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Regulation of Cellular Differentiation in *Caulobacter crescentus*

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INTRODUCTION

Many bacterial species are capable of undergoing a diverse repertoire of complex physiological and developmental changes both in culture and in their natural environments. In recent years, it has been recognized that bacteria can serve as useful experimental systems for understanding the mechanisms underlying basic developmental processes. For example, asymmetric cell divisions are a fundamental aspect of development in diverse organisms, including *Drosophila* species, nematodes, and fungi (reviewed in reference 48). Recent experiments with *Bacillus subtilis* and *Caulobacter crescentus*, two bacterial species that undergo asymmetric cell divisions, have yielded insights into the molecular mechanisms that lead to the establishment of different cell types upon division.

In this review, we focus on the mechanisms that generate asymmetry in the *C. crescentus* predivisional cell, with special emphasis on DNA replication and flagellar biogenesis. Cell division in *C. crescentus* generates two different daughter cells, a motile swarmer cell which possesses a single polar flagellum, and a nonmotile stalk-bearing cell (Fig. 1). *C. crescentus* is a particularly facile experimental organism for the examination of both cell cycle and cellular differentiation events. Pure populations of either swarmer or stalked cells can be easily prepared by density gradient centrifugation. Isolated swarmer cells, when incubated in fresh growth medium, proceed synchronously through the cell division cycle. The progression through the cell division cycle can be easily monitored by light microscopy, and mutant strains can be isolated through either chemical, UV, or transposon mutagenesis. Mutations can be genetically mapped by bacteriophage transduction (21) or physically mapped by pulsed-field gel electrophoresis (22, 23; reviewed in reference 20). The latter technique has benefitted from an alignment of the *Caulobacter* physical and genetic chromosomal maps (22–24).

The progeny swarmer and stalked cell types differ not only in their morphology and motility but also in their programs of gene expression and the capacity to reinitiate DNA replication. Stalked-cell progeny reinitiate chromosome replication almost immediately following cell division (14, 78). The stalked cell functions essentially as a stem cell with an unlimited capacity to divide and generate new swarmer cells. DNA replication in swarmer cells is silenced for a defined period (14, 78). During this time, the swarmer cells are motile and metabolically active.

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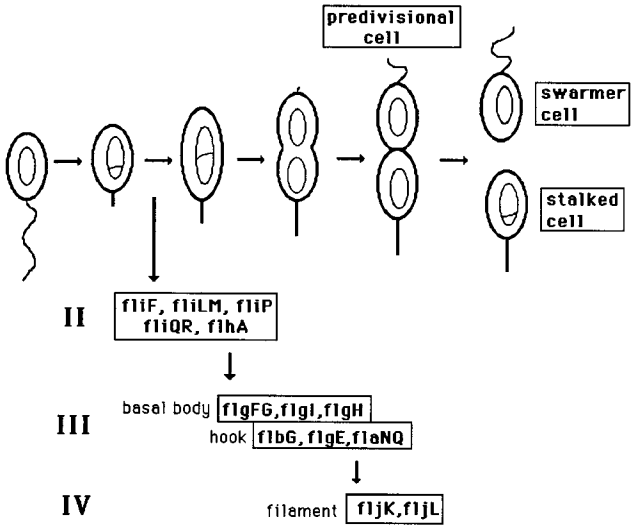

FIG. 1. *C. crescentus* cell division cycle and temporal synthesis of the polar flagellum. The swarmer cell at the far left possesses a single polar flagellum and is unable to initiate chromosomal DNA replication. The chromosome is represented as an oval inside the cell. Following a defined period, a series of global differentiation events occurs in the swarmer cell. The flagellum is shed, a polar stalk is synthesized in its place, and DNA replication initiates. The replicating chromosome is represented by the "theta" structure inside the cells. During cell growth, a flagellum is synthesized and assembled at the pole opposite the stalk. Cell division results in the formation of two distinct cell types, a motile swarmer cell and a nonmotile, replication-competent stalked cell. The temporal pattern of flagellar gene expression is depicted below the cell cycle diagram. (Note that a small fraction of the genes required for flagellar biogenesis is shown.) Shortly after initiation of DNA replication, the flagellar genes begin to be expressed to a defined temporal order. The cellular cue that initiates the expression of early flagellar genes (class II) is not known but is likely to be integrated with the cellular events that trigger chromosomal DNA replication. The expression of early class II genes is required for the expression of class III genes. class III genes encode basal-body structures and the external hook. The expression and successful assembly of these structures are in turn required for the expression of genes which encode flagellins (class IV). See the text for details.

Eventually, in response to an unknown internal cue, the flagellum is shed, a stalk is synthesized in its place, and DNA replication initiates (Fig. 1). As DNA replication proceeds, the stalked cell grows and the flagellar genes begin to be expressed. The flagellar components are specifically targeted to the pole of the cell that is opposite the stalk. Other proteins that are also directed to the incipient swarmer cell pole include the methyl-accepting chemotaxis receptors and pili. Cell division completes the cycle, generating the two distinct cell types. The asymmetric positioning of proteins and compartment-specific gene expression in the predivisional cell are the two major mechanisms that dictate the developmental fate of the progeny cells. In this review we will summarize how each of these mechanisms contributes to the biogenesis of polar structures and the asymmetric programming of chromosomal DNA replication.

DEVELOPMENTAL REGULATION OF CHROMOSOME STRUCTURE AND DNA REPLICATION

One of the most interesting aspects of cellular differentiation in *C. crescentus* is the developmental regulation of chromosomal DNA replication. How can chromosomes, which initially resided in the same cellular compartment, inherit different replication capacities? When is this asymmetry in DNA replication initiation established? Several models have been proposed, including a differential distribution of replication proteins in the swarmer cell and stalked-cell compartments and differences in chromatin structure. Although this problem is still far from being solved, significant experimental progress has occurred in recent years. Notably, the *Caulobacter* chromosomal origin of replication has been isolated and several key genes involved in DNA replication have been identified.

Caulobacter Origin of Replication

The *Caulobacter* chromosomal origin of replication has been cloned and sequenced as an initial step in dissecting the molecular events that regulate DNA replication (80). The region of the *Caulobacter* chromosome that contains the replication origin was initially mapped by pulse-labeling of DNA in synchronized populations followed by conventional (73) or pulsed-field gel electrophoresis (17). The chromosomal origin of replication (Cori) was cloned from a cosmid library prepared from isolated DNA from this earliest labeling region of the chromosome (80). The isolated origin could confer replicative ability to a plasmid which could not normally replicate its DNA in *C. crescentus*. DNA replication of an episome containing the Cori was under similar cell cycle control to the natural origin of replication (80). This experiment established that the *cis*-regulatory elements responsible for temporal DNA replication occur in or near the Cori. The Cori contains sequence elements that are similar to those found in the origins of replication of other bacteria (80). For example, the Cori contains five DnaA boxes, the binding site for the replication initiation protein, DnaA, as well as an *Escherichia coli*-like 13-mer sequence (80; reviewed in reference 98). The conserved DnaA boxes are likely to play a role in replication, because point mutations in one of the boxes prevented a Cori-containing episome from replicating autonomously (80). The Cori also contains an AT-rich DNA sequence, as well as several repeated sequence elements that are not present in the origins of replication of other bacteria. These include seven 8-mer sequences (GGCCTTCC) and nine 8-mer (AAGC CCGG) and five 9-mer ($GTTAAn_7TTAA$) elements. Deletions that remove some of these sequences abolish DNA replication, indicating that these elements may be the binding sites for essential DNA replication proteins (80). Significantly, these critical sequences do not occur in the origins from other bacteria and therefore may be important in regulating the unique temporal pattern of *Caulobacter* DNA replication.

Control of Plasmid Replication and Partitioning

Autonomously replicating plasmids which possess their own origins of replication have also been used to examine the control of DNA replication in *C. crescentus* (78). Plasmids of two different incompatibility types, IncQ and IncP1, were compared. IncQ plasmids encode many of their own replication initiation proteins, including RepA, RepB, and RepC. The Rep proteins have been demonstrated to effectively bypass the requirement for *E. coli* DnaA, DnaB, DnaC, and DnaG (132), factors which are instrumental in controlling the initiation of chromosomal DNA replication (62). In contrast, IncP1 plasmids encode only one replication protein, the product of the *trfA* gene. IncP1 DNA replication is absolutely dependent on host initiation proteins, including DnaA (31). Although these plasmids require different host functions for initiation of DNA replication, their temporal patterns of replication are similar in *C. crescentus* (78). These plasmids replicate in both cell types, including the swarmer cell, where chromosomal DNA replication is silenced, demonstrating that the *trans*-acting functions required for DNA replication are present during all stages of the *Caulobacter* cell cycle. The rate of replication increases

about 10- to 20-fold when the swarmer cells differentiate into stalked cells. The increase in replication rate of the plasmid DNA parallels the timing of initiation of chromosomal DNA replication (78). The increase in DNA replication rate at the swarmer-to-stalked-cell transition, however, indicates that some critical component(s) for DNA replication is in limiting supply in the swarmer cell. Alternatively, the lower rate of plasmid DNA replication in swarmer cells may indicate that replication is repressed or silenced to some extent in the swarmer cell. Plasmids may be able to undergo limited DNA replication in the swarmer cell because their replication origins are more resistant to repression than the chromosomal replication origin.

One possible explanation for the differential programming of the swarmer and stalked chromosomes is that the newly formed chromosomes are "marked" in some way so they may be distinguished by the chromosomal DNA partitioning machinery. In this model, the DNA destined for the swarmer cell is intrinsically different from the DNA destined for the stalked cell, similar to fission yeast cell division, in which there is preferential inheritance of DNA molecules (60). Therefore, the swarmer and stalked cells would preferentially inherit one chromosome over the other at every cell division. To examine if this was occurring, both plasmid and chromosomal DNA-partitioning experiments have been performed (78, 107). Both experiments demonstrated that there was no bias in the inheritance of DNA. Chromosomal and plasmid DNA is partitioned into swarmer and stalked cells by a random process. This suggests that the differential behavior and regulation of chromosomal DNA in the swarmer and stalked cells is determined by the local environment in the swarmer and stalked poles of the predivisional cell and not by intrinsic differences in the properties of the chromosomal DNA.

DNA Replication Proteins

Genetic analysis has been used to identify *Caulobacter* genes involved in cell division and DNA replication. Temperature-sensitive conditional mutants that are affected in either DNA replication initiation or DNA elongation have been identified (109). Use of these conditional mutants in temperature shift experiments has generated valuable information about the ordering of differentiation events within the *Caulobacter* cell cycle. It has been shown that the biogenesis of polar structures such as the flagellum and pili is dependent on DNA replication and that initiation of a new round of replication is dependent on the completion of cell division (51, 93, 109–111). This is in contrast to *E. coli*, which possesses the capacity to initiate a new round of DNA replication before cell division is completed.

dnaC, a gene required for DNA chain elongation, was identified in this mutant screen and has been characterized in some detail (103). (Note that the designation *dnaC* does not constitute identity to the *E. coli dnaC* gene, which is required for initiation of DNA replication.) Cell cycle expression studies, involving either mRNA S1 nuclease protection assays or fusion to a *lacZ* reporter gene, showed that the synthesis of *dnaC* mRNA was under temporal control. Expression is lowest at periods in the cell cycle when DNA replication is not occurring: in swarmer cells and in predivisional cells just before cell division (103). The *Caulobacter* homolog of the *E. coli dnaA* gene has also been recently cloned and sequenced (165). The protein encoded by *dnaA* is the critical factor in triggering the initiation of DNA replication in *E. coli* (reviewed in reference 62). It has been demonstrated that in *C. crescentus*, a *dnaA* box in the origin of replication is essential for replication initiation, implying that the *C. crescentus* DnaA protein may have an essential role in replication initiation. Transcription experiments have shown that *dnaA* in *C. crescentus* is also under temporal control, with lower expression in swarmer cells and a peak of expression in replicating stalked cells (165). Therefore, the temporal control of DNA replication may be attributable, in part, to the periodic synthesis of essential DNA replication proteins.

Several homologs of other *E. coli* replication genes have been identified in *C. crescentus*. In addition to *dnaA*, these include *dnaK*, *dnaJ*, *dnaN*, and *gyrB*. Interestingly, all of these genes map to chromosomal locations near the origin of replication (40, 122, 165). *dnaA*, *dnaK*, and *dnaJ* map within 40 kbp of the origin (40, 165). The *gyrB* and *dnaN* homologs are located approximately 150 kbp from the origin in what appears to be another cluster of genes involved in DNA replication (122). The *dnaK* and *dnaJ* homologs were cloned by virtue of homology to the *E. coli dnaK* and *Drosophila* Hsp70 homologs (40). These genes encode well-characterized heat shock proteins whose function is to act as molecular chaperones in a diverse array of cellular processes. In *C. crescentus*, the *dnaKJ* genes are organized as an operon. Transcription initiates from two distinct promoters: a heat-shock promoter and a σ^{70} promoter (40). Transcription from the σ^{70} promoter is under temporal control and peaks in newly differentiated stalked cells, indicating that the DnaK and DnaJ levels may increase at the time when DNA replication initiates. Interestingly, the *dnaK* gene product and another heat shock protein, Lon protease, are preferentially targeted to the stalked pole of the predivisional cell (121), suggesting a possible role in DNA replication initiation.

The *gyrB* gene, which encodes the DNA gyrase B subunit, has been cloned from *C. crescentus* by complementation of a temperature-sensitive *gyrB* mutation (122). Sequence analysis of the region of the genome adjacent to *gyrB* revealed the *Caulobacter* homologs of *recF* and *dnaN*, as well as another potential open reading frame (*orf1*) (122). The *dnaN* gene encodes the β subunit of DNA polymerase III holoenzyme. The organization of this region of the genome differs from other bacteria, in which the *gyrB* gene often is located adjacent to the *dnaA* gene (reviewed in reference 98). In *C. crescentus*, the *dnaA* gene maps directly adjacent to the origin, which resides approximately 150 kb away (165).

Transcription fusions to a promoterless *lacZ* reporter gene have shown that *orf1*, *recF*, and *gyrB* each possess their own promoter (122). Cell cycle experiments demonstrated that two of these promoters, *orf1* and *gyrB*, were transcribed under temporal control and exhibited an identical pattern of expression. Transcriptional activity was absent in the swarmer cell and began when the swarmer cells differentiated into stalked cells. The absence of expression in swarmer cells is common to many *Caulobacter* promoters, including *dnaC* (103), one of the *dnaKJ* promoters (40), *dnaA* (165), *hemE* (79), and *rpoN* (8) (see below and subsequent sections), and may represent a common global silencing mechanism in which transcription for a subset of promoters is silenced or repressed in swarmer cells and then activated when stalked cell differentiation occurs. In an effort to determine when *gyrB* and *orf1* transcription was silenced, *lacZ* reporter fusions were assayed for compartmentalized transcription in predivisional cells (122). (See the section below on polar transcription of flagellar genes for a detailed explanation of the methods used in this type of experiment.) This experiment demonstrated that *gyrB* and *orf1* transcription was specifically silenced in the swarmer pole of the predivisional cell (122). Therefore, in this case the differ-

ential programming of swarmer and stalked cell gene expression is established in the predivisional cell.

hemE Promoter Activity and DNA Replication

Adjacent to the minimal origin of replication is the promoter for the *Caulobacter* homolog of the *hemE* gene, which encodes uroporphyrinogen decarboxylase (79). Transcriptional activity near the replication origins of both bacteriophages and *E. coli* has been shown to stimulate DNA replication initiation (62). The *Caulobacter hemE* promoter has been examined for its role in influencing the initiation of DNA replication. Two σ^{70}-type promoters, with differing transcriptional activities, have been mapped upstream of *hemE* (79). Within each of these promoter regions are conserved Cori sequence elements, including several 8-mer elements (AAGCCCGG) close to the weaker of the two promoters and two GTTAAn$_7$TTAA elements near the stronger upstream promoter. Mutations in this region that abolish transcription also prevent DNA replication, perhaps suggesting that *hemE* promoter activity may have a role in controlling replication initiation (79). The weaker promoter is expressed continuously throughout the cell cycle, whereas the stronger promoter is not expressed in swarmer cells, exhibiting the same pattern of expression as the *gyrB* and *orf1* promoters. The strong *hemE* promoter is also selectively transcribed in the stalked pole compartment of the predivisional cell (79). It is hypothesized that stalked pole-specific transcription of the strong *hemE* promoter may be one regulatory influence in the differential programming of DNA replication in the predivisional cell (79).

Generation of Asymmetry in Chromosome Structure

Several experiments have suggested that the chromosomes of the swarmer and stalked cell differ in their physical properties (25, 26, 147). Nucleoids (chromosomes plus any associated protein and cell membrane) were isolated from synchronized populations at different times during the cell division cycle and subjected to centrifugation in a sucrose gradient (25, 26, 147). Nucleoids isolated from swarmer cells had an apparent sedimentation coefficient of approximately 6,000S. When the swarmer cells differentiated into stalked cells, the nucleoid sedimentation coefficient decreased to about 3,000S. After chromosome replication and partitioning, the predivisional cell contained one nucleoid with a high sedimentation coefficient, destined for the progeny swarmer cell, and one with a lower sedimentation coefficient, which was inherited by the stalked cell. These experiments indicate that higher-order chromosome structure differs with cell type and that the differences in higher-order structure are generated in the predivisional cell (25, 26, 147). The molecular mechanisms that contribute to the high chromosomal sedimentation coefficient in the swarmer cell are not known. It is possible that the swarmer cell chromosome has a higher degree of superhelical density, resulting in a more extensively folded chromosome, which would sediment more rapidly in a sucrose gradient. Alternatively, the swarmer cell chromosome may be associated with a different complement of histone-like proteins from those associated with the stalked-cell chromosome. It is tempting to speculate that the differences in chromosome higher-order structure contribute to regulation of the different programs of gene expression and DNA replication in the swarmer and stalked cells. In this model, the more compact, faster-sedimenting nucleoid of the swarmer cell would be analogous to eukaryotic heterochromatin in which gene expression is silenced by the higher-order packaging of DNA. It has been suggested (122) that a potential candidate for both compacting DNA and silencing swarmer gene expression is the neutral histone-like protein H-NS. H-NS is a major component of bacterial chromatin and has been shown to compact DNA (140). In several organisms, mutations in the gene that encodes H-NS result in inappropriate expression of specific genes, suggesting that H-NS plays a role mediating negative regulation (42, 49). A differential distribution of a protein such as H-NS between swarmer cell and stalked-cell chromosomes could at least in part be responsible for the differences in chromosome sedimentation coefficient and gene expression in the newly divided daughter cells.

Control of DNA Methylation and the Timing of Replication

Recently, it has been shown that *C. crescentus* possesses a DNA methyltransferase, CcrM (164). This methyltransferase catalyzes the methylation of the adenine in the sequence GANTC. The *ccrM* gene has been cloned and sequenced (165). Sequence analysis demonstrated that the *ccrM* gene was most closely related (49% identity) to *Hin*fM, a DNA methyltransferase from *Haemophilus influenzae*. Expression of the *ccrM* gene is under temporal control; maximal expression occurs in the late predivisional cell, just prior to cell division (143, 164). Interestingly, the *ccrM* promoter possesses significant homology to the temporally transcribed promoters of early flagellar genes (class II genes) (see the section below on cell cycle regulation of class II genes), suggesting that the expression of the methyltransferase and genes encoding flagellar structures is regulated by common elements (143).

The pattern of DNA methylation was assayed during the cell cycle (164). The DNA in *C. crescentus* remains hemimethylated for a defined period following replication and becomes fully methylated later in the cell cycle. This is similar to events that occur in during the *E. coli* cell cycle, although the timing of remethylation differs in *C. crescentus*, where remethylation of DNA parallels the peak of *ccrM* expression. To test whether temporal DNA methylation could regulate cell cycle events, *ccrM* was overexpressed constitutively during the cell cycle (164). Overexpression of *ccrM* decreased the proportion of hemimethylated DNA, caused abnormal cell division, and resulted in some cells undergoing an additional DNA replication initiation event as analyzed by fluorescence-activated cell sorter analysis (164). This result suggests that periodic DNA methylation catalyzed by the *ccrM* gene product influences the timing of DNA replication initiation.

BIOGENESIS OF POLAR STRUCTURES

Pilus Biosynthesis and Assembly

Pili are one of several polar structures that are developmentally expressed in *C. crescentus. Caulobacter* pili are filaments about 4.0 nm in diameter and 1 to 4 μm long that are found at the flagellated pole of the swarmer cell. The filaments are composed of pilin, an 8.0-kDa protein that is rich in hydrophobic amino acids (68, 136). The *Caulobacter* pilin sequence has no homology to pilin sequences from other bacteria (136). Pilin monomers are synthesized under cell cycle control in the early stalked and predivisional cell (135). The pilus structures are assembled in the swarmer cell pole shortly before cell division, suggesting that pilus subunits are stored within the cell until they are recruited for assembly (134). This accumulation of pilin subunits contrasts with the flagellum assembly pathway, in which the subunits are assembled almost immediately following synthesis (11, 12, 16, 46, 69, 72, 83, 84, 101). Immunogold electron microscopy analysis of the subcellular distribution of pilin in a synchronous population of *C. crescen-*

tus showed that unassembled pilin is accumulated in the cytoplasm. The cytoplasmic pool decreases in swarmer cells after cell division (134), and the pili are delivered to the cell surface (137). During the swarmer-cell-to-stalked-cell transition, the pili are retracted from the cell surface, at approximately the same time the flagellum is shed into the environment (68).

Stalk Biogenesis

The polar stalk, which ranges from 0.5 to 3.0 μm in length, is an extension of the cell envelope, containing the outer membrane, inner membrane, and peptidoglycan layer (44, 115). The central region of the stalk rod is apparently continuous with the cell cytoplasm but does not contain ribosomes or DNA (115). The stalk structure is divided by crossbands, which are composed partly of murein arranged in concentric layers that form continuous rings spaced throughout the length of the stalk and are thought to function in supporting the membrane (57, 127). Stalk biosynthesis is initiated at a specific time during the cell division cycle, immediately after the swarmer cell releases the flagellum. The stalk structure is synthesized at the site previously occupied by the flagellum, and elongation continues throughout the cell cycle (141).

Caulobacter stalked cells often attach to each other by the end of their stalks, in a typical "rosette" conformation. The ends of the stalks possess an adhesive structure, the holdfast, that enables the cells to attach to surfaces in the environment (82, 113). The holdfast is present at the base of the flagellum of the swarmer cells, and after the differentiation to stalk cells, it is anchored at the end of the growing stalk at a specific membrane site (105). This adhesive substance is most probably composed of complex polysaccharides (105). Several transposon insertion mutations affecting the production and assembly of the holdfast have been shown to map in four genetic clusters (86). One such cluster involved in the attachment of the holdfast to the cell has been characterized in detail (63). This cluster is composed of the *hfaAB* operon and the *hfaC* gene. The *hfaAB* operon possesses a sequence similar to the consensus for σ^{54} promoters. Sequence comparison suggests that the HfaA protein could encode an adhesion molecule, while HfaB has some amino acid sequence similarity to transcriptional activators of other bacteria (63). While no role has been assigned for HfaC, its maximal expression is dependent on the *hfaAB* gene products (63).

COORDINATION OF CELL DIVISION AND POLAR MORPHOGENESIS

Stalk Biogenesis and Cell Division

Mutants with several different defects in the stalk formation have been isolated (28, 30, 113, 125, 126). Some of these mutants exhibited core-deficient stalks (Cds mutants), others had their stalks released from the cell (Abs mutants), and a third mutant class (Ecs) misplaced the constriction site of cell division, releasing the stalk together with cytoplasmic material (113). A pleiotropic mutation, *pleC*, which results in stalkless cells, a lack of pili and phage receptors, and inactive flagella, has also been described (28) (see the section below for a detailed description of these mutants).

There is genetic evidence that the stalk biosynthesis and cell division are related events. Mutant cells that possess long stalks in complete nutrient medium block stalk synthesis when cell division is inhibited (44). Stalk biogenesis was also inhibited when cell division was blocked by treating cells with low concentrations of penicillin G (149). Stalk biogenesis does not depend on the initiation of DNA replication, as temperature-sensitive DNA elongation mutants develop normal stalks at restrictive temperatures (77, 108).

rpoN and Polar Morphogenesis

rpoN, the gene encoding the σ^{54} subunit of RNA polymerase, was cloned and sequenced because of its pivotal role in the expression *Caulobacter* flagellar genes (8) (see below). *rpoN* null mutations (Tn*5* insertions) have been isolated, indicating that σ^{54} is not essential for cell viability. *rpoN* mutants do not express late flagellar genes and also have a defect in cell division (8). Electron microscopy of these mutants revealed that a significant proportion of the cells were filamentous, with alterations in the position and number of cell division constriction sites, suggesting a defect in the coordination of cell division events. This may indicate that one or several σ^{54}-dependent genes may have a role in regulating the timing of cell division events. Alternatively, the cell division defect seen in *rpoN* mutant strains may be due to the absence of flagellar gene expression. Cell division defects are also observed in almost all strains with mutations in early (class II) flagellar genes (see the section below for more detail). *rpoN* mutants also have shortened stalks under certain growth conditions (8). In wild-type cells, the stalk length increases in response to low phosphate levels (114). *rpoN* mutants cells can also increase stalk length under phosphate limitation, indicating that σ^{54} is not essential for initiation of stalk biosynthesis but is required for growth of the stalk under nutrient-rich conditions (8).

Transcription of the *rpoN* gene is under cell cycle control. The pattern of expression is similar to that observed for many genes involved in DNA replication (see above). There is little expression in swarmer cells, with transcription beginning after differentiation into a stalked cell (8). Transcription increases as the cell cycle progresses, peaking just before cell division. The temporal expression of *rpoN* may have a role in regulating σ^{54}-dependent genes. It is not known, however, whether the timed transcription of *rpoN* actually results in a change in σ^{54} levels during the cell cycle.

Protein Kinases and Polar Morphogenesis

Receptors for bacteriophage ϕCbK are also targeted to the swarmer pole of the *Caulobacter* cell. Mutants resistant to ϕCbK have been isolated (*ple* mutants) and have been shown to be pleiotropically affected in polar morphogenesis (21, 27, 28, 30, 138). By using temperature-sensitive mutants, it has been demonstrated that these genes define two morphogenetic pathways that operate during the *Caulobacter* cell division cycle (138). The *pleA* gene is required for flagellum biogenesis. The *pleC* and *pleD* genes are required for flagellum activation in the late predivisional cell, as well as flagellum and stalk biosynthesis early in the cell cycle, when the swarmer cell differentiates into a stalked cell (138). One of these mutants, *pleC*, which lacks both a stalk and pili, has been characterized in detail. *pleC* mutant strains synthesize a flagellum structure but are not motile, indicating that the expression or activation of some critical flagellum component(s) is also affected. The *pleC* gene product is a putative membrane protein that has homology to the sensor histidine kinases of bacterial two-component regulatory systems (154). In vitro experiments with a LacZ-PleC protein fusion have demonstrated that PleC is capable of catalyzing autophosphorylation (154), indicating that it probably functions as a histidine protein kinase in *C. crescentus*. It is hypothesized that PleC is a component of a sensory transduction pathway whose role is to coordinate cell division and morphogenesis with gene expression (102, 139, 154). Pos-

sible members of this regulatory circuit have been identified through genetic pseudoreversion analysis with *pleC* mutant strains (139). Extragenic suppressors of temperature-sensitive alleles of *pleC* were isolated by screening for the restoration of motility at 37°C. Several of these suppressors possessed a cold-sensitive cell division defect, forming long, filamentous cells at 24°C (139). The mutations mapped to three newly identified cell division genes, *divJ*, *divK*, and *divL*. Like *pleC*, one of these genes, *divJ*, encodes a putative membrane protein that is homologous to a sensor histidine protein kinase (102). The transcription of *divJ* is maximal just before the initiation of DNA replication, at the swarmer-cell-to-stalked-cell transition (102). It is hypothesized that DivJ, PleC, and perhaps DivK and DivL, are components of a phosphorelay system that regulates polar morphogenesis and the cell division cycle (102, 154). This Div/Ple phosphorelay is envisioned to be similar to the phosphorylation cascade that regulates early events in *Bacillus* sporulation. In *Bacillus* cells, the sporulation phosphorylation cascade is triggered by environmental cues. The signals that activate the Div/Ple system of kinases in *C. crescentus* are not known but are presumed to be an internal cell cycle event.

FLAGELLAR BIOGENESIS

Flagellum Structure and Assembly

Flagellar biogenesis is the best-understood and most extensively studied aspect of cellular differentiation in *C. crescentus*. The structure of the polar flagellum has been observed by electron microscopy and is similar to that of flagella of enteric bacteria (142, 150, 153). The flagellum consists of three major components. Within the cell lies a basal body that anchors the flagellum in the cytoplasmic membrane and acts as a rotor (Fig. 2). The basal body, consisting of a series of rings attached to each other by a rod, traverses the cytoplasmic membrane, the peptidoglycan layer, and the outer membrane (142). Attached to the cell-distal portion of the rod is a flexible hook which lies entirely outside of the cell (153). Finally, the rigid flagellar filament is attached to the hook (150).

Many of the genes encoding the structural components of the flagellum have been cloned and sequenced. In this review, the *Caulobacter* flagellar genes are designated by the names of their homologs in enteric bacteria (56). (Table 1 lists both the former and the newly adopted gene designations.) For the most part, flagellar genes have been isolated by complementation of nonmotile mutants. It has been estimated that approximately 50 genes are required for motility in *C. crescentus* (22). This number is similar to that required for flagellum synthesis in enteric bacteria (reviewed in reference 56) and *B. subtilis*. Currently, about half of the genes required for motility in *C. crescentus* have been cloned. The flagellum is thought to be sequentially assembled from the inside to the outside of the cell (Fig. 2 and 3). The MS-ring, encoded by the *fliF* gene, is the first structure to be assembled and resides within the cytoplasmic membrane. The *fliF* gene is the first gene in an early-transcribed operon that also includes *flbD*, a gene encoding a transcriptional activator (12, 104, 119). Another early-transcribed operon, *fliLM*, is genetically unlinked to the *fliF* operon (45) and encodes one of the flagellar switch proteins (162) as well as a protein that is required for flagellar function but not assembly (54). The genes encoding the rod have also been cloned and sequenced (15). The proximal rod in encoded by the *flgF* gene, and the distal portion of the rod is encoded by the *flgG* gene. Both of these genes lie in an operon upstream of the *flgH* gene, which encodes the L-ring (16). The P-ring, which is also an outer ring, is encoded by the genetically

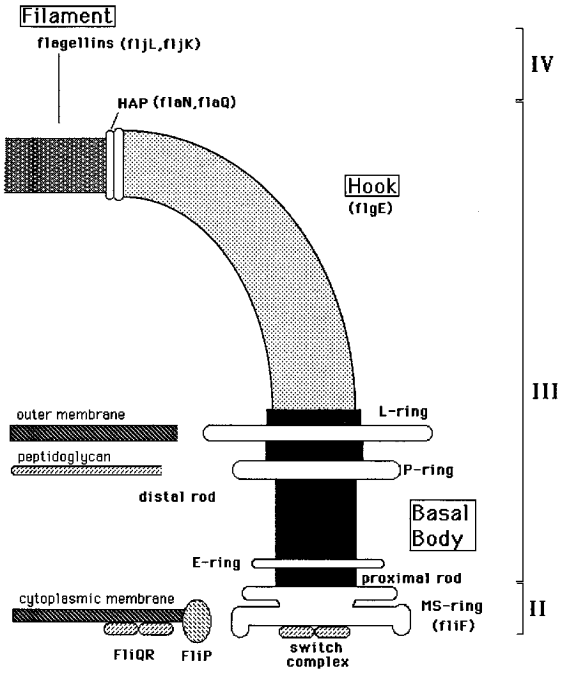

FIG. 2. *C. crescentus* flagellum. A schematic depiction of the *Caulobacter* flagellum is shown. Next to each structure are their names and, in some cases, the name of the gene that encodes them. It is not known if the FliQR and FliP gene products are physically associated with the flagellum or if they are located on the inner or outer membrane. On the far right, the location of each product in the regulatory hierarchy is indicated.

unlinked *flgI* gene (59). The following components assembled into the flagellum are the external hook and filament structures. The hook is composed of monomers of a single polypeptide encoded by the *flgE* gene (55, 70, 100, 131). In enteric bacteria, the base of the hook is attached to the rod by hook-associated proteins (56). These are also involved in linking the hook to the flagellar filament. The known hook-associated protein homologs in *C. crescentus* have been determined by

TABLE 1. *C. crescentus* flagellar genes

Flagellar gene designation		Function	Reference(s)
Former	New		
flaB	*flgG*	Distal rod	15
flaO	*fliF*	M-ring	151
flaK	*flgE*	Hook	101
flaH	*fliP*	Export	53
flaN	*flgK*	HAP[a]	90
flaC	*flgF*	Proximal rod	15
flaP	*flgI*	P-ring	59
flaS	*fliQ*	Export	163
flaS	*fliR*	Export	163
flbD	*flbD*	Transcription	119
flbF	*flhA*	Export	120, 123
flbO	*fliL*	Unknown	162
flbO	*fliM*	Switch	162
flbN	*flgH*	L-ring	16
flgJ	*fljJ*	29-kDa flagellin	84, 85
flgK	*fljK*	25-kDa flagellin	84, 85
flgL	*fljL*	27-kDa flagellin	84, 85

[a] HAP, hook-associated protein.

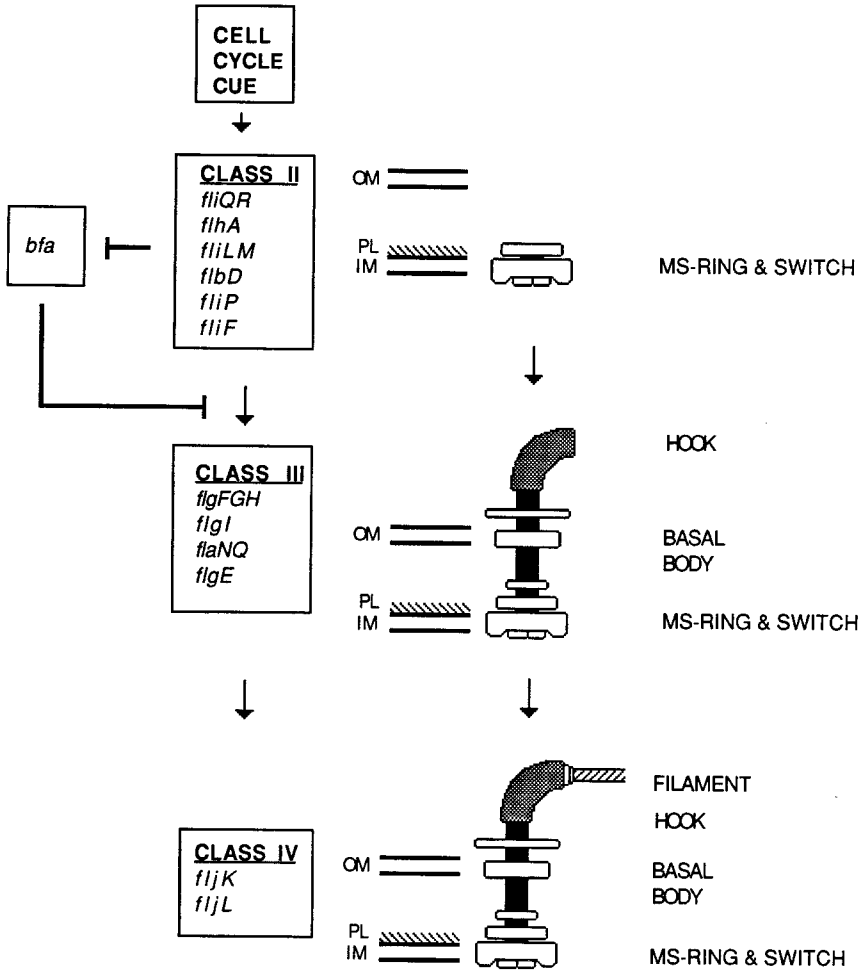

FIG. 3. Flagellar regulatory hierarchy. The genes at the major levels of the regulatory cascade are shown next to the assembled structures they encode. Arrows indicate positive regulation, and lines with bars at their ends indicate negative regulation. The assembly of early structures is coupled to the expression of class III genes. Strains with mutations in class II genes do not transcribe class III genes (10, 84, 94, 101, 161; reviewed in references 7 and 9). Mutations in the *bfa* locus bypass this transcriptional requirement for class II gene products (76). A model of the regulatory loop involving *bfa* is depicted. See the text for details.

DNA sequence analysis and are encoded by the *flaNQ* operon (90). The final structure that is assembled is the rigid flagellar filament which is composed of flagellin monomers. In contrast to enteric bacteria, which have only one flagellin type, *C. crescentus* possesses three flagellins with distinct molecular masses (29, 32, 33, 67, 69, 111, 158). The 25-kDa flagellin is the most abundant and is encoded by genes located on two distinct regions of the chromosome (23, 85). Two other flagellin genes, *fljL* and *fljJ*, encode 27.5- and 29-kDa proteins, respectively, and are located in a cluster that also contains *fljK*, one of the 25-kDa flagellins (84, 85). Immunoelectron microscopy of the flagellar filament has demonstrated that the flagellins themselves are assembled in a distinct order (19). The 29-kDa flagellin is proximal to the hook, followed by the 27.5-kDa flagellin and finally by the 25-kDa flagellin. Although the 25-kDa flagellin is by far the most abundant type in the filament, gene replacement experiments have demonstrated that the minor 27.5- and 29-kDa flagellins are required for normal motility (85).

Gene Products Required for Flagellum Assembly

Several class II flagellar genes, which are required for flagellum biogenesis but are probably not part of the flagellar structure, have been identified in *C. crescentus* and other bacteria. These genes are thought to direct the assembly of the flagellum. The striking finding is that several of these flagellar gene products share significant amino acid identity with virulence factors of both plant and animal pathogens. The product of the *flhA* gene possesses identity to the LcrD protein of *Yersinia* spp. (120, 123). In *Yersinia* spp., LcrD is encoded by one of the low-calcium-response genes, which regulate the expression of virulence genes (Yops) in response to calcium and temperature (reviewed in reference 146). The *Yersinia* Yops are secreted but lack the conventional amino-terminal signal sequence and are believed to be exported by a novel secretory pathway. It is now thought that LcrD is a component of the export machinery for Yops, suggesting that the flagellar homolog, FlhA, functions analogously to export flagellar proteins. The *Caulobacter fliP* gene has recently been sequenced (53) and, like the *E. coli* gene (75), is homologous to virulence factors in *Erwinia*, *Xanthomonas*, *Salmonella*, and *Shigella* spp. (43, 52, 87, 148). In *Salmonella* spp., the *fliP* homolog (*spaP*) is required for efficient invasion of mammalian cells in tissue culture (43). The *spaP* gene product is thought to be required for the export of invasion factors. The *fliQ* and *fliR* genes have also been sequenced recently and have been shown to possess

sequence identity to two other *Salmonella* and *Shigella* Spa proteins (163). The *Salmonella spaL* gene and the *Shigella spa47* gene (43) are similar to *fliI*, another early *Salmonella* flagellar gene (152). The *fliI* homolog in *C. crescentus* has yet to be identified. It is suggested that these genes encode components of a novel protein export system. This conserved system is thought to function in pathogens in the export of virulence factors, and in flagellar biogenesis, to export flagellar components. There is currently no experimental evidence that documents the role of these proteins in export. It also remains to be determined whether this system facilitates the export of all flagellar components or operates at specific steps in flagellum assembly.

Gene products that regulate synthesis and processing of flagellin subunits have also been identified (128, 129). These genes, *flaG*, *flbA*, *flbT*, and *flaF*, map adjacent to the *fljL*, *fljK*, and *fljJ* flagellin genes. Genetic experiments have shown that the *flbA* and *flaG* gene products are required for normal proteolytic processing of the 25-, 27.5-, and 29-kDa flagellins (129). *flaF* and *flbT* function to modulate the level of flagellin expression (128, 129). Strains with mutations in *flaF* have a decreased level of flagellin synthesis, whereas mutations in *flbT* increase the expression of all flagellins. Interestingly, this cluster of genes regulates other swarmer cell-specific functions. *flbT* is required for normal chemotaxis (128), and deletions in this region result in resistance to bacteriophage ϕCbK (129). Both the chemotaxis components (3) and ϕCbK receptors are targeted to the pole of the swarmer cell, suggesting that this gene cluster may have an important role in polar morphogenesis.

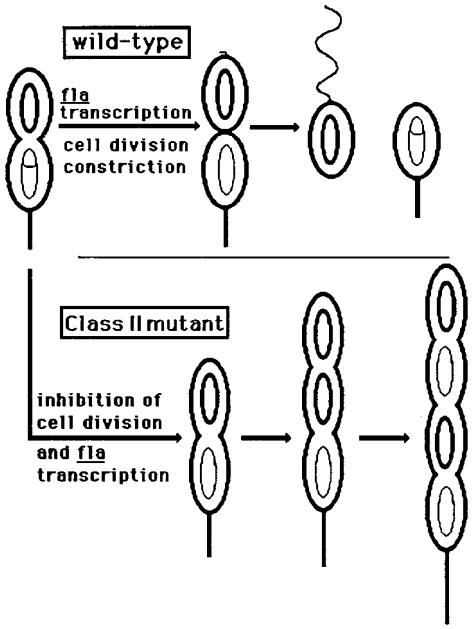

FIG. 4. Early flagellum assembly is required for normal cell division. Shown is a diagram of the effect of mutations in class II flagellar genes on the timing of cell division. In wild-type cells, the timing of the assembly of the flagellum is tightly coupled to the formation of the cell division constriction and subsequent cell division. In mutant cells that do not assemble early flagellar structures, cell division is delayed. Cultures of these mutants contain many long cells, indicating that cell division occurs only after the cell has reached several cell lengths. The cell division delay in response to the absence of flagellar assembly can be viewed as a developmental checkpoint. The operation of this developmental checkpoint ensures the precise coupling between two morphogenetic events: flagellar assembly and cell division.

TRANSCRIPTIONAL REGULATION OF FLAGELLAR BIOGENESIS

Regulatory Hierarchy

Epistasis experiments have shown that flagellar gene expression in *C. crescentus* is subject to a complex regulatory hierarchy (Fig. 3) (10, 84, 94, 101, 161; reviewed in reference 9). This hierarchy is similar to that operating in enteric bacteria, in which the expression of genes encoding early structures is required for late-gene expression (56, 61, 66). In *C. crescentus*, there are four distinct levels in the flagellar regulatory hierarchy (Fig. 3). Level I is envisioned as a cell cycle cue that triggers class II flagellar gene transcription. This level is still somewhat hypothetical, because no mutations have been isolated thus far that abolish class II gene expression.

Class II genes represent the first set of flagellar genes in the regulatory hierarchy (10, 84, 94, 101, 161; reviewed in reference 9). The expression of these genes is absolutely required for the subsequent expression of class III and IV flagellar genes. Class II genes fall into three major groups: (i) those that encode the earliest flagellar structures such as the MS-ring (*fliF*) and the switch proteins (*fliG*, *fliN*, and *fliM*), (ii) those that encode products required for flagellar assembly (*fliP*, *fliQR*, and *flhA*), and (iii) those that encode transcription factors such as *rpoN* and *flbD*. A mutation in any one of these genes results in nonmotile cells, which do not transcribe class III flagellar promoters.

Strains with mutations in class II genes also display striking cell division defects (18, 53, 162, 163). These mutants produce long filamentous cells, indicating that early flagellar assembly events are required for the proper coupling of cell growth to cell division. In class II mutants, cell division is inhibited until the cells reach an abnormal length (Fig. 4). This can be viewed as a developmental checkpoint, in which cell division is delayed until a specific step in flagellar assembly is completed. The mechanism by which cell division is inhibited is not known but quite possibly is exerted through one of the known septum inhibitors in bacteria such as SulA, DicB, or the MinCDE system (reviewed in reference 13). It should be noted that *C. crescentus* does not form a typical septum but, rather, forms a constriction at the site of cell division which eventually pinches inward, forming two cells (116).

Class II flagellar genes are the earliest expressed flagellar genes in the *Caulobacter* cell cycle. One possible consequence of the flagellar regulatory hierarchy is that the order of expression of flagellar genes in the cell cycle reflects their order of assembly. Therefore, class II genes are expressed first, followed by the class III genes and finally by the class IV genes. An experiment in which the temporal expression of the class II *fliF* operon was delayed in the cell cycle resulted in a delay in the expression of class III promoters (99). This experiment clearly demonstrates that the temporal expression of class III genes is dependent on the presence of class II gene products.

The coupling of class III flagellar gene expression to early class II gene expression is one of two steps in the regulatory hierarchy at which flagellum assembly regulates gene expression (Fig. 3). Mutations that bypass the transcriptional requirement for class II gene products have been isolated (76). These mutants, termed *bfa* (for bypass of flagellar assembly), were generated by mutagenesis with ethyl methanesulfonate. The mutants were selected for expression of a *fljL* reporter gene fusion in a *fliQR* mutant strain. The *bfa* mutations mapped to a single chromosomal location and permitted the expression of *fljL* in most of the class II mutants tested, indicating that the negative regulation is common to all mutants. *fljL* was not

expressed in strains with mutations in essential transcription factors such as *rpoN* or *flbD*. The *bfa* mutation relieves the class II gene requirement for all class III genes, including the hook operon, the *flgF* operon, and *flaNQ*. Therefore, a common mechanism regulates the expression of all class III genes in response to early flagellar assembly events. What is the role of *bfa* in nonmutant cells? The wild-type *bfa* gene product might have a role in regulating the timing of class III gene transcription. *bfa* mutants fail to shut off *fljL* transcription at the appropriate time in the cell division cycle (76). In wild-type cells, expression of *fljL* ceases early in predivisional cells (86). In contrast, *fljL* expression continues after cell division in *bfa* mutant strains (76), indicating that *bfa* normally functions to turn off the transcription of late flagellar genes, after flagellum assembly has progressed past some critical point.

The expression of late flagellar genes (class III) is also required for the expression of flagellins (class IV) (45, 84, 161). Regulation at this step of the hierarchy occurs through a post-transcriptional mechanism. Both *fljL* and *fljK* are transcribed in class III mutants, but flagellin protein is not synthesized (76). Furthermore, although *fljL-lacZ* and *fljK-lacZ* operon fusions are expressed in class III mutants, *fljL* and *fljK* protein fusions to β-galactosidase are not expressed, possibly indicating that the mRNA is not translated. Flagellin expression is also regulated at this level of the hierarchy in enteric bacteria. In contrast, the flagellin gene in *Salmonella* spp. is not transcribed in class III mutant strains. Transcription is inhibited by an anti-sigma factor called FlgM (34, 35). FlgM specifically interacts with the σ^{28} subunit of RNA polymerase, which is required for flagellin transcription (106). The inhibitory anti-sigma factor is pumped from the cytoplasm into the growth medium only when assembly of the basal body-hook complex is complete (50).

Cell Cycle Regulation of Early Flagellar Gene (Class II) Transcription

Three class II gene promoters, *fliF*, *fliQR*, and *fliLM*, share similar promoter sequences (18, 88, 144, 151, 162) (Fig. 5). These sequences differ significantly from those of other promoters, prompting the hypothesis that this promoter represents the recognition sequence for a novel sigma subunit of RNA polymerase. This hypothetical sigma factor has been called σ^R, for regulatory sigma factor (144). σ^R probably represents the master regulatory element that triggers the flagellar gene expression cascade. Site-directed mutagenesis has been performed to identify the DNA sequences important for *fliF*, *fliLM*, and *fliQR* transcription (144, 151, 163). These experiments have shown that essentially identical promoter sequences are necessary for temporal transcription. Extensive analysis has been performed on the *fliLM* promoter, revealing an unusual promoter organization (144). Unlike σ^{70} promoter organization, in which there are two important regions (−35 and −10) within the promoter, mutagenesis of the *fliLM* promoter demonstrated three important subregions within the promoter, with an absence of a well-defined typical −35, −10 region (144). These experiments with the *fliLM* promoter have shown that the identified promoter sequence is the sole *cis*-regulatory element that governs *fliLM* transcription (144).

The simplest model to account for the temporal expression of class II genes is that the putative σ^R subunit is expressed periodically during the cell cycle. It is thought that a cell cycle cue is the initiating event in the regulatory cascade. The precise nature of this cell cycle event is not known but is likely to require the initiation of chromosomal DNA replication. Early experiments demonstrated that inhibition of *Caulobacter* chromosomal DNA replication with hydroxyurea resulted in an inhibition of hook and flagellin protein synthesis (107, 132). Similar experiments with promoter probe fusions to *fliF*, *fliQR*, and *fliLM* promoters have shown that inhibition of DNA replication inhibits the transcription of these promoters (18, 144). The effectiveness of hydroxyurea in inhibiting *fliLM* transcription varies during the cell cycle. Treatment of cells before or near the time of initiation of DNA replication had a greater inhibitory effect on *fliLM* transcription than did treatment of cells later in the cell cycle (144). This result is consistent with the idea that an early cell cycle event triggers the expression of an essential transcription factor for class II genes. The occurrence of the σ^R promoter type is not restricted to flagellar genes. The *Caulobacter* DNA methyltransferase gene *ccrM* also possesses a consensus σ^R promoter (143). The transcription of *ccrM* parallels class II flagellar gene transcription and is also requires the initiation of chromosomal DNA synthesis (143), suggesting that the putative σ^R-containing RNA polymerase may regulate other critical cellular functions.

The σ^R subset of class II promoters is also subject to negative regulation. For example, in strains carrying mutations in class II genes, the transcription of σ^R promoters is elevated,

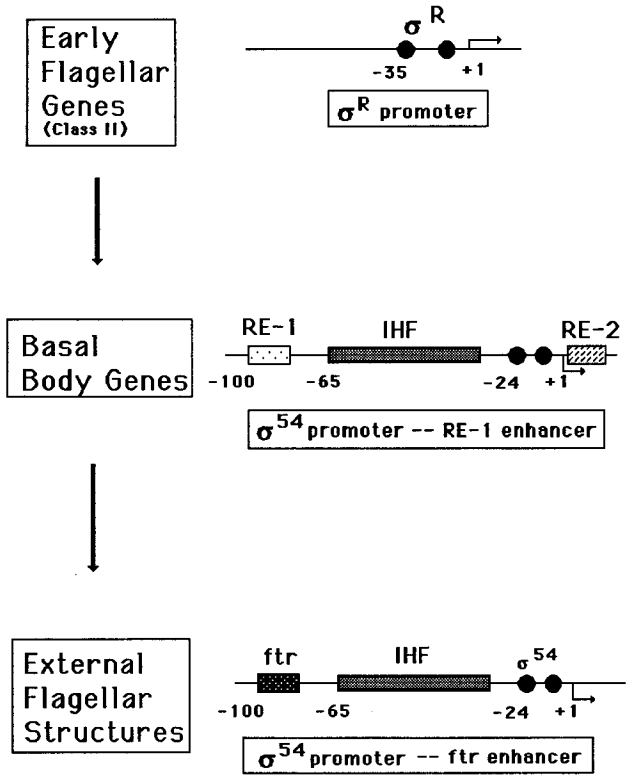

FIG. 5. Different promoter classes operate at each level of the flagellar hierarchy. The major promoter classes of *Caulobacter* flagellar genes are shown in schematic form. Some less abundant flagellar promoter types are not shown (for example, see reference 16). Several early class II genes are transcribed from a conserved, novel promoter sequence which is thought to be recognized by a unique sigma subunit of RNA polymerase tentatively named σ^R for regulatory sigma (18, 144, 151, 162). Late flagellar genes possess σ^{54} promoters and IHF-binding sites (15, 37–39, 59, 81, 84, 88–90, 96). Differential regulation is achieved through the use of unique enhancer elements. Basal body promoters contain an upstream RE-1 enhancer (15, 59, 81) and also, for the *flgF* operon, a downstream RE-2 enhancer (81). Genes encoding external flagellar structures (hook, hook-associated proteins, some flagellins) have ftr enhancers (38, 39, 84, 88–90). The ftr enhancer is the binding site for the FlbD transcriptional activator (6, 91, 160). Late in the cell cycle, transcription of ftr-containing promoters is restricted to the swarmer pole of the predivisional cell (36, 38, 160).

indicating that some type of feedback regulatory loop influences expression. Site-directed mutagenesis of the *fliF* promoter has identified sequences adjacent to the core promoter sequence that are involved in negative regulation (151, 159). Cell cycle experiments demonstrated that transcription of the mutated *fliF* promoter persisted longer in the cell cycle than did transcription from the wild-type promoter (151, 159). Recent experiments have shown that the transcription factor FlbD binds to this promoter and is instrumental in negative regulation (6, 91, 159) (see the section on FlbD, below).

CELL CYCLE REGULATION OF LATE FLAGELLAR GENE TRANSCRIPTION

Three Distinct Enhancer Elements Regulate Late Flagellar Gene Transcription

The expression of class III and IV flagellar genes occurs relatively later in the cell cycle and is dependent on the synthesis of class II gene products (10, 84, 94, 101, 161). Class III and IV promoters have a similar sequence organization (Fig. 5), requiring RNA polymerase containing σ^{54} for transcription (15, 16, 59, 84, 88, 89, 90). Strains harboring Tn*5* insertions in the *rpoN* gene, which encodes σ^{54}, do not express any of these promoters (8). Furthermore, in vitro transcription experiments have shown that the hook and *flaNQ* operon promoters can be transcribed by σ^{54}-containing RNA polymerase holoenzyme in the presence of NtrB and NtrC (96) and FlbD (6). An important characteristic of σ^{54}-containing RNA polymerase is that it can bind to promoters and form a stable, closed transcription complex (reviewed in references 64 and 65). It cannot isomerize this closed complex to an open complex, thereby initiating transcription, unless it interacts with a transcriptional activator. The binding site for the activator protein is an enhancer element that usually lies approximately 100 bp away from the transcription start site. *Caulobacter* flagellar genes have two major different enhancer sequences. The basal body promoters of the *flgF* operon and *flgI* have a conserved enhancer element called RE-1 (15, 59, 81). The promoters of the hook operon, *flaNQ*, *fljL*, and *fljK*, have a conserved enhancer element known as ftr (84, 89, 90).

Extensive mutational analysis has defined the important DNA sequences within the ftr enhancer element (38, 39, 89, 90). As little as a 2-bp change in critical sequences can abolish transcription activity in the hook operon promoter. All ftr-containing promoters have more than one enhancer sequence. For example, the hook operon promoter has an essential ftr element located 120 bp upstream of the transcription start, one ftr element located at 140 bp (6), and two additional ftr elements located approximately 350 bp upstream within the divergent *flaNQ* promoter (89). These two distant ftr enhancers are not essential for transcriptional activity but are required for maximal levels of transcription, indicating that these sequence elements have the capacity to interact over long distances (39). The ftr elements can function as true enhancers, being able to activate transcription whether they are located upstream or downstream of the promoter. The *flaNQ* promoter possesses a single ftr element upstream of the promoter and two ftr elements downstream (39, 90). Mutagenesis of the most proximal downstream enhancer, located at +110 bp, completely abolishes transcription of the *flaNQ* promoter (39, 90).

The *flgF* and *flgI* σ^{54} promoters share a different conserved enhancer sequence called RE-1 (15, 59, 81) (Fig. 5). In addition, the *flgF* promoter contains another *cis*-acting element, called RE-2, is located at +2 bp from the transcription start (81). Site-directed mutagenesis of the upstream RE-1 enhancer did not completely abolish transcription but resulted in a fourfold decrease in promoter activity (81). Mutagenesis was also performed on the downstream RE-2 element to determine if it was also contributing to transcriptional activation. Point mutations in RE-2 also decreased transcriptional activity approximately fourfold (81). Promoters with mutations in both upstream and downstream enhancer elements exhibited a complete loss of promoter activity. Therefore, RE-1 or RE-2 alone can support modest transcription activation, but when they are combined, they can function to synergistically increase the level of transcription (81). This effect is similar to that observed in many eukaryotic promoters, in which the combination of enhancer sequences results in the synergistic activation of transcription. The novel finding here is that a sequence element in close proximity to the transcription start site (at +2 bp) can participate in the synergistic transcriptional activation of a prokaryotic promoter. The *flgH* basal body gene located downstream of the *flgF* operon possesses a third distinct enhancer element known as Rf-2 (16). This gene is also transcribed by the upstream *flgF* promoter (15), and the importance of the internal *flgH* promoter containing the Rf-2 enhancer is not known.

Integration Host Factor and Late Flagellar Gene Expression

Late flagellar gene promoters have a conserved 35- to 50-bp AT-rich region located between the enhancer sequence and the promoter. Gel mobility shift analysis and DNase footprinting experiments have demonstrated that this region contains a binding site for integration host factor (IHF) (37, 38, 81). IHF is a small (20-kDa), sequence-specific DNA-binding protein, which was initially identified as a host requirement for bacteriophage lambda integration in *E. coli* (reviewed in reference 71). In vitro experiments have shown that IHF introduces a large bend when bound to DNA. The DNA bend, estimated at approximately 140°, functions to deliver lambda integrase, bound at high-affinity arm sites, to its lower-affinity core site, where recombination takes place (71). Therefore, the DNA-bending activity of IHF serves to promote long-distance protein-DNA interactions. Depending on the promoter, mutagenesis of the IHF-binding site in flagellar promoters results in a two- to fivefold decrease in transcription when assayed with *lacZ* reporter gene fusions (37, 38, 81). IHF-binding sites have been found in σ^{54} promoters of several bacterial species (124). In vitro transcription experiments with the *Klebsiella pneumoniae nifH* promoter have also shown that IHF is required for maximal levels of transcription (47). The IHF bend is thought to function in delivering activator proteins, bound at the enhancer sequence, to RNA polymerase bound at the promoter (47). This would serve to increase the frequency of interactions between activator and polymerase, resulting in an increase in transcriptional activity. This model would predict that IHF could function to enhance transcription regardless of whether it is located upstream or downstream of the promoter. The only requirement would be that it be located between the promoter sequences and the enhancer. The downstream configuration exists in the *Caulobacter flaNQ* promoter, where IHF binds between the promoter and the 3′ ftr enhancers (38). Consistent with the model of IHF action, mutagenesis of the IHF-binding site in this promoter results in a threefold decrease in transcription of *flaNQ* (38).

Even though the homologous genes encoding IHF have yet to be identified, *C. crescentus* apparently possesses a molecule with properties similar to *E. coli* IHF. Immunoblots of *C. crescentus* cell extracts that reacted with anti-IHF antibody revealed two cross-reacting polypeptides with almost the iden-

tical molecular weight to the *E. coli* IHF (38). In addition, fractionated *Caulobacter* extracts possess an activity that can substitute for *E. coli* IHF in an in vitro lambda recombination assay (38). To facilitate recombination, the *Caulobacter* protein must bind to and bend the DNA in precise locations. The fact that extracts can facilitate integrase-mediated recombination indicates that *Caulobacter* possesses a protein with DNA-binding characteristics that are similar to those of *E. coli* IHF (38). Immunoblots of extracts prepared from synchronized populations show that IHF is expressed periodically during the cell division cycle (38). Predivisional cells have the highest levels of IHF, which disappears after cell division. The peak in IHF levels coincides with the peak in transcriptional activity of late flagellar gene promoters, indicating that modulation of IHF levels may have a role in regulating the temporal transcription of these promoters.

FlbD and the Temporal Expression of ftr-Containing Promoters

Although both IHF and *rpoN* (σ^{54}) are under cell cycle control, it is likely that the proteins that bind to the ftr enhancer sequences are the major transcriptional regulators of late flagellar promoters. Gel mobility shift analysis and Southwestern (DNA-protein) assays have identified two distinct ftr-binding activities (95 and 55 kDa) in cell extracts (39). Both of these binding activities are expressed under cell cycle control and, like IHF, are at maximal levels in the predivisional cell type, where ftr promoter activity is maximal (39). The identity and regulatory significance of the 95-kDa binding activity, called Rf-1, are not known. Recent epistasis experiments with the *fljK* promoter have indicated that the product of the *flbD* gene is the transcriptional activator of ftr-containing promoters and may encode the 55-kDa enhancer-binding activity (160). *fljK*, unlike other ftr promoters, is not subject to regulation by flagellar assembly. Therefore, epistasis experiments involving *fljK* reporter gene fusions can be used to identify genes that encode transcription factors that are essential for flagellar biogenesis. Notably, the *fljK* promoter is expressed in all known class II mutants except strains with Tn*5* insertions in *rpoN* and *flbD* (160). As described above, *rpoN* encodes the σ^{54} subunit of RNA polymerase and is therefore essential for *fljK* transcription. *flbD* encodes a 52-kDa protein that is homologous to a large family of transcriptional activators of σ^{54} promoters (119). The amino-terminal and central domains of FlbD are homologous to the so-called response receiver modules of bacterial two-component regulatory systems (58; reviewed in references 97 and 112). The amino-terminal domain of this class of activators is generally phosphorylated in response to an environmental cue. Phosphorylation serves to modulate the transcriptional activity of the protein. For example, NtrC of enteric bacteria is phosphorylated in response to low cellular nitrogen levels. The phosphorylated form of NtrC activates transcription of operons that encode genes which scavenge nitrogen from the environment (58, 95, 117, 155, 157). The phosphodonor in the case of NtrC is a histidine kinase called NtrB (58, 95, 117, 155, 157), which is a member of a large family of bacterial sensor histidine kinases. The conserved central domain of FlbD is also highly homologous to NtrC and is involved in transcription activation. The carboxyl terminus of the protein contains a helix-turn-helix motif, which is required for DNA binding (119).

Purified FlbD forms a gel mobility shift complex with all ftr-containing promoter DNA (160). In addition, DNase I footprinting shows that FlbD binds specifically to the ftr elements found in these promoters (6, 91). Gel shift competition experiments with wild-type and mutant promoter DNA demonstrated that FlbD requires an intact ftr sequence for binding (160). FlbD could not bind to a promoter with a 2-bp mutation in the ftr element (160). The same mutant promoter was not expressed in vivo, suggesting that a disruption of FlbD binding prevented transcription activation (160).

One mechanism by which FlbD could regulate temporal transcription is if the levels of FlbD changed periodically during the cell division cycle. *flbD* is the last gene in the class II *fliF* operon and therefore is transcribed under cell cycle control. To determine if the level of FlbD was modulated as a function of the cell cycle, a fusion between the entire *flbD* gene and a seven-amino-acid epitope was constructed (160). Epitope-tagged FlbD apparently functioned normally, as it could restore motility in a strain that had a Tn*5* insertion in *flbD* (160). Cells containing the epitope-tagged FlbD were synchronized, and the synthesis of the tagged protein was monitored with a monoclonal antibody to the epitope. Pulse-labeling followed by immunoprecipitation showed that the synthesis of FlbD was under cell cycle control. Surprisingly, immunoblots of the same cell extracts showed that FlbD was present throughout the cell cycle (160). Therefore, if FlbD is responsible for the timed transcription of flagellar genes, its activity must be modulated during the cell cycle. The most likely mechanism for controlling FlbD activity would be through phosphorylation of its amino terminus. Incubation of cells with $^{32}PO_4^{3-}$ and immunoprecipitation of epitope-tagged FlbD showed that FlbD was phosphorylated in vivo (160). Kinase activity could also be demonstrated in vitro when cell extracts were incubated with purified FlbD and [γ-^{32}P]ATP (160).

As outlined above, the amino terminus of FlbD shares sequence identity with a large family of bacterial regulatory proteins, members of so-called two-component regulatory systems (97, 112). The structure of one of these regulators, CheY, has been solved by X-ray crystallography (145). It has been demonstrated that an aspartic acid at position 57 accepts phosphate from the histidine kinase (145). Aspartate 57 is located in an acidic pocket, adjacent to aspartate residues at positions 12 and 13. These residues are conserved in most response regulators and are thought to play a critical role in the phosphorylation reaction through the coordination of Mg^{2+}. Another highly conserved residue is a lysine located at position 109. FlbD possesses only the conserved residue analogous to aspartate 57 (D-54 in FlbD); however, phosphorylation can be demonstrated both in vivo and in vitro (160). Like other response regulators, phosphorylation and dephosphorylation of FlbD require Mg^{2+} ions, although the specific residues involved in coordinating Mg^{2+} are probably different from those in CheY. It is possible that the tertiary structure of the amino terminus of FlbD differs significantly from that of other known response regulators.

Cell extracts prepared from synchronized populations possessed FlbD kinase activity only at the times in the cell cycle when the ftr-containing genes were transcribed (160). Kinase activity peaks in late predivisional cells, which parallels the peak of ftr promoter transcription. These data suggest that FlbD phosphorylation plays a fundamental role in controlling temporal transcription. The identity of the FlbD kinase and the mechanism by which this kinase is activated are not known. One plausible model is that the synthesis of the kinase is under cell cycle regulation. Alternatively, the activity of the kinase may be regulated by some cell cycle event. In most two-component regulatory systems, the kinase phosphorylates the response regulator in response to an environmental or nutritional cue. In *C. crescentus*, the FlbD kinase may be responding to a cell cycle or cell division cue or perhaps to a structural

intermediate in flagellar biogenesis. Constitutively active mutants of FlbD have been used to determine whether FlbD phosphorylation regulates temporal transcription of late flagellar promoters (160). Site-directed mutagenesis was used to change a serine at position 140 in the conserved central domain to a phenylalanine (S140F). This mutation in FlbD is analogous to a well-characterized mutation in NtrC (S160F), which has been shown both in vivo and in vitro to be capable of activating transcription in the absence of the NtrB kinase (155). When the FlbD S140F protein was overexpressed in *C. crescentus*, the cell cycle pattern of *fljK* expression was only slightly altered (160). Most notably, the *fljK* promoter was still not expressed early in the cell cycle, even in the presence of a constitutively active FlbD. This experiment shows that the phosphorylation of FlbD is clearly not the sole regulator of timed transcription. The absence of *fljK* expression in the early phase of the cell cycle might reflect the fact that both *rpoN* and IHF, which are both required for *fljK* transcription, are not expressed early in the cell division cycle.

MECHANISMS OF ASYMMETRIC PROTEIN POSITIONING

Pole-Specific Transcription of Flagellar Genes

Compartment-specific gene expression is one mechanism that serves to localize flagellar gene products to the swarmer pole of the predivisional cell (36, 159, 160). Late flagellar genes continue to be expressed in the predivisional cell after a cell division barrier has formed between the swarmer cell and stalked-cell compartments. This necessitates that late flagellar gene expression be restricted to the swarmer cell compartment, where flagellum assembly is taking place. Milhausen and Agabian (83) demonstrated that the mRNA for the 25-kDa flagellin (*fljK*) segregated to the progeny swarmer cells. Experiments in which the cells were treated with rifampin, an inhibitor of transcription initiation, showed that the *fljK* mRNA present in the progeny swarmer cells was not the result of de novo transcription but, rather, was synthesized before cell division in the predivisional cell type (83). Therefore, *fljK* mRNA was localized to the swarmer pole of the predivisional cell. Three possible mechanisms can account for polar mRNA localization. First, mRNA may be synthesized in both poles of the predivisional cell and then be positioned in the swarmer pole. Second, the mRNA may be selectively degraded in the stalked pole of the predivisional cell. Third, the *fljK* gene may be selectively transcribed in the swarmer pole of the predivisional cell. These possibilities were experimentally explored by using transcription fusions of flagellar genes to promoterless reporter genes (36). In this type of experiment, synchronized populations of cells that carried flagellar transcription fusions were permitted to progress to the late predivisional stage, where proteins were pulse-labeled. The label was chased, and the cells were allowed to divide. After division, the progeny swarmer and stalked cells were separated from each other and the labeled reporter gene product was immunoprecipitated with antibody. The presence of reporter gene in either the swarmer cell or stalked-cell populations indicated the location of synthesis of the reporter gene in the predivisional cell. Therefore, if labeled reporter protein was present in progeny swarmer cells, it indicated that the synthesis of the reporter was restricted to the swarmer pole of the predivisional cell. This type of experiment showed that the *fljK* gene was expressed primarily in the swarmer pole of the predivisional cell (36). In addition, several other genes were shown to be subject to swarmer pole-specific expression, including the hook and the *flaNQ* operons (36, 38). Genes expressed in the swarmer pole encode external flagellar components and possess σ^{54} promoters with IHF-binding sites and ftr enhancer elements (36, 38). The common promoter structure indicated that transcription of these genes may be limited to the swarmer pole. To test this idea, a minimal hook operon promoter of 112 bp (from −120 to −8 bp) was fused to a *neo* reporter gene (36). In this experiment, the promoter sequences were sufficient to drive the polar localization of the *neo* reporter gene, suggesting that localized expression of ftr promoters is a direct consequence of swarmer pole-specific transcription (36).

FlbD-activated promoters are expressed in the swarmer pole of the predivisional cell. FlbD may also be involved in directing swarmer pole-specific expression. Immunofluorescence microscopy, however, has revealed that FlbD is present in both poles of the predivisional cell (160). To test whether the phosphorylation of FlbD played a role in activating pole-specific transcription, strains carrying the constitutive FlbD S140F mutant allele were assayed for compartment-specific expression of flagellins (160). The experiment was performed as described above for transcription fusions. In control cultures that overexpressed wild-type FlbD, labeled flagellin was found only in progeny swarmer cells (160). In contrast, labeled flagellins were present in both swarmer and stalked cell progeny in the strain that carried the constitutively active FlbD, indicating that flagellin was also expressed in the stalked-cell pole of the predivisional cell (160). This experiment suggests that the FlbD is phosphorylated specifically in the swarmer pole of the predivisional cell, resulting in compartment-specific transcription of ftr-containing σ^{54} promoters. Cell cycle phosphorylation experiments indicate that FlbD is phosphorylated before a cell division barrier has formed between the swarmer and stalked poles (160). How does phosphorylated FlbD become restricted to the swarmer pole just before cell division? One possible model is that the kinase itself is targeted to the swarmer pole and that formation of the cell division barrier isolates the FlbD in the stalked pole from the kinase in the swarmer pole (160). Another plausible model is that the kinase is activated by a swarmer pole-specific cue. In this case also, the formation of the cell division barrier is a pivotal event in maintaining the polarity in FlbD activity (Fig. 6). Another formal possibility is that a developmentally regulated phosphatase is specifically localized to the stalked pole. The elucidation of the mechanism of pole-specific activation of FlbD awaits the identification and isolation of the FlbD kinase.

Strains with Tn*5* insertions in *flbD* express early (class II) flagellar promoters at significantly higher levels than do wild-type cells, suggesting that FlbD may either directly or indirectly control the transcription of this class of promoters. Recent evidence indicates that FlbD acts to repress the transcription of the *fliF* operon promoter (6, 159). Purified FlbD can form a DNase footprint on the *fliF* promoter DNA (6, 91, 159). Binding of FlbD to the *fliF* promoter protects two regions, one strong binding site located at −19 to +16 bp from the transcription start site, and another, weaker binding site at −52 to −35 bp (6, 159). Furthermore, methylation interference experiments have demonstrated that FlbD binding requires several sequences that overlap with the σ^{R} promoter sequence (6, 159). Binding of FlbD to these sequences in vivo would be predicted to effectively occlude the binding of polymerase to the promoter and repress transcription. To test this idea, a mutation was introduced in a single base pair shown by methylation interference to be essential for FlbD binding in vitro (159). This mutant promoter, when placed upstream of a reporter *lacZ* gene, expressed β-galactosidase to levels four times higher than the wild-type promoter, suggesting that FlbD neg-

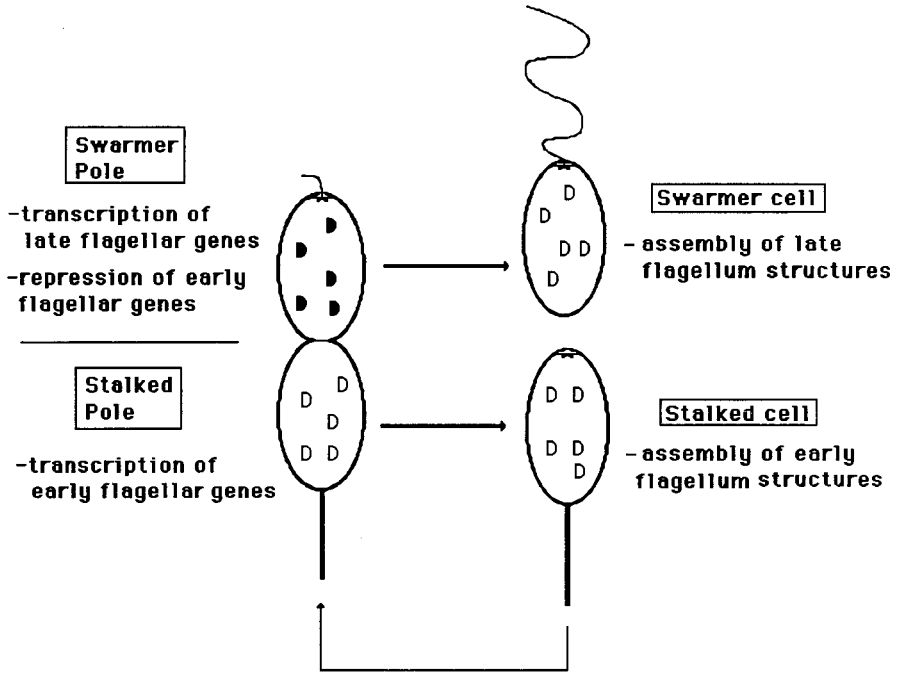

FIG. 6. Swarmer pole-specific activation of FlbD accomplishes two regulatory missions in the *Caulobacter* predivisional cell. Shown is diagram of the relative programs of flagellar gene expression in the swarmer and stalked poles of the predivisional cell and progeny cells. Compartmentalized transcription of late flagellar promoters is a consequence of the swarmer pole-specific activation of FlbD (indicated by a solid D). Activated FlbD also functions to repress the early class II *fliF* promoter in the swarmer pole of the predivisional cell. *fliF* transcription occurs in the stalked pole, where FlbD is inactive (indicated by an open D). Therefore, two different programs of flagellar gene expression are initiated in the swarmer cell and stalked-cell compartments of the predivisional cell and are required for completion of flagellar biogenesis in the progeny cell types. Late flagellar gene expression in the swarmer pole supplies the newly formed swarmer cell with flagellin mRNA. Transcription of the early *fliF* operon prepares the progeny stalked cell for a new round of flagellum biogenesis. Adapted from reference 159 with permission of the publisher.

atively regulates the *fliF* promoter in vivo. In an earlier analysis of this promoter, several mutations 3′ to the −10 region were shown to increase transcription of *fliF* (151). At least two of the mutations described in these experiments would also be predicted to disrupt the binding of FlbD. The predominant effect of FlbD during the cell cycle is to negatively regulate *fliF* transcription in the predivisional cell type just before cell division (159). Mutant *fliF* promoters that cannot be bound by FlbD have an increased level of transcription at the end of the cell cycle (151, 159). In contrast to ftr-containing promoters, the *fliF* promoter has been shown to be expressed predominantly in the stalked-cell compartment of the predivisional cell (38, 159). The mutant *fliF* promoter was expressed in both the swarmer and stalked poles of the predivisional cell, suggesting that FlbD represses *fliF* transcription in a pole-specific manner (159).

The binding of FlbD to the *fliF* promoter is apparently regulated by phosphorylation. It has been shown that phosphorylation of NtrC has two major effects. Phosphorylation increases the ATPase activity of the conserved central domain, which in turn facilitates open-complex formation (155). Phosphorylation can also enhance DNA binding by promoting oligomerization. This effect has been demonstrated for both NtrC (118, 156) and the *B. subtilis* transcription factor Spo0A (5). The NtrC-binding site of the *glnA* enhancer in *Salmonella* species consists of two strong NtrC-binding sites which are separated from each other by three turns of the DNA helix (118). An NtrC dimer binds to each one of these sites. Phosphorylation of NtrC increases cooperative binding to the enhancer by presumably stimulating protein-protein interactions between dimers bound at each site (118). By analogy, the *fliF* promoter region contains two FlbD-binding sites separated by approximately three helical turns (159). The expression of *fliF* was tested in the presence of the constitutively active FlbD mutant to determine if activation enhanced repression. The constitutively active FlbD mutants decreased *fliF* expression two- to threefold compared with that in wild-type cells, indicating that activation of FlbD enhances its binding to the *fliF* promoter (159).

The biological significance of FlbD-mediated repression of *fliF* is not clear. The final gene in the *fliF* operon is *flbD*, indicating that this may be a mechanism to autoregulate the cellular levels of *flbD*. Another possibility is that it may be important to regulate the levels of *fliF* or other flagellar components within this operon. Perhaps inappropriate expression of *fliF* in the swarmer pole has some effect on the polar expression of ftr-containing promoters. The swarmer pole-specific activation of FlbD in late predivisional cells sets the stage for flagellar biogenesis in the progeny stalked and swarmer cell (Fig. 6). Swarmer pole-specific activation of flagellin transcription in the predivisional cell supplies the newly formed swarmer cell with flagellin mRNA. This permits the continued lengthening of the flagellar filament after cell division. On the other hand, stalked pole-specific expression of *fliF* in the predivisional cell readies the newly formed stalked cell for another round of flagellar biogenesis (Fig. 6).

Protein Localization

The newly synthesized flagellar components and chemotaxis machinery are localized to the swarmer pole of the predivisional cell (1, 3, 41, 92; reviewed in reference 130). Although

little is known about the mechanisms underlying flagellar protein localization, the positioning of the chemotaxis components has been examined in some detail. From these experiments, it should be possible to devise some general models for the mechanisms of flagellar protein localization. The chemotaxis machinery in *C. crescentus* is similar to that in enteric bacteria and consists of a methyl-accepting membrane receptor (McpA) as well as the cytoplasmic components required for transducing chemotactic signals to the flagellar motor (2). The chemotaxis components are expressed under cell cycle control in the predivisional cell (2). After synthesis, they are targeted to the swarmer pole (3, 41, 92). This was first demonstrated by assaying methyltransferase activity in newly formed progeny cells. These enzymatic activities were found exclusively in the progeny swarmer cells (41, 92). This is a consequence of protein localization and not localized gene expression, because the chemotaxis components are synthesized in the predivisional cell well before the cell division barrier has formed between the swarmer cell and stalked-cell compartments (2, 41, 92). Pulse-labeled McpA synthesized in the predivisional cell segregates only to the progeny swarmer cells (3). The subcellular location of the chemoreceptor has now been assayed by both immunofluorescence microscopy and immunogold electron microscopy (3). These experiments show that the McpA is localized to a discrete patch of membrane at the flagellated pole of the swarmer and predivisional cells. The highly conserved domain in the carboxyl terminus is required for polar localization. A deleted McpA that lacked the entire carboxyl terminus, including the highly conserved domain, failed to localize to the cell pole (3).

E. coli cells also possess polar-localized Mcp, suggesting that this is apparently a general phenomenon in bacteria (74). In *E. coli*, the Mcp is present at both poles of the dividing cell. Localization to both poles can also be observed in *C. crescentus* if the carboxyl-terminal domain of the receptor is deleted (3, 4). A deletion of 14 carboxyl-terminal amino acids effectively prevented the developmentally regulated proteolytic turnover of McpA (4). The wild-type McpA is normally degraded at the transition from swarmer to stalked cell. The truncated protein does not undergo proteolysis at this time and apparently is not degraded in the stalked pole compartment of the predivisional cell. These experiments show clearly that the appearance of McpA specifically at the swarmer pole of the predivisional cell is attributable to two distinct mechanisms. First, the protein is thought to be synthesized in both halves of the cell and then targeted to both poles of the predivisional cell before a cell division barrier has formed between the swarmer cell and stalked-cell compartments. Then McpA is degraded at the stalked-cell pole by an unidentified protease just before cell division. The signal for degradation is located in the carboxyl terminus of McpA (4). Localization of the truncated McpA to the stalked pole indicates that both poles of the predivisional cell are competent for McpA targeting. The mechanism of targeting McpA and flagellar proteins to the polar region of the membrane is unknown but promises to be of central importance in understanding how asymmetry is generated in the predivisional cell. These experiments should also provide insights into the mechanisms that localize other proteins in bacteria such as DNA replication proteins and the cell division machinery.

CONCLUDING REMARKS

Experimental analysis of the simple developmental program exhibited by *C. crescentus* has generated unique insights into the fundamental mechanisms underlying the control of DNA replication, cell cycle-regulated transcription, and the expression of positional information. One of the most stunning realizations is that bacterial cells are in fact highly organized, with some classes of proteins localized at the cell poles (3, 74). How are proteins targeted to a discrete patch of cell membrane? How are some proteins "sorted" to the pole while others are distributed throughout the cell membrane? Although these questions are important for our understanding of how the flagellum and chemotaxis machinery are organized and assembled, they are also directly relevant to the processes of cell division and chromosome partitioning. In both cases, proteins must be localized to a critical cellular location at a precise time in the cell cycle. The ease with which synchronized populations can be obtained and the intrinsic asymmetry of the *Caulobacter* cell cycle make it an ideal organism in which to examine the temporal and spatial regulation of cell division. Additionally, with the cloning of the *Caulobacter* origin of DNA replication and the identification of several key replication proteins, we are now poised for fundamental experiments dissecting the differential regulation of DNA replication in the swarmer and stalked poles of the predivisional cell.

Lastly, it is becoming increasingly clear that progression through the bacterial cell cycle is dependent on developmental checkpoints. For example, the initiation of DNA replication is required for the transcription of early flagellar genes. Likewise, the expression of middle and late flagellar genes is dependent on the expression and assembly of early flagellar gene products. Early flagellar gene expression is also required for the proper timing of cell division. How are these checkpoints established and regulated? Are there signal transduction pathways that transmit information regarding the completion of cell cycle events? The discovery of response regulator histidine kinases that are required for normal cell division in *C. crescentus* indicates that a signal transduction system of this type may very well exist (102, 139, 154). Future experiments with this simple developmental organism promise to yield even more exciting findings regarding how cells regulate growth and the assembly of cellular structures.

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