

# DnaA couples DNA replication and the expression of two cell cycle master regulators

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Cell cycle progression in *Caulobacter* is driven by the master transcriptional regulators CtrA and GcrA. The cellular levels of CtrA and GcrA are temporally and spatially out-of-phase during the cell cycle, with CtrA repressing *gcrA* transcription and GcrA activating *ctrA* transcription. Here, we show that DnaA, a protein required for the initiation of DNA replication, also functions as a transcriptional activator of *gcrA*, which in turn activates multiple genes, notably those involved in chromosome replication and segregation. The cellular concentration of DnaA is cell cycle-controlled, peaking at the time of replication initiation and *gcrA* induction. Regulated proteolysis of GcrA contributes to the cell cycle variations in GcrA abundance. We propose that DnaA couples DNA replication initiation with the expression of the two oscillating regulators GcrA and CtrA and that the DnaA/GcrA/CtrA regulatory cascade drives the forward progression of the *Caulobacter* cell cycle.

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## Introduction

*Caulobacter crescentus* divides asymmetrically in each cell cycle, giving rise to two different progeny cells with distinct morphological features and cell fates: a stalked cell that can immediately initiate DNA replication and a swarmer cell that cannot initiate DNA replication until it differentiates into a stalked cell (Figure 1A). *Caulobacter*, therefore, must not only coordinate DNA replication with cell division, but also with morphogenetic events. Multiple cell cycle events, such as the G1/S transition, flagellar and pili biogenesis, DNA methylation, chromosome segregation and cytokinesis are mediated by the GcrA and CtrA master regulators, which oscillate out of phase during the cell cycle (Holtzendorff *et al.*, 2004). These events are coordinated, in part, by an interdependent feedback loop between GcrA and CtrA and by the differential methylation state of the chromosome during

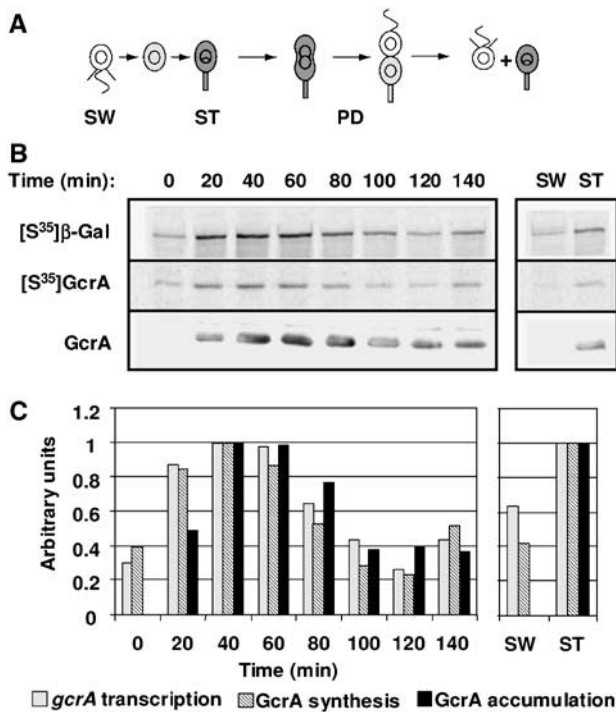
the cell cycle (Reisenauer and Shapiro, 2002; Holtzendorff *et al.*, 2004). Here, we show that DnaA is a critical coordination factor, functioning to allow replication initiation while activating the expression of *gcrA*, with the consequent expression of *ctrA* and genes involved in chromosome replication and segregation.

CtrA is an essential transcription factor and a selective chromosome replication inhibitor in *Caulobacter* (Quon *et al.*, 1996, 1998). CtrA accumulates in swarmer cells, where it directly binds to five sites within the *Caulobacter* origin of DNA replication (*Cori*) and thereby blocks replication initiation. CtrA is cleared from the cell during the swarmer-to-stalked cell differentiation, which is functionally analogous to the eukaryotic G1-to-S transition of the cell cycle. Concurrently, as CtrA is proteolyzed, the *dnaA* and the *himA* genes, which encode the DNA replication initiator protein DnaA and the  $\alpha$ -subunit of the 'histone-like' integration host factor (IHF), are transcribed (Zweiger and Shapiro, 1994; Laub *et al.*, 2000). The DnaA protein, conserved in most eubacteria, directly binds to DNA at DnaA boxes (Fuller *et al.*, 1984). Five DnaA boxes and an exceptional AT-rich region are found in the *Caulobacter Cori* (Marczynski and Shapiro, 1992). DnaA binding to *Cori* has been proposed to unwind the AT-rich region and provide an entry site for protein components of the replisome that then initiates chromosome replication (Bramhill and Kornberg, 1988; Gille and Messer, 1991). An IHF-binding site overlaps one of the CtrA-binding sites in *Cori*. IHF binding to the *Cori* may help displace CtrA from the *Cori* sequence and bend DNA, promoting the initiation of replication (Siam *et al.*, 2003).

Many functions needed for normal progression of the *Caulobacter* cell cycle are regulated by discrete transcription patterns: genes are activated at the time when their function is needed. Whole-genome analysis of messenger RNA (mRNA) accumulation during the *Caulobacter* cell cycle has revealed that the expression of about 19% of the *Caulobacter* genes (~550 out of 3760) are regulated in a cell-cycle-dependent manner (Laub *et al.*, 2000). More than 15% of these cell-cycle-regulated genes are directly regulated by CtrA, which binds to a conserved motif in the promoters of 55 operons (Quon *et al.*, 1996; Laub *et al.*, 2002). CtrA's targets include genetic modules for cell division, DNA methylation, flagellum and pili biosynthesis, chemotaxis and metabolism (Laub *et al.*, 2002). GcrA is another essential regulatory protein controlling 49 cell-cycle-regulated genes, of which eight are also regulated by CtrA (Holtzendorff *et al.*, 2004). GcrA notably controls polar morphogenesis and multiple components of the chromosome replication and segregation machinery. The cellular levels of GcrA are cell-cycle-dependent, both temporally and spatially out-of-phase with CtrA ((Holtzendorff *et al.*, 2004) and Figure 5). Functional complementarities of the conserved CtrA and GcrA proteins allow time- and cell type-specific transcriptional regulation of key cell cycle events, thus providing a motive force for cell cycle

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**Figure 1** Cell cycle expression of *gcrA*. (A) Schematic of the *Caulobacter* cell cycle. Gray indicates accumulation of GcrA. Theta structures indicate replicating DNA. SW, swarmer cell; ST, stalked cell; PD, predivisional cell. (B) From the same synchronized LS4220 (*gcrA*<sup>+</sup> *gcrAP*(-507...+92)-*lacZ*) strain culture, aliquots were taken at the times indicated and were either pulse-labelled with [<sup>35</sup>S]methionine to follow β-galactosidase synthesis or GcrA synthesis using either anti-β-galactosidase or anti-GcrA immunoprecipitation, or immunoblotted with anti-GcrA. Autoradiograms from the immunoprecipitation of labelled β-galactosidase ([<sup>35</sup>S]β-Gal) and GcrA ([<sup>35</sup>S]GcrA), and immunoblots with anti-GcrA (GcrA) are shown. Also shown are autoradiograms and immunoblots from cell extracts from newly divided stalked and swarmer cells harvested at the end of the cell cycle. (C) Histograms of the relative rates of *gcrA* transcription (β-galactosidase synthesis), GcrA synthesis and GcrA accumulation as measured by phosphorimaging or densitometric scanning of immunoblots. Values were normalized to the maximum value of each experiment (at 40 min from 0 to 140 min and in stalked progenies).

progression. The expression and the activity of the CtrA and GcrA master regulators must therefore be tightly regulated.

The cellular accumulation of CtrA is controlled by at least two mechanisms: transcriptional regulation and targeted proteolysis. The *ctrA* gene is transcribed from two promoters: P1 and P2. The *ctrA* P1 promoter can be methylated by the CcrM DNA methyltransferase at a GANTC site and a fully methylated *ctrA* P1 promoter cannot be transcribed (Reisenauer and Shapiro, 2002). At the initiation of replication, the *ctrA* P1 promoter is in the fully methylated state. The *ctrA* P1 promoter can only be transcribed after the passage of the replication fork that generates two hemi-methylated copies of the gene. Thus, *ctrA* transcription is coordinated with the progression of chromosomal replication. Once in the hemi-methylated state, transcription from the *ctrA* P1 promoter is activated by GcrA (Holtzendorff *et al*, 2004). As CtrA accumulates and is activated by phosphorylation, it represses the *ctrA* P1 promoter and activates the strong *ctrA* P2 promoter, leading to a burst of CtrA synthesis in late stalked cells and early predivisional cells (Domian *et al*, 1999),

simultaneously repressing the *gcrA* promoter (Holtzendorff *et al*, 2004). Once the division plane establishes two cellular compartments prior to cell division, CtrA is rapidly proteolyzed by the ClpXP protease (Jenal and Fuchs, 1998) in the stalked compartment of the late predivisional cell and then at the swarmer-to-stalked cell transition, when the cell is preparing to initiate DNA replication (Domian *et al*, 1997; Judd *et al*, 2003).

Even though the cell cycle regulation of *ctrA* expression has been extensively studied, the mechanisms governing the oscillation of CtrA and GcrA concentrations during the cell cycle are not well understood, notably because little is known about the regulation of *gcrA* expression or GcrA stability. Previous microarray analyses showed that *gcrA* mRNA levels are maximal in stalked cells and decrease in predivisional cells (Laub *et al*, 2000), suggesting that *gcrA* transcription may be an important level of regulation of *gcrA* cell cycle expression. It was also shown that CtrA represses transcription of *gcrA* by directly binding to a CtrA-binding site within the *gcrA* promoter (Holtzendorff *et al*, 2004). In this study, we demonstrate that as DnaA accumulates during the swarmer-to-stalked cell transition, it directly activates *gcrA* transcription as the CtrA repressor is being cleared from the cell. Temporally regulated proteolysis further modulates GcrA cellular concentration during the cell cycle. The ‘dual-use’ DnaA protein functions to both enable replisome formation at the origin and to control the transcription of *gcrA*. The accumulation of GcrA, in turn, activates the transcription of genes involved in DNA replication and chromosome segregation, and directly activates *ctrA* transcription. Thus, DnaA serves as a central coordinator of multiple cell cycle events.

## Results

### Cell cycle control of *gcrA* expression

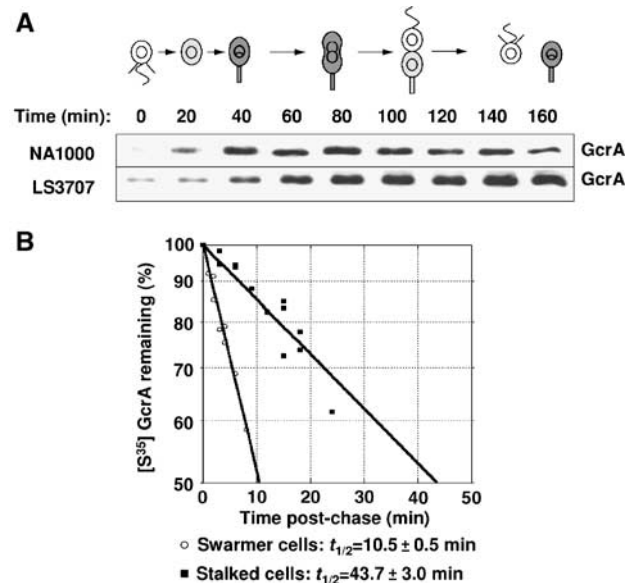
To determine how GcrA protein levels are temporally regulated, each step of GcrA synthesis was analyzed during the *Caulobacter* cell cycle. To measure the transcription of *gcrA*, a *gcrAP-lacZ* transcriptional fusion (Holtzendorff *et al*, 2004) was integrated into the *Caulobacter* chromosome, at a site close to the natural *gcrA* locus (LS4220). Transcription and translation from *gcrAP* results in production of β-galactosidase. We measured newly synthesized β-galactosidase at different time points during the cell cycle. Synchronous cells were pulse-labelled with [<sup>35</sup>S]methionine and β-galactosidase was immunoprecipitated to assay the activity of the *gcrA* promoter (Figure 1). Transcription increased more than two-fold during the swarmer-to-stalked cell transition. The maximum level of transcription was reached in stalked cells and then decreased in the predivisional cells. The cell-cycle-dependent transcription of *gcrA* is comparable to the changes in the steady-state levels of *gcrA* mRNA during the cell cycle (Laub *et al*, 2000), suggesting that the increase in *gcrA* mRNA levels during the swarmer-to-stalked cell transition is the result of increased transcription, rather than stabilization of *gcrA* mRNA. The rate of *gcrA* transcription in separated populations of swarmer and stalked cells was also measured immediately after cell division (at ~150 min) and was found to be significantly higher in stalked than swarmer progeny cells (Figure 1). Overall, these results demonstrate that the transcriptional regulation of *gcrA* contributes to the control of the cell-cycle-dependent accumulation of GcrA.

To measure the temporal synthesis of GcrA, we immunoprecipitated GcrA from the pulse-labelled cell samples from the same synchronous culture used to measure the temporal transcription of *gcrA*. We could thereby determine how much new GcrA protein was synthesized from the natural *gcrA* gene during the pulse (Figure 1). Synthesis of the GcrA protein increased during the swarmer-to-stalked cell transition, reached a maximum in stalked cells, decreased in predivisional cells and was much higher in the stalked than the swarmer progenies after cell division. Since the cell cycle variations of GcrA synthesis are comparable to the cell cycle variations of *gcrA* transcription, we conclude that *gcrA* mRNA translation and stability are not significantly cell cycle regulated, even though the 5' untranslated region of the *gcrA* mRNA is surprisingly long (92 nucleotides long (Holtzendorff *et al*, 2004)).

### The proteolysis of GcrA is cell cycle regulated

The accumulation of the GcrA protein was compared with the efficiency of its synthesis during the cell cycle by immunoblots using anti-GcrA antibodies (Figure 1). Cell-cycle-regulated synthesis of GcrA can approximately account for the increase of GcrA steady-state levels in the stalked cells. Nevertheless, the rapid decrease in GcrA protein levels in predivisional cells, approximately concurrent with the decrease in GcrA synthesis, indicates that GcrA is an unstable protein. To measure GcrA half-life in a mixed cell population, we performed pulse-chase experiments in the wild-type strain grown in exponential phase. We found that the half-life of GcrA is about 24 min (data not shown), which is substantially shorter than the 150 min cell cycle of wild-type *Caulobacter* cells.

The absence of detectable GcrA proteins in swarmer cells (at time = 0 min and after isolation of the swarmer progeny at time = 140 min), despite a basal level of GcrA synthesis (Figure 1), suggests that GcrA is particularly unstable in swarmer cells. If GcrA proteolysis changes during the cell cycle, accumulated levels of GcrA should vary even when GcrA is constitutively synthesized throughout the cell cycle. To test this hypothesis, we used a strain (LS3707) in which the sole copy of *gcrA* is under the control of the chromosomal xylose inducible promoter *PxyIX*, yielding constitutive expression of *gcrA* in the presence of xylose (Holtzendorff *et al*, 2004). Swarmer cells from LS3707 grown in minimal media supplemented with xylose were isolated, and protein samples were collected at different time points throughout the cell cycle in the same medium for immunoblot analysis using anti-GcrA antibodies (Figure 2A). We observed that GcrA accumulation changed during the cell cycle, even under conditions when *gcrA* is transcribed constitutively throughout the cell cycle, with predominant accumulation in stalked and predivisional cells. This result suggests that GcrA is significantly more unstable in swarmer cells than other *Caulobacter* cell types. To ascertain that the weaker accumulation of GcrA in swarmer cells than in stalked cells was due to more rapid proteolysis of GcrA, we determined the half-life of GcrA in independent isolated populations of LS3707 swarmer and stalked cells (Figure 2B). We found that the half-life of GcrA is ~10.5 min in swarmer cells, as compared to ~43.7 min in stalked cells. Since the rate of synthesis of GcrA in wild-type swarmer cells is very low, we were not able to assess accurately the half-life of GcrA in swarmer



**Figure 2** Proteolysis of GcrA is cell cycle regulated. (A) GcrA accumulation when *gcrA* is transcribed constitutively. Immunoblots of cell extracts from a synchronized NA1000 and LS3707 (*ΔgcrA PxyIX::gcrA*) culture using GcrA antibodies at the indicated times of the cell cycle. In the schematic of the *Caulobacter* cell cycle, gray indicates accumulation of GcrA in the NA1000 cell. (B) Stability of GcrA synthesized in swarmer and stalked LS3707 cells. A synchronized population of LS3707 swarmer cells was allowed to proceed through the cell cycle. At 5 (swarmer cells) and 30 min (swarmer-to-stalked cell transition), aliquots of the culture were pulse-labelled with [<sup>35</sup>S]methionine and chased for increasing amounts of time. Aliquots were taken at the times indicated and the remaining radiolabelled GcrA was determined by immunoprecipitation using GcrA antibodies, followed by SDS-PAGE and phosphorimaging. The  $t_{1/2}$  corresponds to the calculated half-life of GcrA in each cell type, along with the calculated standard deviation from three independent LS3707 populations.

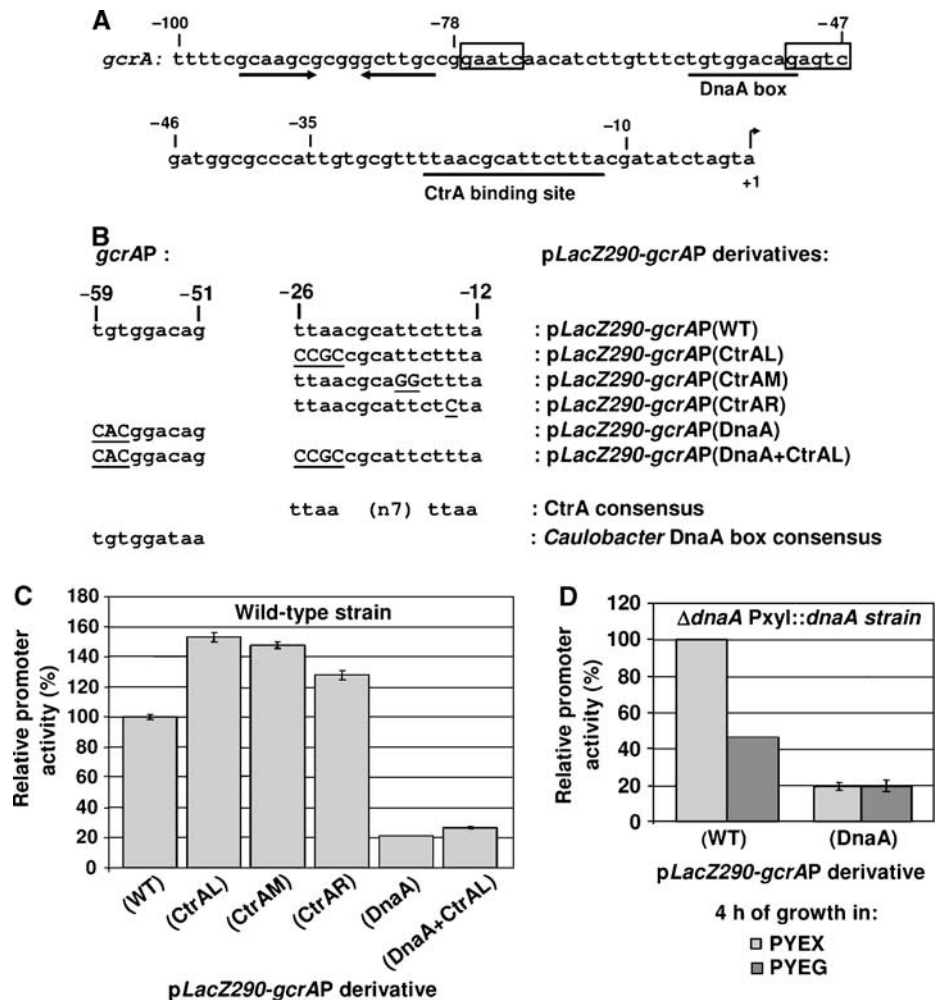
cells from a wild-type strain. Nevertheless, we assessed the half-life of GcrA in stalked cells from a wild-type strain and found it was also about 42 min (data not shown), which confirms that proteolysis of GcrA in the LS3707 strain is comparable to proteolysis of GcrA in wild-type cells. Overall, these results show that GcrA proteolysis is cell cycle regulated, with GcrA being degraded approximately four-fold faster in swarmer than stalked cells.

### Characterization of the *gcrA* promoter

A DNA fragment from -507 to +92 relative to the *gcrA* transcript initiation site (Holtzendorff *et al*, 2004) was used to construct the transcriptional fusion to *lacZ* that we used to study the temporal transcription of *gcrA* shown in Figure 1. We show (in Supplementary data) that all significant regulatory sequences should be included in the -78 to +1 region of the *gcrA* promoter. This sequence notably contains the previously characterized CtrA-binding site (Holtzendorff *et al*, 2004), in addition to a putative DnaA box and two putative DNA methylation sites (Figure 3A).

### CtrA is a negative regulator of *gcrA* transcription

A previous study from our laboratory, assessing the activity of the *gcrA* promoter in a *ctrA401ts* mutant shifted to restrictive temperature, showed that CtrA represses *gcrA* transcription (Holtzendorff *et al*, 2004). Even though CtrA directly



**Figure 3** Opposite effects of CtrA and DnaA on *gcrA* transcription. (A) The nucleotide sequence of the *gcrA* promoter is shown. The transcriptional +1 start site (Holtzendorff *et al*, 2004), the CtrA-binding site (underlined) (Holtzendorff *et al*, 2004), the two putative DNA methylation sites (boxes), the putative DnaA box (underlined) and a putative hairpin structure (two arrows) are indicated. (B) The sequence of the CtrA-binding site and the DnaA box within the *gcrA* promoter (*gcrAP*) are shown and compared to the consensus CtrA-binding site and DnaA box in *Caulobacter*. Mutations introduced in the *gcrAP* are underlined and in capital letters. The names of the *pLacZ290* derivatives carrying the unmodified and the mutated *gcrAP* fused to *lacZ* are also shown. (C) The graph shows the relative  $\beta$ -galactosidase activities from the six plasmids in (B) in an unsynchronized NA1000 strain. (D) The graph shows the relative  $\beta$ -galactosidase activities from *pLacZ290-gcrAP*(WT) and *pLacZ290-gcrAP*(DnaA) in an unsynchronized GM2471 ( $\Delta dnaA::\Omega$  PxyI::*dnaA*) strain, upon depletion of DnaA 4 h after a shift from PYE + xylose (PYEX) to PYE + glucose (PYEG). Activities in Miller units were normalized so that the activity of the *gcrAP*(WT) equals 100% in PYE (NA1000) or PYEX (GM2471), to facilitate comparison. Errors bars indicate the standard deviations (when they were more than 1%).

binds to the *gcrA* promoter, the activation of *gcrA* transcription at restrictive temperature in the *ctrA401ts* strain could partially be an indirect effect of the cell cycle arrest caused by the CtrA depletion. To quantitatively assess the effect of CtrA on *gcrA* transcription in a wild-type strain, we analyzed the activity of three mutant *gcrA* promoters, carrying mutations in the CtrA-binding site in the *gcrA* promoter fused to *lacZ* carried on the low copy number plasmid *pLacZ290* (Figure 3B). The consensus DNA sequence for CtrA binding in *Caulobacter* is TTAA-N7-TTAA (Marczynski *et al*, 1995) and both the CtrA-binding half-sites and the N7 spacing are critical for CtrA-mediated transcriptional regulation (Ouimet and Marczynski, 2000). The first mutant *gcrA* promoter we constructed (*gcrAP*(CtrAL)) carries a 4 base-pair mutation in the left portion of the CtrA-binding site, the second (*gcrAP*(CtrAM)) carries a double base-pair mutation in the middle part of the CtrA-binding site, and the third (*gcrAP*(CtrAR)) carries a single base-pair mutation in the

right portion of the CtrA-binding site. All mutated promoters were cloned in *pLacZ290* and introduced into a wild-type *Caulobacter* strain to compare their activities with the wild-type promoter (*gcrAP*(WT)) by  $\beta$ -galactosidase assays. In all three cases, the activities of the mutated promoters were higher than that of the wild-type *gcrA* promoter, with a 25–50% difference in the activity (Figure 3C). To check whether the mutations we created in the *gcrA* promoter render the *gcrA* promoter independent of CtrA protein levels in the cell, we also introduced these constructs in a *ctrA401ts* strain. After a shift from permissive temperature to restrictive temperature, the activity of the *gcrAP*(CtrAL) did not change significantly, unlike the activity of the wild-type *gcrA* promoter, which increased significantly (data not shown). We conclude that the mutation we introduced in the left portion of the CtrA-binding site of the *gcrA* promoter (*gcrAP*(CtrAL)) renders it independent of CtrA protein levels in the cell. The activities of the *gcrAP*(CtrAR) and the *gcrAP*(CtrAM) in

a *ctrA401ts* strain at restrictive temperature slightly increased, but more moderately than the wild-type promoter (data not shown), showing that these two mutated promoters are still slightly sensitive to CtrA protein levels in the cell, which probably explains why the effect of these two mutations on *gcrA* transcription are more moderate than the mutation in the left portion of the CtrA-binding site (Figure 3C). Overall, these results confirm that CtrA is a significant repressor of *gcrA* transcription. Yet,  $\beta$ -galactosidase assays using *lacZ* transcriptional fusions measure transcriptional levels in log phase cultures, but not temporal control.

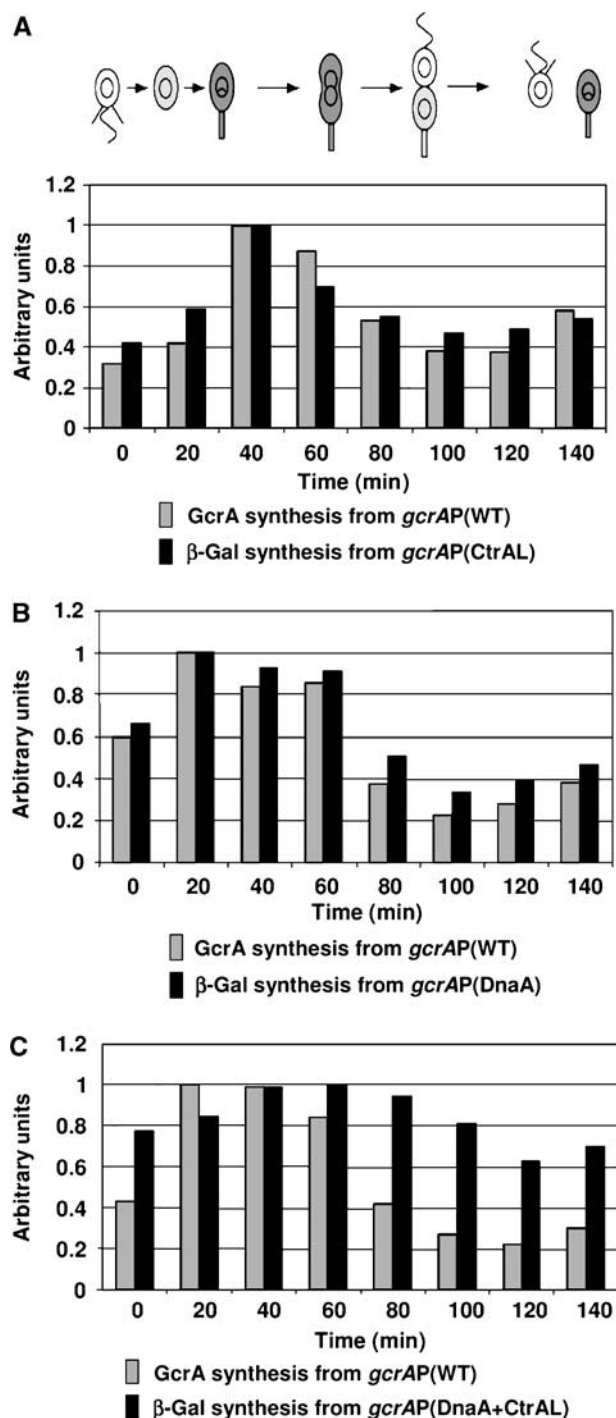
In order to understand the role of CtrA in the regulation of *gcrA* transcription during the cell cycle, we integrated the apparently CtrA-independent *gcrAP*(CtrAL)-*lacZ* transcriptional fusion into the *Caulobacter* chromosome. As was done for the wild-type promoter (Figure 1), transcription and translation from the *gcrAP*(CtrAL) at different time points of the cell cycle were compared by pulse-labelling cell samples from a synchronous culture with [<sup>35</sup>S]methionine and immunoprecipitating the  $\beta$ -galactosidase (Figure 4A). Surprisingly, we found that transcription from the mutated *gcrAP*(CtrAL) promoter is still cell cycle regulated. We also immunoprecipitated GcrA from the same pulse-labelled samples, which allowed us to accurately compare GcrA synthesis from the wild-type *gcrA* promoter to  $\beta$ -galactosidase synthesis from the mutated promoter, during the same synchronization experiment. This experiment shows that the level of transcription from the mutated *gcrAP*(CtrAL) is still cell cycle controlled. We conclude that the degradation of the CtrA repressor is not solely responsible for the increase in *gcrA* transcription during the swarmer-to-stalked cell transition, suggesting that other regulatory elements are also involved in the temporal control of *gcrA* transcription.

#### DnaA is critical for the induction of *gcrA* transcription

The *gcrA* promoter has a putative DnaA box about 50 base pairs upstream of its +1 transcriptional start site (Figure 3A), which matches seven of the nine base-pair *Caulobacter* consensus DnaA box sequence (Marczynski and Shapiro, 1992). DnaA is a dual function protein: it is essential for the initiation of DNA replication and it acts as a transcription

factor in *Escherichia coli* (for a review, see Messer and Weigel, 1997), *Bacillus subtilis* (Goranov *et al*, 2005) and *Caulobacter* (Hottes *et al*, 2005).

To determine whether DnaA is a regulator of *gcrA* transcription, we used a strain (GM2471) in which the sole copy of *dnaA* is under the control of the chromosomal xylose-inducible promoter *PxylX*. The *gcrAP-lacZ* fusion on plasmid pLacZ290-*gcrAP*(WT) was assayed in strain GM2471 (Figure 3D). At 4 h after this strain was shifted from PYEX to PYEG to deplete DnaA, the activity of the *gcrA* promoter decreased by more than 50%. Thus, DnaA is directly or indirectly involved in the activation of the *gcrA* promoter.



**Figure 4** Effects of CtrA and DnaA on *gcrA* cell cycle transcription. Cell cycle activities of the mutated *gcrA* promoters were measured by immunoprecipitation of  $\beta$ -galactosidase from pulse-labelled samples of synchronized *Caulobacter* cultures. To compare with the activity of the *gcrAP*(WT), radiolabelled GcrA was also immunoprecipitated from the same synchrony. Relative rates of *gcrA* transcription from the mutated *gcrA* promoter and GcrA synthesis from the wild-type *gcrA* promoter at the indicated times of the cell cycle were measured by phosphorimaging and were normalized so that the maximum value of each experiment equals 1. The averages of the results of multiple independent experiments are shown. In the schematic of the *Caulobacter* cell cycle, gray indicates accumulation of GcrA in the NA1000 cells. (A) The LS4224 (*gcrA*<sup>+</sup> *gcrAP*(CtrAL)-*lacZ*) strain was used to compare the cell cycle activity of the *gcrAP*(CtrAL) with the cell cycle activity of the *gcrAP*(WT). (B) The NA1000 (*gcrA*<sup>+</sup>) strain carrying the pLacZ290-*gcrAP*(DnaA) plasmid was used to compare the cell cycle activity of the *gcrAP*(DnaA) with the cell cycle activity of the *gcrAP*(WT). (C) The NA1000 (*gcrA*<sup>+</sup>) strain carrying the pLacZ290-*gcrAP*(DnaA + CtrAL) plasmid was used to compare the cell cycle activity of the *gcrAP*(DnaA + CtrAL) with the cell cycle activity of the *gcrAP*(WT).

To test whether the activation of *gcrA* transcription by DnaA requires the presence of the DnaA box in the *gcrA* promoter, we analyzed the transcription of a mutant *gcrA* promoter (*gcrAP*(DnaA)), in which the DnaA box has been disrupted (Figure 3B). The *gcrAP*(DnaA) was cloned in the *pLacZ290* and introduced in a wild-type *Caulobacter* strain to compare its activity with the wild-type promoter (*gcrAP*(WT)) by  $\beta$ -galactosidase assays. We observed a striking 80% loss of activity of the *gcrAP*(DnaA) as compared to the wild-type promoter (Figure 3C). Similarly, when the construct was introduced in the GM2471 strain grown in PYEX, the activity of the *gcrAP*(DnaA) was also five-fold lower than the activity of the wild-type promoter (Figure 3D). Since the mutated *gcrAP*(DnaA) has the same activity in GM2471 grown for 4 h in either xylose or glucose containing media, we conclude that the mutations we introduced in the DnaA box renders the mutated *gcrAP*(DnaA) independent of the DnaA protein levels in the cell. The dependence of *gcrA* transcription on DnaA and on the presence of an intact DnaA box in the *gcrA* promoter provides evidence that the induction of *gcrA* transcription by DnaA is direct and requires binding of DnaA to the *gcrA* promoter.

To understand the effect of DnaA on the temporal control of *gcrA* transcription, we analyzed the transcription and translation from the *gcrAP*(DnaA) at different time points of the cell cycle by pulse labelling cell samples from a synchronous culture and immunoprecipitating the  $\beta$ -galactosidase (Figure 4B). We then compared GcrA synthesis from the wild-type *gcrA* promoter to  $\beta$ -galactosidase synthesis from the DnaA-independent *gcrAP*(DnaA) promoter during the same synchronization experiments. This experiment shows that the low-level activity from the DnaA-independent promoter is still cell cycle controlled, parallel to our results with the CtrA-independent promoter and with the wild-type *gcrA* promoter.

#### ***Caulobacter* integrates the negative control by CtrA and the positive control by DnaA to regulate *gcrA* cell cycle transcription**

We observed that the temporal regulation of the mutated *gcrAP*(CtrAL) and *gcrAP*(DnaA) promoters are equivalent to the wild-type *gcrA* promoter. This result suggests that CtrA and DnaA both contribute to the control of the temporal transcription of *gcrA*. Another possibility is that a third regulator is responsible for *gcrA* temporal regulation. To discriminate between these two possibilities, we constructed a *gcrA* promoter double mutant (*gcrAP*(DnaA + CtrAL)) that is mutated in both the left portion of the CtrA-binding site and in the DnaA box (Figure 3B). This mutated promoter was cloned in *pLacZ290* and introduced into a wild-type *Caulobacter* strain and its activity was compared to strains with single promoter mutants:  $\beta$ -galactosidase assays were carried out in all three strains (Figure 3C). Like the *gcrAP*(DnaA), we observed that the activity of the *gcrAP*(DnaA + CtrAL) is much lower than the activity of the wild-type promoter when introduced in a wild-type *Caulobacter* strain (Figure 3C), showing that DnaA can activate the CtrA-independent *gcrAP*(CtrAL). Yet, the activity of the *gcrAP*(DnaA + CtrAL) is about 26% higher than the activity of the *gcrAP*(DnaA) (Figure 3C), showing that CtrA can repress the basal transcription from the DnaA-independent *gcrAP*(DnaA). These observations suggest that CtrA and

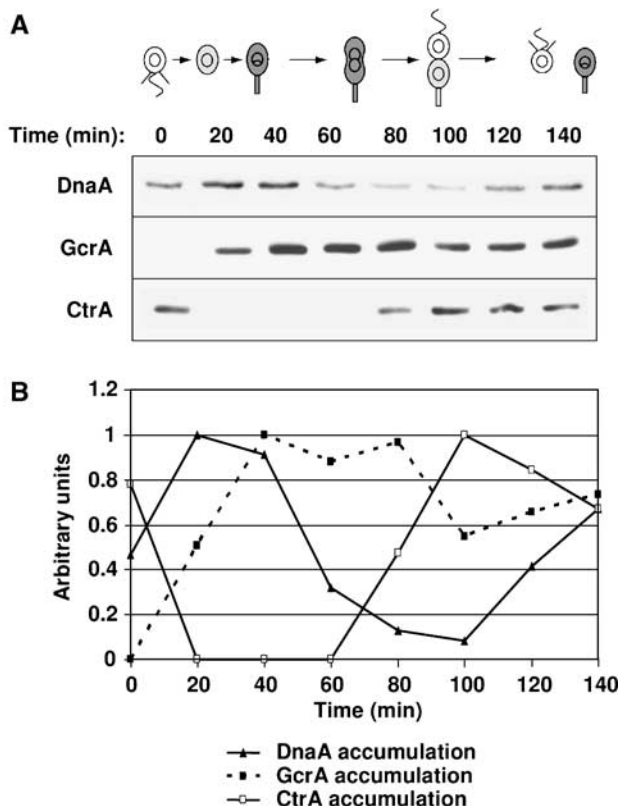
DnaA independently contribute to the level of expression of the *gcrA* promoter but that their activities may function together to provide correct temporal regulation.

To test this hypothesis, we compared the temporal activity of the *gcrAP*(DnaA + CtrAL) double mutant fused to *lacZ* with that of the wild-type *gcrA* promoter by immunoprecipitation experiments from samples pulse labelled at different time points in a synchronized cell culture (Figure 4C). Unlike that observed with the single mutants *gcrAP*(CtrAL) (Figure 4A) or *gcrAP*(DnaA) (Figure 4B), the cell cycle regulation of the double mutant *gcrAP*(DnaA + CtrAL) is significantly attenuated as compared to the wild-type promoter (Figure 4C). Indeed, we observed that the maximum change in the activity of the *gcrAP*(DnaA + CtrAL) over the course of a cell cycle is about 1.5-fold, versus about five-fold for the maximum change in the activity of the *gcrAP*(WT) over the course of a cell cycle. This result argues that *Caulobacter* integrates the activation of *gcrA* transcription by DnaA and the repression of *gcrA* transcription by CtrA to maintain strong cell cycle regulation of *gcrA* transcription. Although these experiments show that either DnaA or CtrA is required for the strong temporal control of *gcrA* transcription, other regulatory elements may also contribute to this control, since transcription from the *gcrAP*(DnaA + CtrAL) double-mutant promoter is not totally constant throughout the cell cycle.

If DnaA, in combination with CtrA, contributes to the temporal control of *gcrA* transcription, then active DnaA should accumulate during the swarmer-to-stalked cell transition and before GcrA accumulates. Indeed, we observed that DnaA protein levels are cell cycle regulated and that DnaA starts to accumulate at the swarmer-to-stalked cell transition about 20 min before GcrA (Figure 5). Since DnaA functions to allow DNA replication initiation at this same time period (Zweiger and Shapiro, 1994), DnaA likely carries out a dual function at that time in the cell cycle.

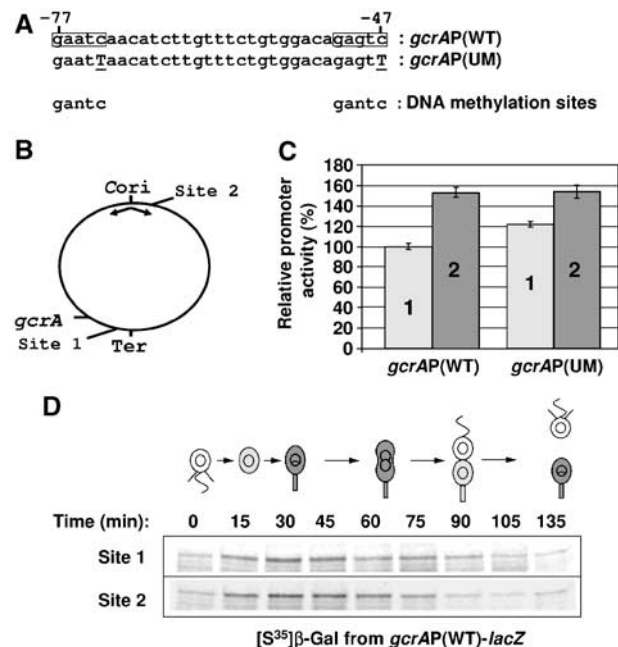
#### **The two methylation sites in the *gcrA* promoter are minor contributors to *gcrA* regulation**

In addition to a DnaA box and a CtrA consensus sequence, the *gcrA* promoter contains two GANTC sequences (Figure 3A) that are the recognition motifs for the CcrM DNA methyltransferase in *Caulobacter* (Zweiger *et al*, 1994). We explored the possibility that the methylation state of these two sites affects the activity of the *gcrA* promoter, as it is the case for the *ctrA* P1 promoter (Reisenauer and Shapiro, 2002). Because the native *gcrA* gene is located next to the terminus of the *Caulobacter* chromosome, it remains in the fully methylated state till near the end of chromosome replication (Figure 6B). To determine whether the position of *gcrA* on the chromosome, and thus its methylation state, affects the level of *gcrA* transcription, we integrated the *gcrAP-lacZ* reporter at a site next to the origin of replication on the chromosome (at the *hrcA* locus, named site 2, in strain LS4221) and compared its activity to the same reporter integrated at a site next to the terminus of replication on the chromosome (at the *trpE* locus, named site 1, in strain LS4220). Previous studies have demonstrated that DNA methylation at the two sites used in this study varies during the cell cycle (Stephens *et al*, 1996; Marczyński, 1999). GANTC sites near *Cori* (site 2) become hemimethylated soon after the initiation of DNA replication and remain hemimethylated until the end of



**Figure 5** DnaA is cell cycle regulated, accumulating before GcrA. (A) Immunoblots of cell extracts from a synchronized NA1000 culture using DnaA, GcrA and CtrA antibodies at the indicated times in the cell cycle. Cultures were adjusted to the same  $A_{660}$  for all lanes of each gel. (B) DnaA (plain triangles, plain line), GcrA (plain squares, dashed line) and CtrA (open squares, plain line) protein levels at the indicated times of the cell cycle from densitometry graphs of the DnaA, GcrA and CtrA immunoblots. Values were normalized so that the maximum value of each immunoblot equals 1. In the schematic of the *Caulobacter* cell cycle, gray indicates accumulation of GcrA.

S phase when the DNA methyltransferase CcrM is present and active. In contrast, GANTC sites near the terminus (site 1) are hemimethylated only for a short period at the end of S phase.  $\beta$ -Galactosidase assays showed that the activity of the wild-type *gcrA* promoter fused to *lacZ* integrated near the terminus (site 1) is  $\sim 35\%$  lower than its activity when integrated near *Cori* (site 2) (Figure 6C). To determine what proportion of this 35% difference in activity reflects simple changes in the copy number of the reporter during DNA replication, we also constructed a mutant *gcrA* promoter that cannot be methylated [*gcrAP*(UM)], by generating a single base pair mutation in each of the two GANTC sequences in the *gcrA* promoter (Figure 6A). The activity of the mutated *gcrAP*(UM)-*lacZ* reporter integrated near the terminus (site 1) was  $\sim 20\%$  lower than its activity when integrated near *Cori* (site 2) (Figure 6C), revealing the difference due to change in the copy number between the two integration sites. These results suggest that the difference in the timing of methylation of the two GANTC sequences in the *gcrA* promoter near the *Cori* or the terminus, results in a 15% difference (35% total difference minus 20% difference due to change in the copy number) in the activity of the *gcrA* promoter. Overall, we propose that full methylation of the *gcrA* promoter partially represses *gcrA* transcription.



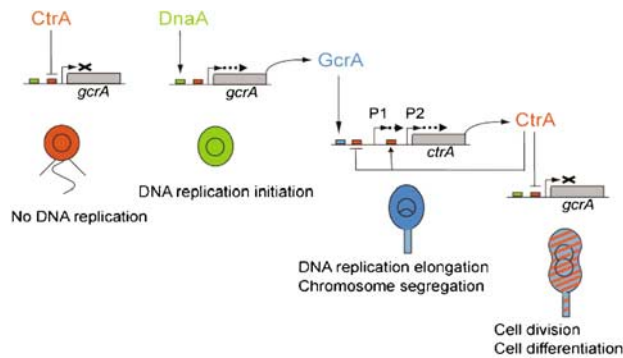
**Figure 6** Effect of *gcrAP* methylation on *gcrA* transcription. (A) The sequences of the two putative methylation sites (boxes) within the *gcrA* promoter (*gcrAP*) are shown and compared to the consensus DNA methylation sites in *Caulobacter*. Mutations introduced in the *gcrAP*(-507... +92) methylation sites are underlined and in capital letters. (B) Diagram of the *Caulobacter* chromosome showing the locations of the origin of replication (*Cori*), the terminus region (*Ter*), the *gcrA* gene and the *trpE* (site 1) and *hrcA* (site 2) integration sites. (C) Activities of the wild-type *gcrAP*(WT) fused to *lacZ* and integrated at site 1 or site 2 (strains LS4220 and LS4221, respectively) and the unmethylatable *gcrAP*(UM) fused to *lacZ* integrated at site 1 or site 2 (strains LS4222 and LS4223, respectively) were determined by  $\beta$ -galactosidase assays. The activities of the control LS3321 and LS3323 strains were subtracted from the activities of the *gcrAP-lacZ* constructs. Activities were normalized so that the activity of the *gcrAP*(WT) at site 1 equals 100%. Errors bars indicate the standard deviations from three independent experiments. (D) Cell cycle *gcrAP*(WT) activities at site 1 and site 2 were measured by immunoprecipitation of  $\beta$ -galactosidase from pulse-labelled samples of synchronized LS4220 and LS4221 strains cultures, respectively. Autoradiograms from the immunoprecipitations of labelled  $\beta$ -galactosidase are shown. In the schematic of the *Caulobacter* cell cycle, gray indicates accumulation of GcrA in the NA1000 cells.

To test whether the modest effect of DNA methylation on *gcrA* transcription affected the temporal regulation of *gcrA* transcription, we assayed transcription levels at multiple time points in a synchronized culture. We compared expression from the *gcrA* promoter integrated at site 1 to the promoter integrated at site 2, by pulse-labelling cell samples from the two synchronous cultures with [<sup>35</sup>S]methionine and immunoprecipitating the  $\beta$ -galactosidase (Figure 6D). We found that temporal transcription from the *gcrA* promoter fused to *lacZ* was not affected by the chromosomal position of the *gcrA* promoter reporter. However, the effect of methylation on the temporal regulation of *gcrA* transcription may be hidden by the strong temporal regulation of the wild-type *gcrA* promoter by DnaA and CtrA.

## Discussion

By studying the regulation of *gcrA* expression, we have uncovered an unexpected and critical link between the oscillating GcrA and CtrA global cell cycle regulators and





**Figure 7** Model for the control of the *Caulobacter* cell cycle by sequential accumulation of the DnaA, GcrA and CtrA global cell cycle regulators. In swarmer cells, CtrA represses *gcrA* transcription and GcrA protein is very unstable, so GcrA does not accumulate. During the swarmer to stalked cell differentiation, CtrA is degraded and DnaA accumulates, which allows *gcrA* transcription to be turned on. Since GcrA is more stable in stalked cells, GcrA can accumulate efficiently. When cell division is initiated, *ctrA* transcription is turned back on (notably by accumulated GcrA), and DnaA is degraded and probably inactivated. Accumulation of CtrA and disappearance of active DnaA turns off *gcrA* transcription in predivisional cells. In the schematic of the *Caulobacter* cell cycle, red, green and blue indicate accumulation of CtrA, DnaA and GcrA, respectively.

DNA replication control. We show that the DnaA replication initiation factor activates the transcription of *gcrA*, which encodes a central component of the regulatory circuit that drives the cell cycle (Figure 7). DnaA thereby acts to coordinate the initiation of DNA replication with the expression of genes required for DNA replication and segregation, cell division and cell differentiation.

#### Cell-cycle-dependent regulation of GcrA

Transcription of *gcrA* is very low in swarmer cells and increases during the swarmer-to-stalked cell transition. It reaches a maximum in the stalked cells, before it decreases to a low level again in predivisional cells. At the end of the cell cycle, transcription of *gcrA* rapidly resumes in the stalked progeny but remains very low in the swarmer progeny (Figure 1). The following evidence demonstrates that the CtrA cell cycle response regulator directly represses *gcrA* transcription: first, depletion of CtrA causes an increase in *gcrA* transcription (Holtzendorff *et al*, 2004). Second, CtrA directly binds to the *gcrA* promoter (Holtzendorff *et al*, 2004). Third, disruption of the CtrA-binding site in the *gcrA* promoter by targeted mutagenesis renders *gcrA* transcription non-responsive to CtrA depletion and causes an increase in *gcrA* transcription (Figure 3). Since CtrA is present and active in swarmer cells and in predivisional cells, but not in stalked cells (Domian *et al*, 1997, 1999), we hypothesized that the burst in *gcrA* transcription that we observed in stalked cells was due to the degradation of its repressor CtrA. However, here we show that CtrA is not the only regulator of *gcrA* cell cycle transcription since the mutant *gcrA* promoter that is not responsive to CtrA, is still cell cycle controlled (Figure 4A). Indeed, we demonstrate that transcriptional activation of *gcrA* by the DnaA protein, in concert with the negative effect of CtrA, regulates the temporal control of *gcrA* transcription. Transcription of *gcrA* is dramatically reduced when the DnaA protein is depleted (Figure 3D). Additionally, a mutant *gcrA*

promoter, which is not responsive to DnaA cellular levels, has a transcriptional activity that is five-fold lower than the wild-type *gcrA* promoter (Figure 3). We observed that the DnaA cellular concentration is cell cycle regulated and is highest during the swarmer-to-stalked cell transition (Figure 5), when DNA replication also initiates. The model we propose for the temporal regulation of GcrA cellular levels during the *Caulobacter* cell cycle is presented in Figure 7. The low levels of the inducer DnaA and the presence of the repressor CtrA in the swarmer cells yield a basal level of *gcrA* transcription. Furthermore, the few GcrA proteins synthesized are rapidly proteolyzed to prevent any accumulation of GcrA in swarmer cells (Figure 2). During the swarmer-to-stalked cell transition, the repressor CtrA is removed and the inducer DnaA accumulates (Figure 5), leading to a significant increase in *gcrA* transcription. In addition, the turnover of the newly synthesized GcrA proteins in the stalked cells is four-fold slower than in swarmer cells (Figure 2B), which allows the rapid accumulation of GcrA proteins in stalked cells. As the cells elongate approaching cell division, GcrA accumulation turns on *ctrA* transcription. CtrA accumulation and concomitant reduction in DnaA levels together turn off *gcrA* transcription in the predivisional cell (Figure 7).

#### Multilayer systems tightly control the cell cycle

One striking feature of this model is that multiple pathways regulate GcrA accumulation and that they function in concert to affect the temporally controlled accumulation of GcrA.

First, the temporally regulated transcription of *gcrA* is controlled by the combined effects of CtrA and DnaA, integrating the negative control by CtrA and the positive control by DnaA (Figure 3). To affect the strong temporal regulation of *gcrA* transcription, both CtrA-mediated and DnaA-mediated regulation pathways have to be disrupted at the same time. Moreover, CtrA and DnaA may not be the only regulators of *gcrA* cell cycle transcription, since the cell cycle control of a DnaA and CtrA-independent *gcrA* promoter is greatly attenuated, but not abolished (Figure 4C). We present evidence that the methylation state of the *gcrA* promoter mildly influences the expression of *gcrA* (Figure 6C) and this may provide the additional regulation for complete cell cycle control.

Second, the cell cycle accumulation of GcrA is controlled both by transcriptional regulation and by regulated proteolysis. We showed that GcrA accumulation still exhibits cell cycle control when *gcrA* is transcribed constitutively (Figure 2A). This result suggests that the control by regulated *gcrA* transcription is partly redundant with the control by regulated GcrA proteolysis, to fine-tune the cellular amounts of GcrA during the cell cycle. Many other essential *Caulobacter* proteins are subject to multiple mechanisms of control. The master regulator CtrA (Domian *et al*, 1997, 1999; Reisenauer and Shapiro, 2002; Judd *et al*, 2003; Holtzendorff *et al*, 2004), the cell division protein FtsZ (Kelly *et al*, 1998), the replication initiator DnaA (Zweiger and Shapiro, 1994; Gorbatyuk and Marczyński, 2005) and the DNA methyltransferase CcrM (Stephens *et al*, 1995, 1996; Wright *et al*, 1996) are other examples of essential proteins regulated by differential transcription and differential proteolysis during the *Caulobacter* cell cycle. Since the proteolysis of DnaA and GcrA is coincident, occurring specifically in swarmer cells, a common mechanism may regulate their degradation.



**Table I** Strains and plasmids used in this study

Plasmids	Relevant characteristics or construction	Source or reference
pNPT228	pLitmus28-derived integration vector	MRK Alley
pHP45Ω	Vector carrying a Spec <sup>R</sup> /Strep <sup>R</sup> cassette (Ω)	Prentki and Krisch (1984)
PBOR	2-kb <i>EcoRI</i> fragment from pHP45Ω ligated into <i>EcoRI</i> -digested pBluescript	C Stevens
p <i>LacZ290</i>	<i>lacZ</i> transcriptional fusion vector	Gober and Shapiro (1992)
p <i>LacZ290-gcrAP</i> (WT) = p <i>LacZ290-gcrAP</i>	<i>gcrAP</i> (−507... +92)- <i>lacZ</i> in pRK <i>lac290</i>	Holtzendorff et al (2004)
p <i>LacZ290-gcrAP'</i> (WT)	<i>gcrAP</i> (−100... +92)- <i>lacZ</i> in pRK <i>lac290</i>	This study
p <i>LacZ290-gcrAP''</i> (WT)	<i>gcrAP</i> (−78... +92)- <i>lacZ</i> in pRK <i>lac290</i>	This study
p <i>LacZ290-gcrAP</i> (CtrAL)	<i>gcrAP</i> (CtrAL)- <i>lacZ</i> in pRK <i>lac290</i>	This study
p <i>LacZ290-gcrAP</i> (CtrAM)	<i>gcrAP</i> (CtrAM)- <i>lacZ</i> in pRK <i>lac290</i>	This study
p <i>LacZ290-gcrAP</i> (CtrAR)	<i>gcrAP</i> (CtrAR)- <i>lacZ</i> in pRK <i>lac290</i>	This study
p <i>LacZ290-gcrAP</i> (UM)	<i>gcrAP</i> (UM)- <i>lacZ</i> in pRK <i>lac290</i>	This study
p <i>LacZ290-gcrAP</i> (DnaA)	<i>gcrAP</i> (DnaA)- <i>lacZ</i> in pRK <i>lac290</i>	This study
p <i>LacZ290-gcrAP</i> (DnaA + CtrAL)	<i>gcrAP</i> (DnaA + CtrAL)- <i>lacZ</i> in pRK <i>lac290</i>	This study
pNPT228- <i>gcrAP-lacZ</i>	4-kb <i>Bam</i> HI- <i>Dra</i> I fragment from p <i>LacZ290-gcrAP</i> ligated into <i>Bam</i> HI- <i>Eco</i> RV-digested pNPT228	This study
pNPT228- <i>gcrAP-lacZ-Ω</i>	2-kb <i>Bam</i> HI fragment from pBOR ligated into <i>Bam</i> HI-digested pNPT228- <i>gcrAP-lacZ</i>	This study
pNPT228- <i>gcrAP</i> (UM)- <i>lacZ</i>	4-kb <i>Bam</i> HI- <i>Dra</i> I fragment from p <i>LacZ290-gcrAP</i> (UM) ligated into <i>Bam</i> HI- <i>Eco</i> RV-digested pNPT228	This study
pNPT228- <i>gcrAP</i> (UM)- <i>lacZ-Ω</i>	2-kb <i>Bam</i> HI fragment from pBOR ligated into <i>Bam</i> HI-digested pNPT228- <i>gcrAP</i> (UM)- <i>lacZ</i>	This study
pNPT228- <i>gcrAP</i> (CtrAL)- <i>lacZ</i>	4-kb <i>Bam</i> HI- <i>Dra</i> I fragment from p <i>LacZ290-gcrAP</i> (CtrAL) ligated into <i>Bam</i> HI- <i>Eco</i> RV-digested pNPT228	This study
pNPT228- <i>gcrAP</i> (CtrAL)- <i>lacZ-Ω</i>	2-kb <i>Bam</i> HI fragment from pBOR ligated into <i>Bam</i> HI-digested pNPT228- <i>gcrAP</i> (CtrAL)- <i>lacZ</i>	This study
Strains	Relevant genotype	Source or reference
<i>E. coli</i>		
S17-1	294::RP4-2(Tc::Mu)(Km::Tn7)	Simon et al (1983)
<i>C. crescentus</i>		
NA1000	Synchronizable derivative of CB15	Evinger and Agabian (1977)
GM1258	NA1000 <i>trpE</i> :: <i>Tn5Ω</i> -MP	Marczynski (1999)
LS3323	NA1000 <i>trpE</i> :: <i>Tn5Ω</i> ::pNPT228- <i>lacZ-Ω</i>	Reisenauer and Shapiro (2002)
LS4220	NA1000 <i>trpE</i> :: <i>Tn5Ω</i> ::pNPT228- <i>gcrAP</i> (−507... +92)- <i>lacZ-Ω</i>	This study
LS4222	NA1000 <i>trpE</i> :: <i>Tn5Ω</i> ::pNPT228- <i>gcrAP</i> (UM)- <i>lacZ-Ω</i>	This study
LS4224	NA1000 <i>trpE</i> :: <i>Tn5Ω</i> ::pNPT228- <i>gcrAP</i> (CtrAL)- <i>lacZ-Ω</i>	This study
LS2293	NA1000 <i>hrcAΩ</i>	Roberts et al (1996)
LS3321	NA1000 <i>hrcAΩ</i> ::pNPT228- <i>lacZ-Ω</i>	Reisenauer and Shapiro (2002)
LS4221	NA1000 <i>hrcAΩ</i> ::pNPT228- <i>gcrAP</i> (−507... +92)- <i>lacZ-Ω</i>	This study
LS4223	NA1000 <i>hrcAΩ</i> ::pNPT228- <i>gcrAP</i> (UM)- <i>lacZ-Ω</i>	This study
GM2471	NA1000 Δ <i>dnaA</i> ::Ω P <i>xylX</i> :: <i>dnaA</i>	Gorbatyuk and Marczynski (2001)
LS3707	NA1000 Δ <i>gcrA</i> ::Ω P <i>xylX</i> :: <i>gcrA</i>	Holtzendorff et al (2004)

### Parallel systems regulate *gcrA* expression and the initiation of DNA replication

The regulation of *gcrA* expression is parallel to the regulation of the initiation of replication. First, chromosome replication is initiated coincident with the transcription of *gcrA*, during the swarmer-to-stalked cell transition. Second, *Cori* and the *gcrA* promoter contain CtrA-binding sites, DnaA boxes and DNA methylation sites (Figure 3A) (Marczynski and Shapiro, 2002; Holtzendorff *et al*, 2004). Third, the activities of *Cori* and the *gcrA* promoter are activated by DnaA and repressed by CtrA (Figure 3) (Marczynski and Shapiro, 2002; Holtzendorff *et al*, 2004). Thus, the cell is using similar mechanisms of regulation to coordinate the initiation of chromosome replication with the synthesis of GcrA. However, the regulation of *gcrA* is not identical to the regulation of the *Cori*. For example, the IHF protein activates the initiation of chromosome replication (Siam *et al*, 2003), but does not influence *gcrA* transcription (data not shown).

### The sequential accumulation of three essential regulators control the *Caulobacter* cell cycle

GcrA concentration oscillates not only with CtrA concentration, but also with DnaA concentration during the *Caulobacter* cell cycle (Figure 5). CtrA notably acts as a silencer of the chromosome replication origin, an inhibitor of *ftsZ* transcription and a regulator of multiple genes required for pili and flagella biogenesis (Quon *et al*, 1996, 1998; Kelly *et al*, 1998; Laub *et al*, 2002). GcrA notably activates *podJ* and *pleC* that are required for polar morphogenesis and regulates genes needed for DNA replication elongation and chromosome partitioning, like *dnaQ*, *dnaB*, *dnaC* and *gyrA* (Holtzendorff *et al*, 2004). Further analysis now shows that GcrA does not significantly affect *dnaA* transcription, in contrast to previous results (Holtzendorff *et al*, 2004). Like CtrA, *Caulobacter* DnaA is an essential dual function protein (Gorbatyuk and Marczynski, 2001): it regulates the initiation of DNA replication and it is a transcription factor. The DnaA regulon in *Caulobacter* has recently been characterized showing that DnaA regulates the transcription of multiple genes, notably involved in nucleotide biosynthesis and DNA replication (Hottes *et al*, 2005). Overall, the DnaA/GcrA/CtrA cascade during the *Caulobacter* cell cycle defines the timing of expression of multiple genes encoding proteins with diverse functions needed for progress through the cell cycle (Figure 7). The activation of *gcrA* transcription by DnaA is a novel critical regulatory pathway, which coordinates DNA replication initiation with cell division and cell differentiation functions regulated by GcrA and CtrA.

## Materials and methods

### Bacterial strains, synchronization and growth conditions

*Caulobacter* strains were grown in peptone-yeast extract (PYE) complex media or M2 minimal salts + 0.2% glucose (M2G) minimal media (Ely, 1991) at 30°C (or 28°C for experiments with LS3707). For GM1258 derivative strains, M2G was supplemented with 0.01% of tryptophan. When indicated, xylose (0.3% for GM2471 or 0.03% for LS3707) or glucose (0.2%) was added to the media to induce or

repress the *PxyIX* promoter, respectively. Strains and plasmids used are listed in Table I. Antibiotics used include oxytetracycline (1 µg/ml), kanamycin (5 or 25 µg/ml), spectinomycin (25 µg/ml), streptomycin (5 µg/ml) and nalidixic acid (20 µg/ml). Plasmids were mobilized from *E. coli* S17-1 into *Caulobacter* by bacterial conjugation or introduced by transformation. Synchronized cultures of *Caulobacter* were obtained by centrifugation in a Ludox density gradient followed by isolation of swarmer cells (Evinger and Agabian, 1977). Swarmer cells were released into minimal medium for cell cycle studies.

### Site-directed mutagenesis and truncation of the *gcrAP*

See Supplementary data.

### Construction of the *gcrAP-lacZ* chromosomal integrants

Plasmids pNPT228-*gcrAP-lacZ*-Ω, pNPT228-*gcrAP*(UM)-*lacZ*-Ω and pNPT228-*gcrAP*(CtrAL)-*lacZ*-Ω were integrated into the chromosomal Ω cassette of strains LS2293 and/or GM1258 by a single integration event.

### Promoter activity assays

The β-galactosidase activity (in Miller Units) of strains containing the promoter-*lacZ* plasmids or integrated promoter-*lacZ* transcriptional fusions was assayed in log-phase cultures (Miller, 1972). β-galactosidase activities represent the average of at least three independent assays.

### Immunoblot analysis

GcrA and CtrA proteins were resolved on 15% SDS-PAGE and DnaA proteins were resolved on 8% SDS-PAGE (Sambrook *et al*, 1989), and electrotransferred to a polyvinylidene fluoride membrane (Millipore). Immunodetection was performed with polyclonal antibodies. Anti-DnaA, anti-CtrA and donkey anti-rabbit conjugated to horseradish peroxidase (Jackson ImmunoResearch) serums were diluted 1:10 000. Anti-GcrA serum was diluted 1:2000. A chemiluminescent reagent (PerkinElmer) and Kodak Bio-Max MR films were used. Images were processed with Photoshop (Adobe) and relative band intensities were determined using ImageQuant software (Molecular Dynamics).

### Cell cycle transcription and synthesis analysis

Aliquots (1 ml) of cells grown in M2G were removed and labelled with 10 µCi of [<sup>35</sup>S]methionine (Amersham) for 2 or 5 min. Cells were collected and lysed. Equivalent counts of radiolabelled proteins were then used for immunoprecipitation using anti-GcrA or anti-β-galactosidase serums. Resulting samples were resolved by SDS-PAGE and labelled protein bands were quantified using the ImageQuant software. Their relative activity was normalized so that the maximum value equals 1. Details of protocols and composition of buffers used are in the Supplementary data.

### GcrA half-life determination

The stability of GcrA was determined by pulse-chase experiments. Cells grown in M2G or M2GX were labelled with 10 µCi/ml of [<sup>35</sup>S]methionine for 3 or 5 min and then chased with 1 mM unlabelled methionine and 0.2 mg/ml casamino acids. Cells from 1 ml of culture were collected at the indicated times by centrifugation and freezing on dry ice. Labelled GcrA protein was immunoprecipitated as described above, resolved by 12.5 or 15% SDS-PAGE and quantified as described above. Data fitting to determine GcrA half-life is described in Supplementary data.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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