JOURNAL OF BACTERIOLOGY, Apr. 1995, p. 1662–1669 Vol. 177, No. 7
0021-9193/95/$04.00+0

Coordinate Cell Cycle Control of a *Caulobacter* DNA Methyltransferase and the Flagellar Genetic Hierarchy

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Received 25 October 1994/Accepted 14 January 1995

The expression of the *Caulobacter ccrM* gene and the activity of its product, the M.*Ccr* II DNA methyltransferase, are limited to a discrete portion of the cell cycle (G. Zweiger, G. Marczynski, and L. Shapiro, J. Mol. Biol. 235:472–485, 1994). Temporal control of DNA methylation has been shown to be critical for normal development in the dimorphic *Caulobacter* life cycle. To understand the mechanism by which *ccrM* expression is regulated during the cell cycle, we have identified and characterized the *ccrM* promoter region. We have found that it belongs to an unusual promoter family used by several *Caulobacter* class II flagellar genes. The expression of these class II genes initiates assembly of the flagellum just prior to activation of the *ccrM* promoter in the predivisional cell. Mutational analysis of two M.*Ccr* II methylation sites located 3′ to the *ccrM* promoter suggests that methylation might influence the temporally controlled inactivation of *ccrM* transcription. An additional parallel between the *ccrM* and class II flagellar promoters is that their transcription responds to a cell cycle DNA replication checkpoint. We propose that a common regulatory system coordinates the expression of functionally diverse genes during the *Caulobacter* cell cycle.

Site-specific methylation of chromosomal DNA has been observed in a wide range of organisms, from bacteria to plants and humans. DNA methylation can have a critical role in the regulation of protein-DNA transactions involved in numerous cellular processes, including transcription (5, 8, 28), repair of mutational lesions (18, 25), and transposition (2, 35). The methylation state of the origin of replication is an important factor regulating the initiation of chromosomal replication in *Escherichia coli* (29). Analysis of the M.*Ccr* II DNA methyltransferase (MTase) in the bacterium *Caulobacter crescentus* revealed that chromosomal methylation has an important role in growth and development in this organism (47). (The M.*Ccr* II DNA MTase protein was referred to by Zweiger et al. [47] as CcrM. We have changed the name herein to conform to accepted nomenclature for DNA methyltransferases. There does not appear to be a cognate restriction enzyme for the M.*Ccr* II recognition site [GAnTC].) To gain insight into the role of DNA methylation in *Caulobacter* cell differentiation, we have focused in this work on understanding how M.*Ccr* II activity is controlled and have found that a common regulatory system appears to coordinate expression of M.*Ccr* II with other cell cycle-regulated events.

C. crescentus exhibits a distinctive cell cycle modulated pattern of DNA adenine methylation (47). GAnTC sites become hemimethylated upon passage of the replication fork, and remethylation of the newly synthesized strand in both new chromosomes is delayed until shortly before cell division, when replication is at or near completion. The onset and duration of the hemimethylated state for a particular site is thus dependent on its position on the chromosome relative to the origin of replication. Transcription of the gene (*ccrM*) encoding the GAnTC-specific DNA MTase (M.*Ccr* II) occurs only in the predivisional cell, suggesting that the delay in remethylation could be due to an absence of MTase for the majority of the cell cycle. Placing *ccrM* expression under the control of a constitutive promoter (P_{lac}) demonstrated that proper temporal control of DNA methylation is essential for normal *Caulobacter* development (47). Strains with continuously fully methylated chromosomes exhibited unusually long cells with frequent twists or pinches, indicative of aberrant cell division processes, and showed significant relaxation of the control of initiation of chromosomal replication. The parallels between M.*Ccr* II and the *E. coli* Dam MTase with respect to DNA replication are noteworthy, as *E. coli* strains overexpressing Dam initiate chromosomal replication more frequently than wild-type cells, and in an uncoordinated fashion (4, 9). It is not yet known why aberrant DNA methylation affects morphology, cell division, or DNA replication in *C. crescentus*.

C. crescentus generates two distinct cell types at each cell division, a motile swarmer cell and a stalked cell. These cell types exhibit fundamental differences in both morphology and function. An important functional distinction between these cell types is that chromosomal replication initiates only in the stalked cell. Morphologically, the most dramatic distinctions are in the polar appendages, the flagellum and the stalk. The fundamental asymmetry that yields dissimilar progeny cells appears to be dependent, at least in part, on transcriptional events that occur in the predivisional cell, the stage preceding separation of the swarmer and stalk cell progeny (reviewed in reference 7). The hierarchy controlling the ordered transcription of flagellar genes has served as a model for developmental control of gene expression in the predivisional cell (10, 16, 27, 44). As yet, however, the signals and factors responsible for the cell cycle-controlled initiation of the flagellar regulatory cascade have not been identified, and there is essentially no information on how the expression of nonflagellar genes, such as *ccrM*, might be controlled in the predivisional cell. A clear understanding of temporal and spatial regulation of transcription is essential for understanding the expression of asymmetry during the cell cycle.

Characterization of the *ccrM* promoter region revealed striking similarities in sequence and structure between *ccrM* and class II flagellar gene promoters, whose temporal expression

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TABLE 1. Strains and plasmids

Strain or plasmid	Description
Strains	
NA1000	Synchronizable derivative of *C. crescentus* CB15 (15)
LS1	NA1000, P_{lac}-directed constitutive transcription of *ccrM* (47)
LS178	NA1000, chromosomal integration of P_{ccrM}-*lacZ* reporter (47)
LS1435	NA1000 *dnaC303* (temperature-sensitive DNA elongation mutant)
Plasmids	
pCS104	pRKlac290 + P_{ccrM} (−75 to +233)
pCS105	pRKlac290 + P_{ccrM} (−92 to +233)
pCS106	pRKlac290 + P_{ccrM} (−11 to +233)
pCS129	pRKlac290 + P_{ccrM} (−608 to +15)
pCS130	pRKlac290 + P_{ccrM} (−24 to +156)
pCS133	pRKlac290 + P_{ccrM} (−67 to +233)
pCS134	pRKlac290 + P_{ccrM} (−608 to −38)
pCS138	pRKlac290 + P_{ccrM} (12-bp *Eco*RI linker insertion at −37)
pCS140	pRKlac290 + P_{ccrM} (−92 to +156)
pCS142	pRKlac290 + P_{ccrM} (−45 to +7)
pCS143	pRKlac290 + P_{ccrM} (−45 to +41)
pCS144	pRKlac290 + P_{ccrM} (−92 to +7)
pCS145	pRKlac290 + P_{ccrM} (−45 to +156)
pCS148	pRKlac290 + P_{ccrM} (−45 to +19)
pCS149	pSelect + P_{ccrM} (−608 to +156)
pCS150	pSelect + P_{ccrM} (−608 to +156, in opposite orientation than insert in pCS149)
pCS155	pRKlac290 + P_{ccrM} (−36AC→TG mutant)
pCS156	pRKlac290 + P_{ccrM} (−28CTAA→AATT mutant)
pCS157	pRKlac290 + P_{ccrM} (−12ACA→CGG mutant)
pCS164	pRKlac290 + P_{ccrM} (+11T→C, +16A→G mutant
pCS165	pRKlac290 + P_{ccrM} (+16A→G mutant)
pCS166	pRKlac290 + P_{ccrM} (+11T→C mutant)
pCS177	pRKlac290 + P_{ccrM} (−36AC→TG mutant, +11T→C, +16A→G)
pCS178	pRKlac290 + P_{ccrM} (−12ACA→CGG mutant, +11T→C, +16A→G)
pGZ22	pRKlac290 + P_{ccrM} (−608 to +156)
pGZ25	pRKlac290 + P_{ccrM} (−37 to +156)

has been described previously (12, 39, 41, 46). Furthermore, as is the case for flagellar class II promoters, *ccrM* transcription is rapidly repressed when chromosomal DNA replication is inhibited. We propose that transcription of *ccrM* is controlled by the same system responsible for cell cycle regulation of early flagellar genes and suggest that this system may be integral to global coordination of the temporal program of gene expression in the cell cycle.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *Caulobacter* strain LS1435 was generated by first transducing a *trpE*::Tn*5* insertion into PC2179 (*dnaC303* [31]), using phage φCr30. (*trpE* is approximately 10% linked to *dnaC* by transduction.) A transducing lysate was produced from the *dnaC303 trpE*:Tn*5* strain and used to infect NA1000. Temperature-sensitive Km^r Trp^- isolates were identified. Finally, a φCr30 transducing lysate from NA1000 was used to restore the strain to tryptophan prototrophy. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth (37) supplemented with ampicillin (50 μg/ml), tetracycline (10 μg/ml), or kanamycin (50 μg/ml) as necessary. *C. crescentus* strains were grown at 30°C in either peptone-yeast extract (PYE) or M2 minimal salts-glucose medium (M2G) (13) supplemented with tetracycline (2 μg/ml) or kanamycin (20 μg/ml) as necessary.

Culture synchronization. *Caulobacter* cultures for synchronization were grown in M2G medium, and swarmer cells were isolated by Ludox density gradient centrifugation (15). Swarmer cells were released into fresh M2G medium at 30°C at an optical density at 600 nm of 0.3 to 0.4. Progression through the cell cycle was monitored microscopically. The initial swarmer cell populations contained less that 2% predivisional cells. Samples for immunoprecipitation were labelled and prepared as described previously (39).

Construction of promoter reporters and assay of promoter activity. Cloning of the *ccrM* promoter region in plasmid pBGST18 was described by Zweiger et al. (47). The *ccrM* locus was originally cloned as an *Nla*III fragment whose sequence begins 35 bp 5′ to the start codon. Approximately 1 kb of additional upstream DNA was cloned by cleavage of LS1 genomic DNA with *Sac*I or *Nco*I, followed by religation, transformation into *E. coli*, and selection for plasmid-encoded kanamycin resistance. LS1 contains an integrated copy of plasmid pGZ1, which is not cut by *Sac*I or *Nco*I; the resulting plasmids therefore contain DNA extending to chromosomal *Sac*I and *Nco*I sites 5′ and 3′ to the *ccrM* coding region. Subclones of the upstream region in pBluescript (Stratagene) derivatives were used for sequencing by the dideoxynucleotide method (38), using Taquence DNA polymerase and 7-deaza-dGTP to reduce compression artifacts. Deletion fragments were generated either by PCR or by the exonuclease III-mung bean nuclease method for generating unidirectional deletions in Bluescript. The sequences of oligonucleotides used for PCR were as follows, with underlined bases being sites at which mutations were introduced to generate restriction sites: ccrmecor1, (−104)CTAGACCTTTGAATTCCTTCAACTTTG(−77), used to generate a 5′ end at −92 after cutting with *Eco*RI; ccrMPCR3, (−57)GCGCCT GAATTCCCGTGGTTA(−37), 5′ end at −45 after cutting with *Eco*RI; ccrMPCR4 (reverse strand), (+21)CGGAATCATGAAGCTTCCCTGG(+1), 3′ end at +7 after cutting with *Hin*dIII; ccrmut4 (reverse strand), (+33)CAAGAA AGGGATCCGAATCATG(+12), 3′ end at +19 after cutting with *Bam*HI; ccrMPCR5 (reverse strand), (+52)CTTCATGGATCCCACGTC(+36), 3′ end at +41 after cutting with *Bam*HI; and ccrmpstpe (reverse strand), (+160)CAG CTGCAGATTATAGGG(+143), 3′ end at +156 after cutting with *Pst*I.

Oligonucleotide-mediated site-directed mutations in the *ccrM* promoter region were generated by using the Altered Sites system (Promega). The *ccrM* promoter region was cloned into the pSelect vector as a 0.8-kb *Bam*HI-*Hin*dIII fragment, generating pCS149. Mutagenic oligonucleotides (Operon Technologies) were as follows: ccr-35mut, CCGTGGTTATGGGCCCGCTAAC; ccr-10mut, CGTCTCTCACGGCCGGATTTAC; and ccr-20mut, AACGGCCCGA ATTCCACGTCTCTCA. Oligonucleotide annealing and in vitro DNA synthesis conditions were as recommended by Promega. All deletion constructions and mutagenesis products were sequenced as described above.

Mutations in the inverted repeat 3 (IR3) element were generated by PCR using the ccrMPCR3 primer for the 5′ end and a primer designated IR3PCRmut [(+33)CAAGAAAGGGATCCGAAYCATGRGTCTTCC(+4); mutant bases underlined) for the 3′ end. This primer is identical to ccrmut4 in generating a *Bam*HI site immediately 3′ to the IR3 element for cloning. At two positions in IR3, a 50% mixture of bases was used (T and C at +16, A and G at +11) in order to recover single and double mutants in IR3 at the M.*Ccr* II methylation sites. To recover the IR3 mutations in combination with the −36AC→TG or −12ACA→CGG mutation, the pCS155 and pCS157 plasmids containing these promoter mutations were used as template for PCR with the ccrPCR3 and IR2PCRmut oligonucleotides.

To assay transcriptional activity, putative promoter-containing DNA fragments and mutant promoters were inserted into the multiple cloning site of the pRKlac290 vector to generate transcriptional fusions to *lacZ* (39). The resulting clones were mated from the *E. coli* S17-1 host strain into *Caulobacter* strains (13). β-Galactosidase activity was measured at 30°C as described by Miller (24), using log-phase cultures grown in PYE containing tetracycline. Assays were done at least in duplicate with a minimum of two independent cultures for each promoter construct.

Primer extensions. RNA was isolated by hot phenol-sodium dodecyl sulfate (SDS) lysis (36) from mid-log-phase NA1000 grown in PYE medium. RNA preparations were extracted twice each with phenol and chloroform-isoamyl alcohol and then precipitated with ethanol. RNA concentration was determined by A_{260}. Several oligonucleotide primers were used for extension reactions; the experiment shown in Fig. 2 used oligonucleotide ccrmpe (TTCATCTGCTC GATGCAGTC) (Operon Technologies). Primers were end labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Primer annealing to RNA (5 to 20 μg) and extension reactions using Superscript Moloney murine leukemia virus reverse transcriptase (Gibco BRL) were carried out essentially as described by Sambrook et al. (37), with the exceptions that the extension buffer provided by the manufacturer was used and no postextension RNase digestion was done. Reaction products were analyzed by denaturing gel electrophoresis on 6% acrylamide gels. For direct calibration of extension product sizes, dideoxynucleotide sequencing reactions were carried out with identical primers and run in adjacent lanes.

Inhibition of DNA synthesis. Treatment of M2G cultures with hydroxyurea (3 mg/ml, final concentration), sampling, and immunoprecipitation were as described previously (39). Osley et al. (33) have shown that DNA synthesis in the *dnaC303* mutant is blocked within minutes of a temperature shift to 37°C. To examine the effects of the mutation on *ccrM* gene expression (see Fig. 6), plasmid pCS140 was mated into LS1435. Cultures were grown to mid-log phase in M2G medium and shifted to 37°C. At 15-min intervals, 1-ml cultures were pulse-labelled with [^{35}S]methionine and processed as previously described for hydroxyurea experiments (39).

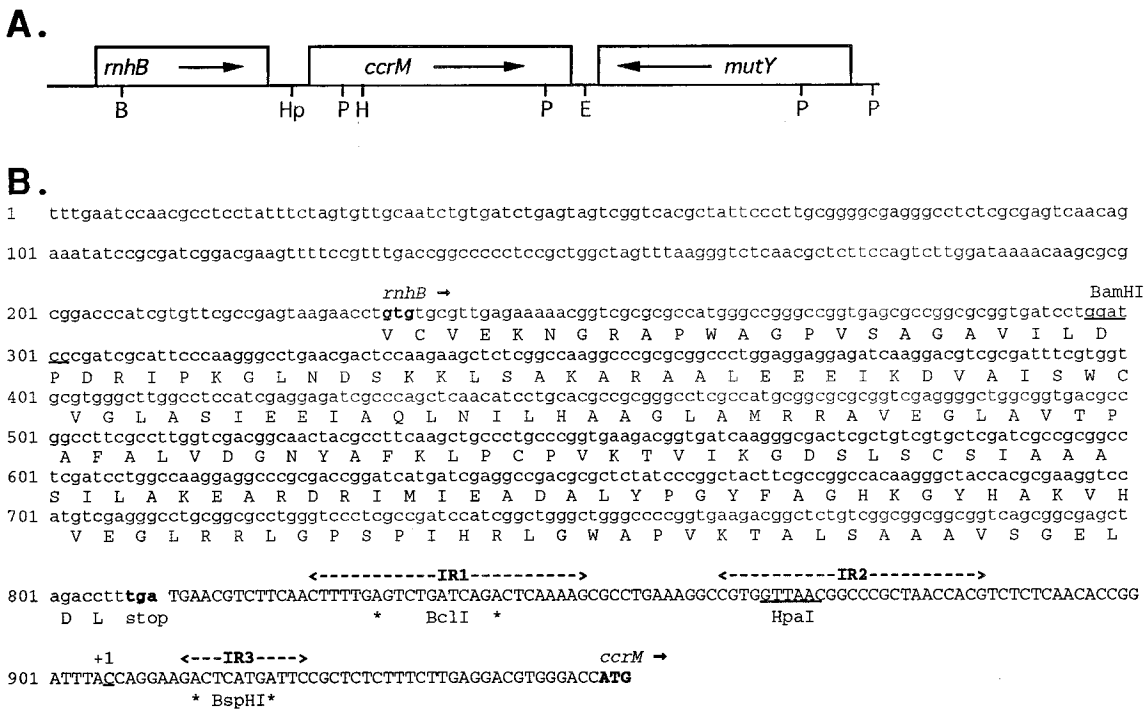


FIG. 1. The *ccrM* upstream region. (A) Schematic and partial restriction map of *ccrM* and adjacent genes. Restriction site abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; P, *Pst*I. (B) Sequence of 951 bp upstream of *ccrM*. The predicted amino acid sequence of the putative *rnhB* gene is indicated beneath the coding region. The GUG (Val) start codon (nucleotides 232 to 234) is inferred from several observations: a nonsense codon (UAA) defines the 5′ end of this open reading frame three codons upstream of the GUG; the homology with the *E. coli rnhB* product begins two codons downstream (GUU, Val); a reasonable consensus Shine-Dalgarno sequence begins 12 bp upstream of this codon; and GUG is the most frequently used initiation codon in *C. crescentus* after AUG. The full sequence of the *ccrM* coding region has been published (47), and the predicted ATG start codon is indicated. The intergenic region between the predicted *rnhB* stop codon and the *ccrM* start codon contains three inverted repeat motifs (IR1, IR2, and IR3). IR1 and IR3 contain M.*Ccr* II methylation sites (GAnTC), and the methylated adenine on the coding strand is indicated by an asterisk. The transcriptional start site for *ccrM* (see Fig. 2) is underlined and indicated as +1.

RESULTS

The *ccrM* promoter region. The DNA MTase gene, *ccrM*, was shown to be expressed only in predivisional cells, using an integrated transcriptional fusion to *lacZ* (47). To begin to identify factors that control *ccrM* transcription, it was necessary to identify and characterize the *ccrM* promoter region. The sequence of 951 bp upstream of the *ccrM* start codon is shown in Fig. 1. The majority of this sequence constitutes an open reading frame encoding a predicted 212-amino-acid polypeptide highly homologous (52% identity over 182 residues) to the *E. coli rnhB* gene product, RNase HII (22, 40). There is a 141-bp intergenic region between the *rnhB* stop codon and the predicted *ccrM* ATG codon with several interesting features. Four M.*Ccr* II methylation sites are seen in this region. One pair is part of a 26-bp perfect inverted repeat (IR1), beginning 10 bp downstream of the *rnhB* stop codon. A second pair of methylation sites generates a 10-bp inverted repeat (IR3), centered 34 bp upstream of the *ccrM* start codon. Between IR1 and IR3 is a 25-bp inverted repeat, IR2, which lacks methylation sites.

A DNA fragment extending from the *Bam*HI site 650 bp upstream of the *ccrM* translational start codon to a *Hin*dIII site 187 bp downstream (Fig. 1) exhibited cell cycle-regulated promoter activity. However, a fragment extending from the *Bam*HI site to an *Hpa*I site located between the *rnhB* and *ccrM* coding sequences lacked promoter activity, suggesting that the *ccrM* promoter overlaps or is downstream of the *Hpa*I site in the intergenic region. Primer extension analysis indicated that the major 5′ end of *ccrM* mRNA is 45 bp 5′ to the start codon, between IR2 and IR3 (Fig. 2A). The DNA sequence immediately upstream of this position does not resemble common bacterial promoter motifs, including those recognized by the *Caulobacter* σ^{70} and σ^{54} subunits. However, this region is strikingly similar to certain *Caulobacter* class II flagellar promoters, particularly that of the *fliLM* operon (39, 45) (Fig. 2B). Class II flagellar promoters are also temporally controlled, though initiation of transcription tends to be earlier than for *ccrM* and reaches a peak at 0.6 to 0.7 division units. The family of class II promoters typified by P_{fliL} is characterized by sequence conservation between −20 and −30 relative to the transcription start site, with additional conservation around −35 and −10. The location of the *ccrM* start site and the class II sequence similarity within the intergenic region are shown in Fig. 2C.

Structure of the *ccrM* promoter. To define the extent of sequences necessary for *ccrM* expression and cell cycle regulation, the intergenic region was progressively deleted from either direction and the remaining DNA was fused to *lacZ* on a low-copy-number plasmid (pRKlac290). As shown in Fig. 3A, deletions made from the 5′ side to −45 (pCS145), just upstream of IR2, maintained strong promoter activity, indicating that IR1 is not essential for P_{ccrM} activity. Promoter activity then dropped fourfold upon deletion to −37 (the edge of the class II promoter sequence similarity) and was eliminated by deletion to −24 (the center of the class II similarity). From the 3′ side, deletion of sequences between +156 and +41 increased β-galactosidase levels threefold (pCS143). Further deletion to the 3′ edge of IR3 (+19, pCS148) maintained high activity, but removal of the 3′ half of IR3 (to +15, pCS129) reduced activity to less than 10%, and complete deletion of

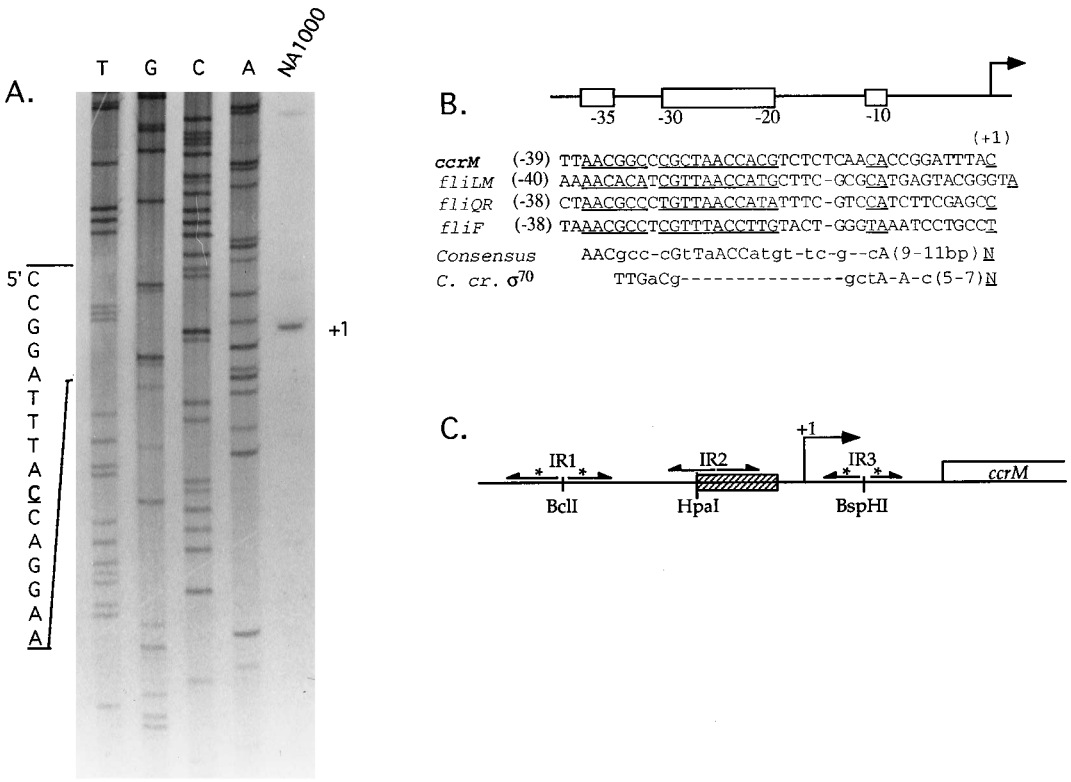


FIG. 2. Identification of the *ccrM* transcript start site. (A) Determination of the 5′ end of *ccrM* mRNA by primer extension. Reaction conditions and primer sequence are described in Materials and Methods. The first four lanes show a sequencing ladder generated from pCS150 by using primer ccrmpe; the fifth lane shows primer extension products generated by using 10 μg of NA1000 RNA as the template. (B) Comparison of the region upstream of the start site of P_{ccrM} with the class II flagellar operon promoters and the *Caulobacter* σ^{70} promoter consensus sequence (23). The class II consensus was derived from the four promoters shown at the top. Capital letters indicated conservation in all four promoters; lowercase letters indicate conservation in three of four. (C) A schematic of the intergenic region indicating the position of the apparent transcription start site relative to the sequence motifs described in the text. The hatched box represents the region of similarity to class II flagellar promoters, the inverted repeat motifs are represented by divergent arrows, and M.*Ccr* II methylation sites are marked by asterisks.

IR3 (to +7, pCS142 or pCS144) eliminated β-galactosidase activity. IR3 thus may be a positive regulatory motif necessary for P_{ccrM} activity, which is particularly intriguing in that it is downstream of the transcription initiation site. The increase in reporter activity upon deletion of sequences between +41 and +156 may be indicative of a negative regulatory element in this region; a similar effect on *lacZ* reporter activity was noted for deletions in this region of the *fliL* operon (39). It must be noted, however, that changes in β-galactosidase activity observed with the various 3′ deletions cannot be definitively attributed to changes in promoter activity, as each of these constructs alters the mRNA sequence and potentially affects its structure, stability, and translational efficiency.

The functional importance of many of the conserved bases in the class II flagellar promoters has been demonstrated by examining the effects of point mutations on promoter activity (*fliF* [41], *fliL* [39], and *fliQ* [46]). To determine whether the sequence similarity between the *ccrM* upstream region and class II promoters reflects shared functional properties, mutations were introduced into the *ccrM* sequence and assayed for effects on promoter activity (Fig. 3B). A 2-bp substitution at −36 and −35 reduced activity by 70%, while a 4-bp substitution in the central conserved region (−28 to −25) and a 3-bp substitution at −12 to −10 both reduced activity by greater than 90%. These results are consistent with the effects of mutations in the analogous regions of the *fliL*, *fliQ*, and *fliF* promoters and are consistent with the same factors recognizing P_{ccrM} and class II promoters. A 3-bp change at −47 to −50 had no effect on promoter activity. A 12-bp insertion at −38 reduced promoter activity by 75%, consistent with the reduced activity of the deletion to −37 (Fig. 3). This insertion interrupts the IR2 element but does not change the region conserved with class II flagellar promoters, implying that additional nucleotides in IR2 immediately upstream of the conserved sequence are relevant to promoter function.

To determine the minimal promoter sequence necessary for temporal regulation of *ccrM* expression, P_{ccrM}-*lacZ* transcriptional fusions were examined by immunoprecipitation of β-galactosidase from culture samples pulse-labelled during synchronous growth. Figure 4 compares the temporal expression of a transcriptional fusion integrated at the *ccrM* locus on the chromosome (strain LS178) with expression of two plasmids with P_{ccrM}-*lacZ* fusions, pCS105 (−92 to +233) and pCS148 (−45 to +19, the smallest fully active promoter fragment). In all cases P_{ccrM} is induced at 0.5 to 0.6 division units and peaks at 0.7 to 0.8 units. Thus, the information necessary for cell cycle regulation of P_{ccrM} is contained between −45 and +19. The only difference evident among these constructs is that the plasmid constructs show higher activity around the time of cell division. The significance of this is unclear; it could reflect mildly relaxed control of transcription initiation, a time lag in eliminating the overexpressed plasmid-encoded fusion mRNA, or increased stability of the fusion mRNA.

Function of M.*Ccr* II methylation sites in the *ccrM* leader region. The IR3 element immediately downstream of the *ccrM* transcription start site contains two M.*Ccr* II methylation sites,

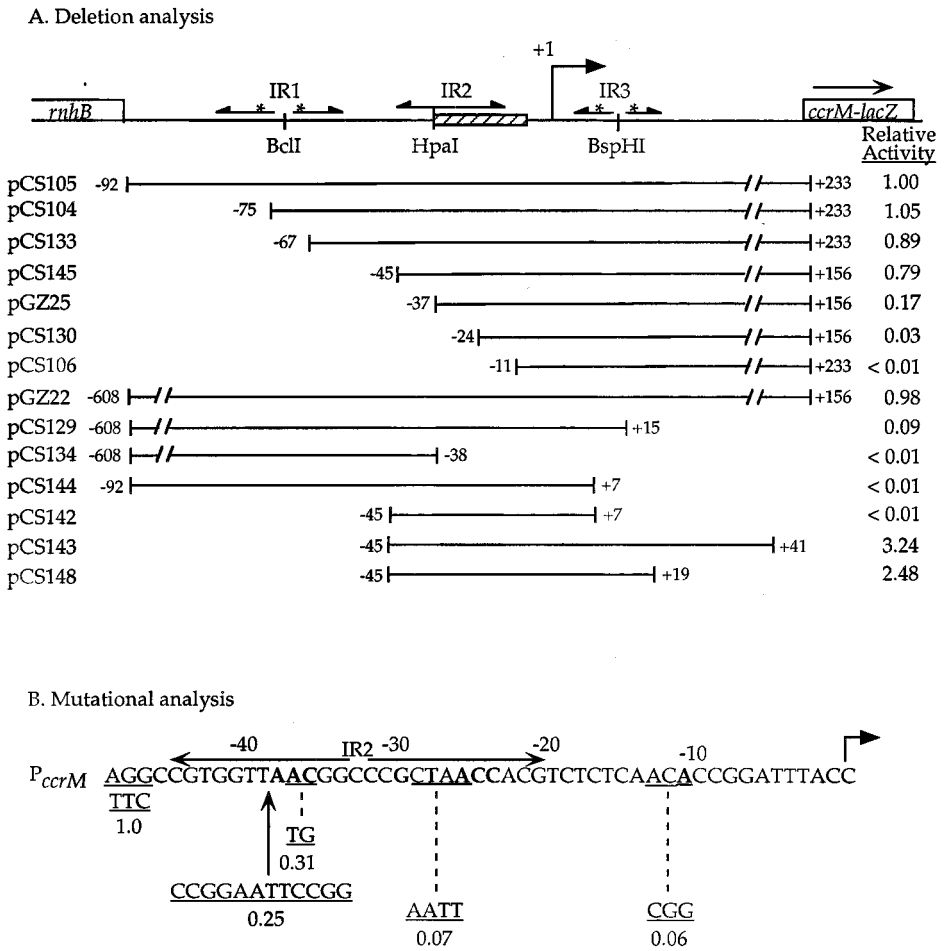


FIG. 3. Deletion and mutational analysis to define the functional *ccrM* promoter. (A) Deletion analysis. A schematic of the intergenic region between *rnhB* and *ccrM* is shown for orientation, with symbols as described in the legend to Fig. 2. The 5′ and 3′ endpoints of each deletion fragment are indicated, with numbering relative to the *ccrM* transcription start site. The fragments were cloned into pRKlac290 upstream of a promoterless *lacZ* reporter to generate the plasmids named at the left. β-Galactosidase assays (24) were done at 30°C, using mid-log-phase plasmid-bearing NA1000 cultures. Each construct was assayed at least twice, with a variation between assays of ≤10%. The activity of plasmid pCS105, defined here as 1.00, is 3,705 Miller units. The background activity due to the pRKlac290 vector (150 U) has been subtracted from each value. Activities reported as <0.01 were within 40 U of background and could not be accurately determined. (B) Effects of mutations in P_{ccrM} on promoter activity. Site-directed mutations were constructed on a 0.8-kb *Bam*HI-*Hin*dIII fragment, cloned as transcriptional fusions to *lacZ* in pRKlac290, and assayed for β-galactosidase activity. Nucleotides which are conserved in class II flagellar promoters (see Fig. 2B) are in boldface. Mutant bases are shown beneath the wild-type sequences being replaced. The relative β-galactosidase activity resulting from each of the mutant promoters is indicated beneath the mutant bases.

suggesting a possible role for methylation in autogenous regulation of promoter activity. Further impetus to investigate the role of IR3 came from the observation that at least two other class II flagellar promoters, P_{fliL} and P_{fliQ}, also contain M.*Ccr* II sites shortly downstream of the transcript start site (45, 46); the role of these sites, if any, has not been defined. Because null mutations in *ccrM* have not been isolated, it is not possible to examine promoter activity in the absence of methylation. As an alternative, mutations which eliminated one or both methylation sites were generated in IR3 and examined in the context of the minimal promoter fragment (−45 to +19). The effects of these mutations on the activity and temporal regulation of P_{ccrM} are shown in Fig. 5. Mutations in either arm of IR3 increased P_{ccrM} activity slightly, and elimination of both methylation sites (pCS164) had an additive effect (Fig. 5A). To ensure that these mutations were not elevating activity by generating a new promoter, the double IR3 mutation (+11T→C, +16A→G) was combined with mutations in the −35 or −10 region of P_{ccrM}. In both constructs, promoter activity was significantly reduced relative to that of pCS164, indicating that elevated activity of pCS164 still requires the functional domains of P_{ccrM}. Examination of the cell cycle expression pattern of the pCS164 mutant promoter lacking both methylation sites showed a normal time of induction in the predivisional cell but significantly increased expression in the swarmer cell.

To determine if constitutive methylation of GAnTC sites in the IR3 region affects the activity and timing of P_{ccrM}, promoter activity from pCS148 in strain LS1 was examined in synchronized cultures. LS1 is a strain in which M.*Ccr* II protein is constitutively expressed from a P_{lac} promoter, resulting in full methylation of chromosomal DNA throughout the cell cycle (47). In LS1/pCS148, P_{ccrM} activity in mixed cultures is reduced about 20%. P_{ccrM} is induced at the normal time in the cell cycle in synchronous LS1 cultures but is repressed more efficiently in swarmer cells (Fig. 5B). In summary, the effects of eliminating methylation of IR3 (by mutation) or methylating IR3 at all times (in LS1) indicate that the methylation state of IR3 is not crucial for induction of *ccrM* expression but appears to contribute to the efficient shutdown of P_{ccrM} in the progeny swarmer cell.

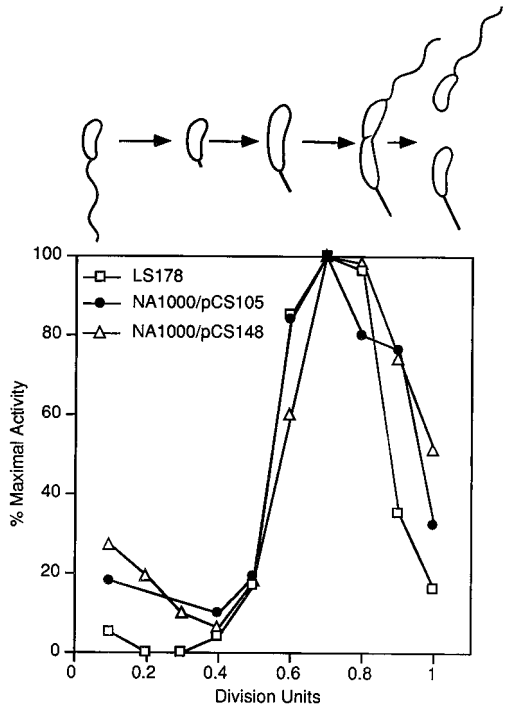


FIG. 4. The minimal *ccrM* promoter is cell cycle regulated. Swarmer cells were isolated as described in Materials and Methods and released into minimal M2G medium. The quality of the synchrony and duration of the cell cycle were monitored microscopically. Samples were labelled with [^{35}S]methionine at the indicated times. β-Galactosidase synthesis was assayed by immunoprecipitation and gel electrophoresis, followed by exposure of the dried SDS-polyacrylamide gels to a storage phosphor screen and quantitation with a Molecular Dynamics PhosphorImager. P_{ccrM}-*lacZ* activity from LS178 (a chromosomal *lacZ* fusion) is indicated by open squares, NA1000/pCS105 (−92 to +233) is shown by filled circles, and NA1000/pCS148 (−45 to +19) is shown by open triangles.

P_{ccrM} responds to a DNA replication block. Class II flagellar gene transcription is inhibited when chromosomal replication is blocked (12, 39). To determine whether P_{ccrM} responds to this DNA replication checkpoint as well, cultures containing plasmids with P_{ccrM}-*lacZ* fusions were treated with hydroxyurea, an inhibitor of deoxynucleotide synthesis. DNA synthesis is reduced by 60 to 80% within 15 min under the conditions used, but bulk transcription continues (11, 34), and expression of constitutive promoters such as *neo* (12) and *rsaA* (39) is unaffected. Under these conditions, P_{ccrM} activity declined by 80 to 90% within 1 h (Fig. 6). This rate of shutoff of P_{ccrM} is similar to that observed with P_{fliL} and other class II promoters and is slightly faster than the rate of shutoff of the genes encoding the flagellins, which reside lower (class IV) in the transcriptional hierarchy (Fig. 6).

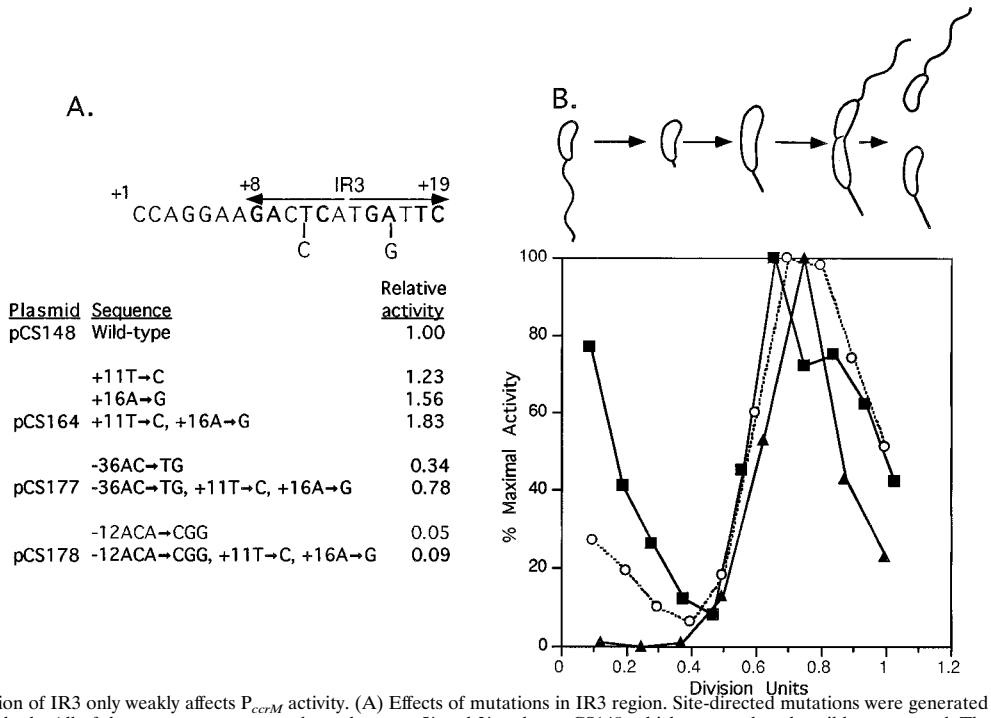
To further demonstrate a DNA replication checkpoint in the control of *ccrM* expression, a temperature-sensitive DNA replication mutant strain was used. Strain LS1435 contains a temperature-sensitive allele of the *dnaC* locus (*dnaC303* [31]) which results in cessation of DNA chain elongation at 37°C. (Note that the *Caulobacter dnaC* locus has not been sequenced [30] and is not necessarily a homolog of the enteric *dnaC* gene.) Shifting LS1435 cultures from the permissive temperature (28°C) to the restrictive temperature (37°C) resulted in rapid inhibition of both P_{ccrM} and flagellin expression (Fig. 6), demonstrating that functionally diverse *Caulobacter* genes respond to a DNA replication checkpoint.

DISCUSSION

Regulation of P_{ccrM} and class II flagellar promoters. The activation of the *ccrM* promoter is a critical event controlling the appearance and activity of the M.*Ccr* II DNA MTase in the predivisional stage of the *Caulobacter* cell cycle. Analysis of the *ccrM* promoter region revealed striking similarities between P_{ccrM} and class II flagellar promoters (12, 39, 41), and *ccrM* and class II flagellar promoters respond similarly to a DNA replication checkpoint. A sequence upstream of the *ccrM* transcript start site strongly resembles a family of class II promoters which includes those of the *fliLM*, *fliQR*, and *fliF* operons (39). The deleterious effects of mutations in P_{ccrM} are consistent with the effects of mutations in the *fliLM* and *fliQR* promoters, as substitutions at −35 and −10, as well as between −24 and −28, severely reduced activity (39, 46). The functional importance of the conserved region between −20 and −30 is particularly diagnostic of *Caulobacter* class II promoters; its importance in P_{ccrM} is significant evidence linking it to the flagellar promoters. The *ccrM* promoter sequence, however, differs from the other class II promoters in that the most highly conserved part of the promoter, between −35 and −20, is part of a larger inverted repeat element, IR2. (There is no noticeable conservation of sequence upstream of −38 in these promoters.) Whether the entire IR2 sequence constitutes a functional element, such as a transcription factor binding site, remains unclear; the deleterious effects of a deletion and an insertion in the portion of IR2 5′ to the conserved region support some role for this sequence.

Class II genes are the first flagellar genes expressed, beginning midway through the cell cycle and peaking at roughly 0.6 division units. P_{ccrM} activity, however, rises and peaks slightly later in the cell cycle (by 0.1 to 0.2 units) than other class II promoters, and class II flagellar promoters are also subject to intraclass regulation not observed with the *ccrM* promoter. Mutations in class II genes result in elevated class II gene expression (usually by 1.5- to 3-fold) (27, 44), whereas P_{ccrM} activity does not increase, and in fact M.*Ccr* II protein levels are slightly reduced (38a). Inactivation of class II promoters under normal conditions may be linked to completion of an early stage of flagellar assembly. The observation that P_{ccrM} does not respond in the same fashion as other class II promoters to failed flagellar assembly may reflect the necessity of unlinking DNA methylation from motility. These differences between P_{ccrM} and class II flagellar promoters (timing and response to flagellar assembly) suggest that these promoters are subject to separate as well as common regulatory mechanisms. This situation is analogous to the differences between *Caulobacter* class III flagellar operon promoters and the class IV *fljK* (27-kDa flagellin) promoter. The *fljK* promoter appears to have the same functional elements (binding sites for $E\sigma^{54}$ polymerase, integration host factor, and the FlbD transcriptional activator) as most class III operons. Nevertheless P_{fljK} remains active in class II flagellar mutants, with the exception of *rpoN* and *flbD* strains, indicating that its activity is independent of the early flagellar assembly cues controlling class III promoters (43). (Note that failure of flagellar assembly in class II or class III still blocks expression of the 27-kDa flagellin through translational regulation [17].)

The IR1 and IR3 inverted repeats, each of which contain M.*Ccr* II methylation sites, were possible candidates for roles in autogenous regulation of P_{ccrM} activity. GAnTC sites should occur on average once in 256 bp, but in this case four sites occur in the 100 bp surrounding P_{ccrM}. We were encouraged to investigate the role of IR3 methylation in regulation of P_{ccrM} by recent results with the *E. coli papB* promoter, in which



Plasmid	Sequence	Relative activity
pCS148	Wild-type	1.00
	+11T→C	1.23
	+16A→G	1.56
pCS164	+11T→C, +16A→G	1.83
	-36AC→TG	0.34
pCS177	-36AC→TG, +11T→C, +16A→G	0.78
	-12ACA→CGG	0.05
pCS178	-12ACA→CGG, +11T→C, +16A→G	0.09

FIG. 5. Methylation of IR3 only weakly affects P_{ccrM} activity. (A) Effects of mutations in IR3 region. Site-directed mutations were generated by PCR as described in Materials and Methods. All of the promoter constructs have the same 5′ and 3′ ends as pCS148, which was used as the wild-type control. The sequence of the IR3 region is indicated above the table for reference. The wild-type activity (pCS148), defined as 1.00, was 9,780 Miller units. (B) Expression of P_{ccrM} during the cell cycle under various methylation conditions. P_{ccrM}-*lacZ* fusions were used to monitor timing as described in the legend to Fig. 4. ○, = NA1000 (wild-type host)/pCS148 (wild-type P_{ccrM}); ▲, LS1 (constitutive methylation)/pCS148; ■, NA1000/pCS164 (both methylation sites in IR3 mutated).

alternative methylation of two Dam (GATC) sites acts as an on/off switch (6). We also observed that deletion of IR3 eliminated P_{ccrM} activity, though IR1 was not necessary for activity. (It should be noted that for deletions of the region 3′ to the start site, we have not established a direct correlation between β-galactosidase activity and transcription initiation; these constructions alter the sequence of the *ccrM-lacZ* RNA and may affect β-galactosidase levels by altering the stability or translational efficiency of the mRNA.) However, mutational inactivation of one or both of the methylation sites in IR3 actually somewhat increased P_{ccrM} activity, implying that methylation might aid in repression of P_{ccrM}. Examination of the cell cycle expression of a mutant promoter lacking both IR3 methylation sites revealed that it is not shut off in the swarmer cell as tightly as wild-type P_{ccrM}. Thus, methylation appears to influence the temporally controlled inactivation of *ccrM* transcription. This is consistent with the observation that there is tighter shutdown of *ccrM* transcription in swarmer cells in a strain in which IR3 is always fully methylated, though it cannot be concluded that this effect is solely due to methylation at IR3, as constitutive methylation occurs throughout the chromosome and may affect *ccrM* transcription indirectly. Further dissection of P_{ccrM}, and ultimately development of an in vitro transcription system, is necessary to clearly define the role of IR3 in promoter function.

A DNA replication checkpoint. *Caulobacter* flagellar gene expression is rapidly turned off in response to a block in chromosomal DNA replication (12, 33, 39). We have shown here that P_{ccrM} expression is similarly inhibited when DNA synthesis is blocked by hydroxyurea or in a temperature-sensitive DNA synthesis mutant, demonstrating parallel regulation of P_{ccrM} and class II flagellar promoters. The completion of DNA synthesis and repair of damaged DNA are frequently and perhaps universally used as checkpoints controlling progression to the later stage(s) of the cell cycle (26). In the fission yeast *Schizosaccharomyces pombe*, for example, passage from S phase to the G_2/M stages of the cell cycle is controlled by maturation-promoting factor (MPF). Unreplicated DNA or damaged DNA blocks the activation of MPF through pathways affecting

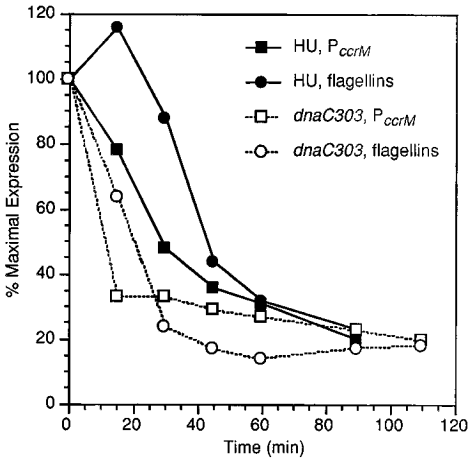


FIG. 6. P_{ccrM} is repressed by inhibition of DNA synthesis. NA1000/pCS140 and LS1435 (*dnaC303*)/pCS140 cultures were grown to mid-log phase (optical density at 600 nm of 0.3 to 0.4) in M2G medium. At time zero, 1-ml samples of each culture were labelled for 5 min with 3 μCi of [^{35}S]methionine. Hydroxyurea (HU; to 3 mg/ml) was then added to the NA1000 culture (closed symbols). The LS1345 (*dnaC303*) culture was shifted to 37°C (open symbols). Labelling was repeated at the indicated intervals. β-Galactosidase and flagellins were immunoprecipitated and quantitated as described in the legend to Fig. 4.

the activity of Cdc2, a component of MPF (1, 14, 42). In *E. coli*, DNA damage is sensed by the SOS system, which when induced prevents cell division by inhibiting the activity of the essential septation factor FtsZ (3, 19). In *Bacillus subtilis*, expression of genes involved in sporulation requires both undamaged DNA (20) and the initiation and completion of a round of chromosomal replication after sporulation has been induced (21). It has now been demonstrated that the expression of numerous *Caulobacter* genes normally activated during the S phase of the cell cycle is sensitive to inhibition of DNA replication. It would not be surprising to find that expression of *Caulobacter* genes involved directly in cell division is subject to this checkpoint as well, as pinching, the earliest visible indicator of imminent cell division, does not occur when DNA synthesis is blocked (31). Identifying the factors interacting with P_{ccrM} and class II flagellar promoters will be an important step in understanding the interactions between chromosomal replication, cell cycle progression, and gene expression.

ACKNOWLEDGMENTS

We thank the members of the Shapiro laboratory for helpful discussions and critical reading of the manuscript, and we thank Greg Marczynski for construction of the LS1435 strain. We thank Ann Reisenauer in particular for technical and editorial assistance.

This work was supported by National Institutes of Health grants GM32506 (to L.S.) and GM14179 (to C.M.S.).

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