to the efficient

inactivation of DnaA by the HdaA/DnaN complex immediately following the initiation of DNA replication. The feedback regulation of *hdaA* transcription by DnaA likely makes the cell cycle control system more robust to accidental variations or mutations.

Control of the initiation of DNA replication in different bacteria. The RIDA mechanism was first identified in *E. coli*, where it prevents the overinitiation of replication (22). However, there are significant differences between the regulatory networks that control the initiation of chromosome replication in *E. coli* and in *Caulobacter*. One striking difference is that the *Caulobacter* CtrA inhibitor of the initiation of DNA replication is not conserved in *E. coli*. Instead, *E. coli* has a unique SeqA protein that binds to the hemimethylated origin of replication to prevent further the overinitiation of DNA replication (42). A second difference is that the DnaA protein is unstable in *Caulobacter* cells (15), while it is very stable in *E. coli* cells (47). A third difference is that the promoter of the *hda* gene in *E. coli* does not contain any obvious DnaA boxes, whereas in *Caulobacter*, DnaA activates the transcription of *hdaA* (Fig. 5). A fourth interesting difference is that the levels of DnaA remain unchanged when *hdaA* is depleted in *Caulobacter* cells (data not shown), while the levels of DnaA decrease in Hda-deficient *E. coli* cells (39), due to the autorepression of *dnaA* transcription by DnaA-ATP (43). The last known difference is that the overexpression of *hdaA* is not deleterious in *Caulobacter* cells (data not shown) unlike the overexpression of *hda* in *E. coli* cells, which induces the SOS response and cell division defects (1).

The use of an inhibitor of the initiation of DNA replication that interacts with the β-clamp of DNA polymerase is not restricted to gram-negative bacteria. Indeed, the conserved YabA protein from *Bacillus subtilis* also acts as a negative inhibitor of replication initiation and forms a ternary complex with DnaA and DnaN in vivo, although YabA shares no homology with Hda (36). This finding suggests that there is an evolutionary conserved need for a temporally precise mechanism that controls the initiation of bacterial DNA replication.

ACKNOWLEDGMENTS

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