caused by the suppression of presynaptic Ca^{2+} current at the calyx-MNTB synapse. Direct modulation of the exocytotic machinery by mGluRs has been suggested from the reduced frequency of spontaneous miniature synaptic current by mGluR agonists (*11*); however, miniature frequency does not always display a direct relation to evoked transmitter release during presynaptic modulation (*23*). Although the possibility that washout may influence mechanisms downstream of Ca^{2+} currents cannot be excluded from this whole-cell study, our results strongly suggest that presynaptic Ca^{2+} channels are the main target for the mGluR-mediated presynaptic inhibition at the calyx of Held. Because P/Q-type Ca^{2+} channels mediate fast synaptic transmission at a variety of central synapses (*17*) and mGluRs are widely distributed in central presynaptic terminals (*24*), modification of the P/Q-type calcium channel may be a general mechanism underlying presynaptic modulation by mGluRs.

REFERENCES AND NOTES

1. S. Nakanishi, *Neuron* **13**, 1031 (1994); D. D. Schoepp and P. J. Conn, *Trends Pharmacol. Sci.* **14**, 13 (1993).
2. I. D. Forsythe and J. D. Clements, *J. Physiol. (London)* **429**, 1 (1990); A. Baskys and R. C. Malenka, *ibid.* **444**, 687 (1991).
3. Y. Hayashi *et al.*, *Nature* **366**, 687 (1993).
4. F. Sladeczek, A. Momiyama, T. Takahashi, *Proc. R. Soc. London Ser. B* **253**, 297 (1993).
5. K. Kobayashi, T. Manabe, T. Takahashi, *Science* **273**, 648 (1996); M. Yokoi *et al.*, *ibid.*, p. 645.
6. T. Takahashi, A. Momiyama, S. G. Cull-Candy, *J. Physiol. (London)* **446**, 549P (1992).
7. K. J. Swartz and B. P. Bean, *J. Neurosci.* **12**, 4358 (1992); Y. Sahara and G. L. Westbrook, *ibid.* **13**, 3041 (1993).
8. D. M. Lovinger, E. Tyler, S. Fidler, A. Merritt, *J. Neurophysiol.* **69**, 1236 (1993).
9. M. Yoshino and H. Kamiya, *Brain Res.* **695**, 179 (1995).
10. M. Barnes-Davies and I. D. Forsythe, *J. Physiol. (London)* **488**, 387 (1995).
11. J.-C. Poncer, H. Shinozaki, R. Miles, *ibid.* **485**, 121 (1995); I. Llano and A. Marty, *ibid.* **486**, 163 (1995).
12. B. Katz and R. Miledi, *ibid.* **192**, 407 (1967); N. Kawai and A. Niwa, *ibid.* **305**, 73 (1980).
13. A. R. Martin and G. Pilar, *ibid.* **168**, 443 (1963); E. F. Stanley and G. Goping, *J. Neurosci.* **11**, 985 (1991); H. Yawo and A. Momiyama, *J. Physiol. (London)* **460**, 153 (1993).
14. I. D. Forsythe, *J. Physiol. (London)* **479**, 381 (1994).
15. J. G. G. Borst, F. Helmchen, B. Sakmann, *ibid.* **489**, 825 (1995).
16. Transverse slices (150 μm thick) of superior olivary complex were prepared from Wistar or Lister Hooded rats killed by decapitation at the age of 8 to 18 days [F. A. Edwards, A. Konnerth, B. Sakmann, T. Takahashi, *Pfluegers Arch.* **414**, 600 (1989); I. D. Forsythe and M. Barnes-Davies, *Proc. R. Soc. London Ser. B* **251**, 143 (1993)]. The MNTB neurons and calyces were viewed with an upright microscope with a 63× objective (Zeiss) through a charge-coupled device camera. Each slice was superfused with artificial cerebrospinal fluid (aCSF) containing 120 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.25 mM Na_2HPO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM *myo*-inositol, 2 mM sodium pyruvate, 0.5 mM ascorbic acid, and 4 mM lactic acid (pH 7.4 with 5% CO_2 and 95% O_2); when measuring Ca^{2+} currents, 10 mM tetraethylammonium (TEA) chloride, 1 μM TTX, 10 μM bicuculline, and 0.5 μM strychnine were also included. The postsynaptic patch pipette contained 97.5 mM potassium gluconate, 32.5 mM KCl, 10 mM Hepes, 5 mM EGTA, and 1.0 mM $MgCl_2$ (pH 7.3). *N*-(2,6-diethylphenylcarbamoylmethyl)-triethylammonium bromide (QX314, 5 mM) was routinely included in the postsynaptic pipette solution to suppress action potential generation. The presynaptic pipette solution contained 110 mM CsCl, 40 mM Hepes, 0.5 mM EGTA, 1 mM $MgCl_2$, 2 mM adenosine triphosphate (ATP), 0.5 mM guanosine triphosphate (GTP), 12 mM phosphocreatinine, and 10 mM TEA (pH adjusted to 7.4 with CsOH); 10 mM cesium glutamate was added in replacement with equimolar CsCl in some experiments. For recording presynaptic potassium currents, TEA was omitted from aCSF solution and the pipette was filled with the postsynaptic solution including ATP, GTP, and phosphocreatinine at the above concentrations. Recordings were made at room temperature (22° to 25°C). The liquid junction potential between the pipette and aCSF (+2 mV) was not corrected. The electrode resistances were 5 to 10 megohms for the postsynaptic pipette and 7 to 12 megohms for the presynaptic pipette. Series resistances (10 to 30 megohms) were compensated by 50 to 90%. Records were low-pass filtered at 2 to 10 kHz and digitized at 5 to 27 kHz by means of a CED 1401 interface (Cambridge Electronic Design). Leak currents were subtracted for presynaptic currents by a P/N protocol.
17. T. Takahashi and A. Momiyama, *Nature* **366**, 156 (1993); D. B. Wheeler, A. Randall, R. W. Tsien, *Science* **264**, 107 (1994); S. Sivaramakrishnan and G. Laurent, *J. Neurosci.* **15**, 6576 (1995).
18. T. Takahashi, T. Tsujimoto, K. Onodera, I. D. Forsythe, M. Barnes-Davies, data not shown.
19. G. J. Augustine and M. P. Charlton, *J. Physiol. (London)* **381**, 619 (1986).
20. R. Anwyl, *Brain Res. Rev.* **16**, 265 (1991).
21. L.-G. Wu and P. Saggau, *Neuron* **12**, 1139 (1994); *J. Physiol. (London)* **485**, 649 (1995); J. S. Dittman and W. G. Regehr, *J. Neurosci.* **16**, 1623 (1996).
22. S. A. Siegelbaum, J. S. Carmado, E. R. Kandel, *Nature* **299**, 413 (1982).
23. W.-m. Fu and M.-m. Poo, *Neuron* **6**, 837 (1991); Y. Hori, K. Endo, T. Takahashi, *J. Physiol. (London)* **492**, 867 (1996).
24. H. Ohishi *et al.*, *Neuron* **13**, 55 (1994); *J. Comp. Neurol.* **360**, 555 (1995); R. Shigemoto *et al.*, *Nature* **381**, 523 (1996).

25. We thank M. Farrant, T. Manabe, and K. Kobayashi for comments on early drafts and Y. Ofune for DCG-IV. Supported by a grant-in-aid for scientific research from the Ministry of Education of Japan, the Wellcome Trust, and a travel grant from Yamada Science Foundation. I.D.F. is a Wellcome Senior Research Fellow in Basic Biomedical Sciences.

1 May 1996; accepted 13 August 1996

Identification of an Asymmetrically Localized Sensor Histidine Kinase Responsible for Temporally and Spatially Regulated Transcription

James A. Wingrove and James W. Gober*

Caulobacter crescentus undergoes asymmetric cell division, resulting in a stalked cell and a motile swarmer cell. The genes encoding external components of the flagellum are expressed in the swarmer compartment of the predivisional cell through the localized activation of the transcription factor FlbD. The mechanisms responsible for the temporal and spatial activation of FlbD were determined through identification of FlbE, a histidine kinase required for FlbD activity. FlbE is asymmetrically distributed in the predivisional cell. It is located at the pole of the stalked compartment and at the site of cell division in the swarmer compartment. These findings suggest that FlbE and FlbD are activated in response to a morphological change in the cell resulting from cell division events.

The generation of asymmetry is a critical event in the developmental programs of many organisms, including bacteria, yeast, and multicellular organisms (*1*). Asymmetrical cell divisions can arise either as a consequence of external influences on the cell or as a result of intrinsic signals built into the architecture of the cell. An intrinsically governed asymmetric cell division in the bacterium *Caulobacter crescentus* produces two morphologically distinct progeny cells: a stalked cell and a motile swarmer cell with a single polar flagellum (*2*). Several genes encoding external flagellar components are preferentially transcribed in the swarmer compartment of the late predivisional cell (*3*). Compartmentalized expression of late flagellar genes is attributable to the swarmer pole–specific activation of the transcriptional regulator protein FlbD (*4*). Activated FlbD also functions as a swarmer pole–specific repressor of the early *fliF* promoter (*5*, *6*). FlbD is homologous to a large family of two-component response regulators (*6*), members of which are active when phosphorylated on a conserved aspartate residue (*7*). The activating phosphate is typically obtained from sensor histidine kinases that are capable of undergoing autophosphorylation, often in response to an external environmental cue. Once phosphorylated, histidine kinases serve as phosphodonors for their cognate response regulators.

A potential candidate histidine kinase

Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, CA 90095–1569, USA.

*To whom correspondence should be addressed.

for FlbD is encoded by *flbE*, which lies within the same operon as *flbD* (Fig. 1A) and is essential for motility and the expression of late flagellar genes (6, 8). *flbE* encodes a 25-kD, apparently non–membrane-bound protein with no sequence homology to other known bacterial flagellar proteins. Detailed analysis of the *flbE* sequence, however, revealed that FlbE shares regions of homology with two-component histidine kinases (7, 8) (Fig 1A). To investigate whether FlbE functions as the cognate kinase for FlbD, we purified histidine-tagged FlbE for biochemical assays (9) (Fig. 1B). Purified His-FlbE was incubated in the presence of [γ-^{32}P]ATP (adenosine triphosphate) to determine whether FlbE can autophosphorylate (*10*). In this reaction, a phosphorylated product corresponding to the molecular mass of FlbE was formed, indicating that FlbE autophosphorylates. The rate of FlbE autophosphorylation was slow and reached maximal phosphorylation after 120 min, perhaps indicating that FlbE requires a cofactor or additional proteins to enhance the rate of autophosphorylation.

Phosphoamidite residues such as phospho-histidine, -arginine, or -lysine show a distinct pattern of acid-base stability, in which the phosphorylated residues are relatively base-stable and acid-labile (*11*). Phosphorylated FlbE was unstable under acidic conditions, whereas it retained its phosphate when incubated under basic conditions (*11*) (Fig. 1C), suggesting that FlbE is phosphorylated on a histidine residue—results supported by its homology to two-component histidine kinases. To examine whether phosphorylated FlbE could serve as a phosphodonor for FlbD, we incubated [^{32}P]FlbE under the same conditions as above and then added purified FlbD to a final concentration of 60 μM (*10*) (Fig. 1D). The results demonstrate that the phosphorylation of FlbD is dependent on the presence of phospho-FlbE in the reaction mixture. Purified FlbE protein in which the conserved His^{83} residue was changed to an Ala (H83A) is incapable of autophosphorylation (9, *10*) (Fig. 1D). To test whether phosphorylation of FlbD required the conserved Asp^{52} residue, we used a mutant FlbD containing an Asp^{52}→Glu mutation (D52E) in a phosphorylation assay with FlbE. Purified mutant FlbD cannot be phosphorylated by FlbE (9, *10*) (Fig. 1D). [This mutant protein also contains a Ser^{140}→Phe (S140F) mutation within the central domain. This change does not affect the ability of the protein to acquire phosphate from its kinase.] These results indicate that FlbE can function as a kinase for FlbD.

To determine whether FlbE is responsible for the compartment-specific activation of FlbD, we took advantage of the fact that FlbD serves as a swarmer compartment–specific repressor of the *fliF* promoter (5, 6). Active FlbD specifically inhibits the expression of the *fliF* promoter by binding to the promoter region (5, 6). If FlbE is required for swarmer pole–specific activation of FlbD, then strains lacking FlbE should exhibit a loss of repression. The *flbE* mutant strain UC3003 (*12*) exhibited a three- to fourfold increase in *fliF* promoter expression as assayed with a *lacZ* reporter fusion [9854

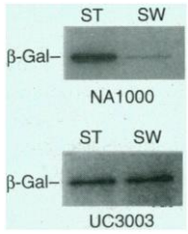

Fig. 2. FlbE is required for swarmer-compartment repression of the *fliF* promoter. Wild-type (NA1000) or Δ*flbE* (UC3003) *C. crescentus* swarmer cells containing a *fliF-lacZ* transcriptional fusion were synchronized by Ludox density centrifugation. Swarmer cells were placed in fresh M2 medium and were permitted to progress to the late predivisional stage (120 to 150 min). Proteins were labeled with ^{35}S-trans-label, the label was chased with nonradioactive methionine, and the cells were allowed to divide. After division, the progeny swarmer and stalked cells were isolated by Ludox centrifugation, and labeled β-Gal was immunoprecipitated. After SDS-PAGE, the labeled proteins were visualized by fluorography. The presence of labeled protein in the progeny cells indicates the location of synthesis in the predivisional cell.

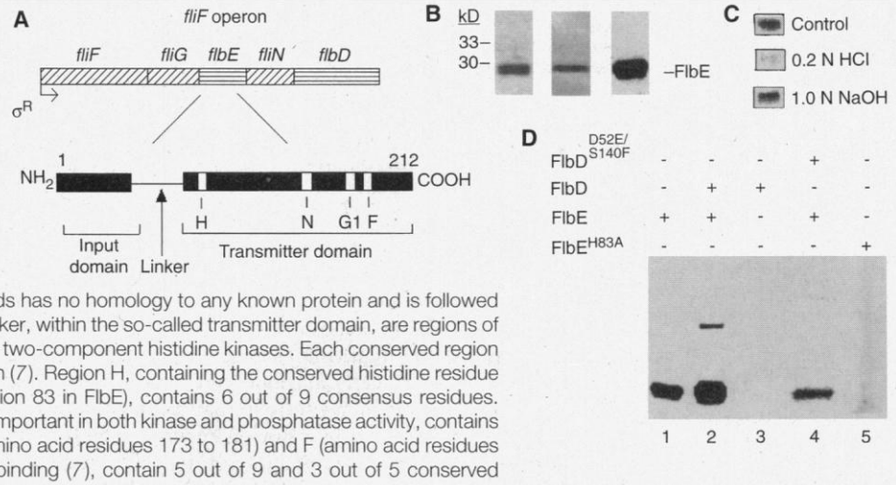

Fig. 1. Identification of FlbE as a histidine kinase. (**A**) Schematic diagram of the *C. crescentus fliF* operon. *fliF* encodes the flagellar M-ring, *fliG* and *fliN* encode flagellar switch proteins, and *flbD* encodes a σ^{54} transcriptional regulator that activates the expression of class III and IV flagellar promoters (5, 6). *flbE* is predicted to encode a 25-kD polypeptide with no homology to known flagellar structural components. Shown below the operon is a block diagram of the predicted *flbE* amino acid sequence denoting the relative locations of regions similar to other two-component histidine kinases. The NH_2-terminal domain of ~52 amino acids has no homology to any known protein and is followed by a glutamine-rich linker-like sequence. After the linker, within the so-called transmitter domain, are regions of sequence similar to the transmitter domains of other two-component histidine kinases. Each conserved region is designated with a letter after Kofoid and Parkinson (7). Region H, containing the conserved histidine residue known to be the site of autophosphorylation (position 83 in FlbE), contains 6 out of 9 consensus residues. Region N (amino acid residues 142 to 153), an area important in both kinase and phosphatase activity, contains 8 out of 12 consensus residues (7). Regions G1 (amino acid residues 173 to 181) and F (amino acid residues 184 to 188), thought to be involved in nucleotide binding (7), contain 5 out of 9 and 3 out of 5 conserved residues, respectively. Another potential nucleotide binding region, G2, appears to be missing from FlbE. The spacing of these regions within the protein also appears to be conserved in FlbE with respect to other histidine kinases. Sequences outside of these conserved regions vary among histidine kinases. In addition, there are also examples of histidine kinases that apparently lack one or more of these conserved regions, and yet are capable of functioning as histidine kinases in two-component systems (7). (**B**) (Left) Coomassie blue–stained gel showing the purified preparation of His-tagged FlbE. (Middle) Immunoblot of purified FlbE with monoclonal antibody directed against the His-tag leader sequence. (Right) Autophosphorylation assay (*10*). Phosphoimage of phospho-FlbE after incubation with [γ-^{32}P]ATP. FlbE does not autophosphorylate in the presence of [γ-^{32}P]GTP (guanosine triphosphate) or [α-^{32}P]dATP (*22*). (**C**) Assay of acid-base stability of phospho-FlbE (*11*). The autophosphorylation assay was done as described (*10*). The protein was subjected to gel electrophoresis, and the radioactive band was excised and exposed to either 0.2 N HCl, 1.0 N NaOH, or buffer as a control. After incubation, the gel slices were subjected to PhosphoImager analysis. FlbE phosphate is acid-labile and base-stable, indicating the presence of a phosphoamidite group such as phospho-histidine (*11*). (**D**) Phospho-FlbE can stimulate the phosphorylation of FlbD (*10*). His-FlbE or His-FlbE^{H83A} was incubated with [γ-^{32}P]ATP for 120 min at 30°C. Purified FlbD or FlbD$^{D52E/S140F}$ was then added to one of the reaction mixtures, and incubation continued for 30 min. Each reaction was then subjected to PAGE and analyzed by PhosphoImager. Lane 1, FlbE alone; lane 2, FlbE followed by the addition of FlbD; lane 3, FlbD alone with [γ-^{32}P]ATP; lane 4, FlbE followed by the addition of FlbD$^{D52E/S140F}$; and lane 5, FlbE^{H83A} alone.

U of β-galactosidase (β-Gal) in the Δ*flbE* strain compared with 2514 U in wild-type cells]. This increase in expression is comparable to that observed in mutant strains lacking FlbD (*5*, *8*) and is similar to results obtained by Newton and co-workers (*8*). To address whether this increase in promoter expression was due to the loss of swarmer pole–specific repression, we assayed compartment-specific expression of a *fliF-lacZ* reporter fusion in a synchronized population of *C. crescentus* predivisional cells (*13*). The wild-type strain exhibited a normal pattern of *fliF* promoter expression; the promoter was expressed at high levels in the stalked compartment and at relatively low levels in the swarmer compartment (Fig. 2). In marked contrast, the *flbE* mutant strain exhibited equal levels of *fliF* promoter expression in both swarmer and stalked compartments (Fig. 2). These results suggest that FlbE is responsible for the swarmer compartment activation of FlbD.

FlbD phosphorylating activity is under cell cycle control, peaking at the time when FlbD responsive genes are either activated or repressed (*4*). The levels of epitope-tagged FlbE (FlbE-M2) (*14*), however, are relatively constant throughout the cell cycle (*15*). Thus, the temporal regulation of FlbD-dependent genes cannot be ascribed to fluctuations in the levels of FlbE. FlbE may be activated in response to cell cycle cues as well as to an asymmetrically generated signal in the predivisional cell. FlbE may be specifically activated by a swarmer compartment–specific signal or, alternatively, may be held inactive by a signal found in the stalked compartment. One attractive possibility is that FlbE might respond to a cell cycle change in cellular morphology associated with cell division. For example, during sporulation in *Bacillus subtilis*, the serine phosphatase SpoIIE, which controls the inactivation of an anti–σ factor within the forespore compartment, is specifically localized to the forespore septum (*16*). We reasoned that the subcellular position of FlbE, like that of SpoIIE, might reflect its role in compartmentalized gene expression.

The subcellular distribution of FlbE-M2 was assayed by immunofluorescent microscopy (*17*). The distribution of FlbE-M2 varied according to the cell type. In swarmer cells, distinct regions of localization were generally not observed, although a few cells exhibited weak polar localization (Fig. 3H). After the cells had progressed to the predivisional stage, many had FlbE concentrated both at a single pole and at the mid-site of the cell, in the compartment opposite that of the polarly localized protein (Fig. 3, A and C). Most predivisional cells (82%) exhibited midcell localization either alone or in combination with localization at the pole (see legend to Fig. 3). In cells containing fluorescently marked membranes (*17*), which includes the stalk, the polarly local-

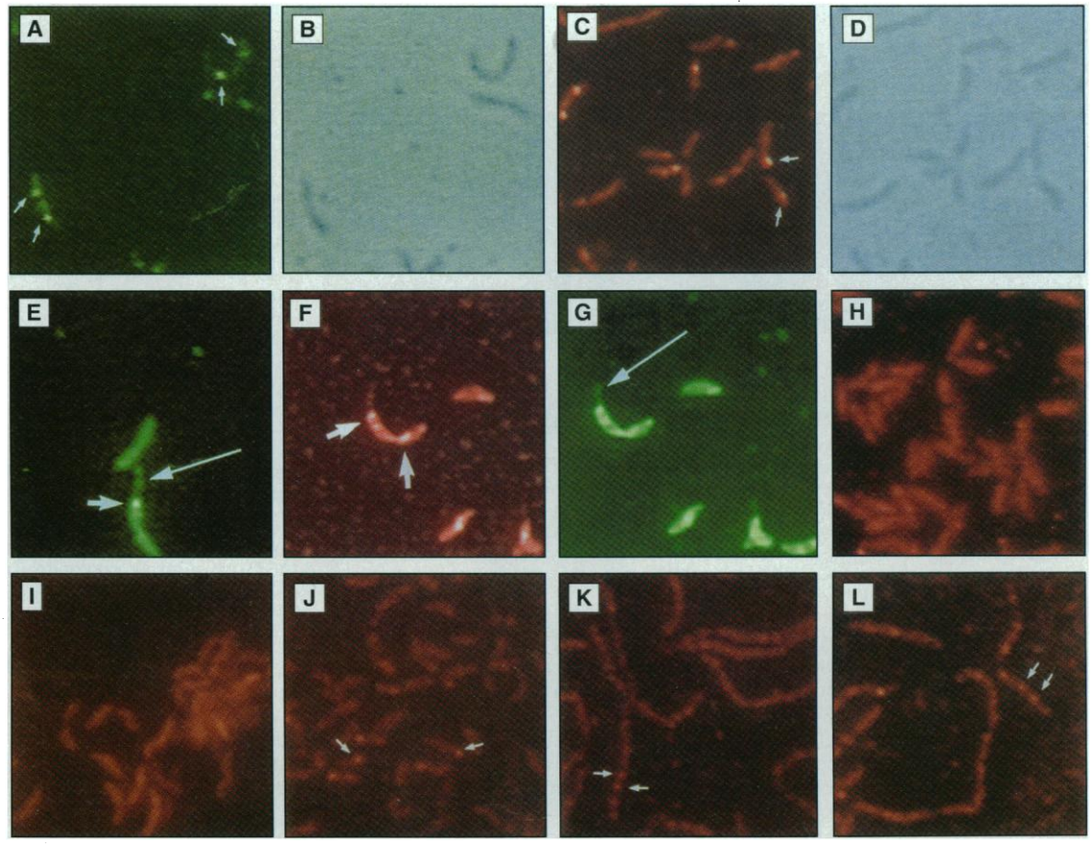

Fig. 3. Localization of FlbE by fluorescence microscopy (*18*). (**A**) Immunolocalization of epitope-tagged FlbE (FlbE-M2) showing staining of fluorescein-conjugated secondary antibody (bright staining areas). The arrows indicate staining at the midcell sites and the poles. (**B**) Phase-contrast light micrograph of the same field as in (A). (**C**) Immunolocalization of epitope-tagged FlbE (FlbE-M2) showing staining of streptavidin–Texas red bound to biotinylated secondary antibody. The arrows indicate staining at the midcell sites and the poles. Out of 78 predivisional cells observed, 18% exhibited staining only at the pole. Most predivisional cells exhibited localization of FlbE-M2 to the midcell site. Sixty-four percent showed staining at both the midcell and pole, and an additional 18% stained at the midcell only. (**D**) Phase-contrast light micrograph of the same field as in (C). (**E**) Immunolocalization of epitope-tagged FlbE (FlbE-M2) showing staining of fluorescein-conjugated secondary antibody (bright staining areas) in the presence of DiA. Stalks are indicated by narrow, long arrows. (**F**) Immunolocalization of epitope-tagged FlbE (FlbE-M2) showing staining of streptavidin–Texas red bound to biotinylated secondary antibody in cells costained with DiA. Arrows indicate localized FlbE. (**G**) Visualization of membrane with DiA, showing the same field as in (F). Stalks are indicated by the narrow, long arrow. (**H**) Immunolocalization of epitope-tagged FlbE (FlbE-M2) showing staining of streptavidin–Texas red bound to biotinylated secondary antibody in a purified swarmer cell population. (**I**) β-Gal staining of streptavidin–Texas red bound to biotinylated secondary antibody. (**J**) Immunolocalization of epitope-tagged methyl-accepting chemotaxis receptor (McpA-M2) showing staining of streptavidin–Texas red bound to biotinylated secondary antibody. Arrows indicate polar localization. (**K** and **L**) Immunolocalization of epitope-tagged FlbE (FlbE-M2) showing staining of streptavidin–Texas red bound to biotinylated secondary antibody in class II flagellar mutant *fliP*::Tn5 (SC1048) and Δ*fliF* (LS1298) (*23*), respectively. Arrows indicate regions of localization along the cell length.

ized protein resided within the stalked compartment, whereas FlbE-M2 at the midcell site appeared to be within the smaller swarmer compartment (Fig. 3, E to G). As controls, we assayed the distribution of two proteins. β-Gal was found evenly distributed in all cell types (Fig. 3I), whereas a methyl-accepting chemotaxis receptor–M2 fusion (*18*) was shown to be polarly localized (Fig. 3J).

We also examined the subcellular localization of FlbE-M2 in class II flagellar mutant backgrounds that have a characteristic defect in proper cell division, resulting in the formation of filamentous cells. In both SC1048 (a *fliP* mutant strain) and LS1298 (a strain lacking the M-ring), FlbE-M2 localized to discrete regions that might be potential cell division sites, in addition to being found at the pole (Fig. 3, K and L). These results suggest that FlbE is recognizing some cellular "landmark" found at both the pole and midcell site.

We next examined whether the localization of FlbE was responsible for the activation of the transcription factor FlbD. Because the NH_2-terminal input domain of this class of protein is responsible for sensing environmental cues, we reasoned that a dominant negative mutant of FlbE could be constructed by fusing this domain to a partially deleted β-Gal gene (*19*). The resulting fusion had no β-Gal activity. This *flbE-lacZ* fusion (Δ6Z) was introduced into wild-type *C. crescentus* on a multicopy plasmid (*20*). As with epitope-tagged FlbE, the fusion protein was localized to the midcell and the poles of predivisional cells and appeared to decrease the localization of FlbE-M2 when expressed in the same strain (Fig. 4A). This result indicates that the NH_2-terminal input domain is sufficient to direct the localization of FlbE and can compete for binding with FlbE-M2. We then examined whether expression of the fusion protein affected the expression of FlbD-regulated promoters (Fig. 4B). Cells containing the fusion protein exhibited at least a 50% decrease in the expression of *fljK* and *fljL*, two promoters that are activated by FlbD. Likewise, the *fliF* promoter, which is repressed by FlbD, showed an increase in expression (Fig. 4B). Pole-specific transcription of *fljK* was then assayed in cells expressing the FlbE-LacZ fusion (Fig. 4C). In wild-type cells, expression of a *fljK-lacZ* transcription fusion in the swarmer compartment was ~ninefold as high as that in the stalked cell compartment of predivisional cells. In contrast, expression of the *fljK-lacZ* reporter fusion in the swarmer compartment of cells containing the FlbE-LacZ fusion protein was only threefold as high as that in the stalked cell compartment. Thus, cells expressing the FlbE-LacZ fusion are impaired in their ability to activate swarmer pole–specific transcription of FlbD-dependent promoters. These results suggest that the expression of the FlbE-LacZ fusion protein interferes with the activation of FlbD by competing with wild-type FlbE for binding at specific sites in the predivisional cell.

In bacterial two-component regulatory systems, an environmental cue often triggers a sensor histidine kinase to autophosphorylate. By analogy, we propose that FlbE senses an internal cue, a cell cycle alter-

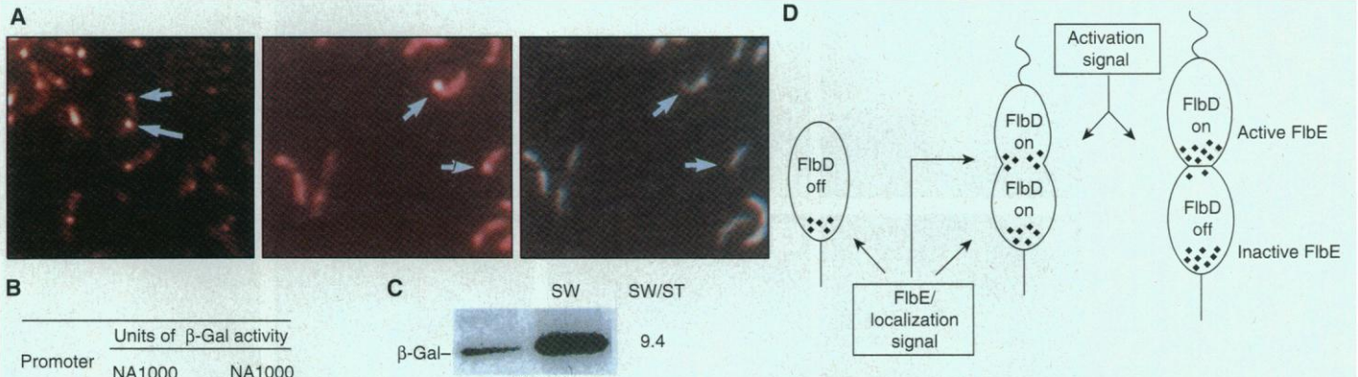

Fig. 4. Localization of FlbE is required for proper spatial expression of FlbD-regulated promoters. (**A**) (Left) Immunolocalization of a fusion protein consisting of the NH_2-terminal 52 amino acids of FlbE with a truncated β-Gal gene (Δ6Z) showing staining of streptavidin–Texas red bound to biotinylated secondary antibody. Arrows indicate localization at the midcell and pole. This fusion is recognized by the monoclonal antibody to β-Gal but is enzymatically inactive. (Middle) Immunolocalization of Δ6Z showing staining of streptavidin–Texas red bound to biotinylated secondary antibody in the presence of FlbE::M2 being expressed from the plasmid pMR4. Arrows indicate localization at the pole. (Right) Same as middle panel, visualizing the distribution of FlbE::M2 being recognized by fluorescein-conjugated secondary antibody. Note the decrease of FlbE::M2 localization in the presence of Δ6Z. The positive localization controls for this experiment are shown in Fig. 3, E and F. (**B**) Effect of FlbE::LacZ (Δ6Z) on expression levels of FlbD-regulated promoters. The Δ6Z fusion was introduced into wild-type NA1000 cells containing either *fljK-lacZ*, *fljL-lacZ* (two promoters activated by FlbD) or *fliF-lacZ* (a class II promoter repressed by FlbD) transcription fusions (*23*). β-Gal activity was assayed in each of these strains. Expression levels in these strains are compared to those obtained in wild-type NA1000 cells expressing only wild-type *flbE*. Unit activity represents the mean values obtained with three cultures on which the assay was performed in duplicate. (**C**) Effect of FlbE::LacZ (Δ6Z) on swarmer compartment expression of *fljK*. Swarmer compartment expression of a *fljK-lacZ* transcriptional fusion was assayed in wild-type cells (NA1000) or cells containing the Δ6Z fusion. The assay was performed as described in Fig. 2. The ratio of labeled β-Gal in the progeny swarmer and stalked cells was obtained by densitometry. (**D**) Model depicting the relation between FlbE localization and FlbD activation. In predivisional cells, FlbE becomes concentrated at the midcell site and the pole. We hypothesize that the FlbE located at the midcell site becomes active to transfer phosphate to FlbD, because most predivisional cells (82%) exhibit staining at the midcell location (see text). Late in the cell cycle, the midcell-localized FlbE is trapped in the swarmer compartment. This conclusion is based on membrane and stalk staining and the relative size of the two compartments late in the cell cycle. Therefore, later in the cell cycle after the formation of the cell division plane, active FlbE at the midcell site phosphorylates only those molecules of FlbD within the swarmer compartment. FlbE localized at the stalked pole is apparently unable to transfer phosphate to FlbD. It is possible that FlbE is held in an inactive conformation at this pole or that an as-yet-unidentified stalked pole–specific phosphatase activity may exist. We hypothesize that these events result in swarmer compartment–specific expression of late flagellar promoters and repression of the early *fliF* operon promoter.

ation in the cell morphology, which results in the activation of temporal and spatial transcription of flagellar genes (Fig. 4D). We envision that the FlbE input domain associates with a protein or cellular structure involved in cell division. This idea is consistent with the observed midcell and stalked pole localization pattern. Cell division machinery is likely to be present at both cellular locations: at the midcell, where cell division actually occurs, and at the stalked pole, where stalk biogenesis requires new cell wall growth. Late in the cell cycle, we hypothesize that FlbE located at the midcell is activated and, as a consequence of cell division, is eventually trapped in the swarmer compartment of the predivisional cell (Fig. 4D). FlbE localized to the stalked pole is apparently unable to activate FlbD. One possibility is that localization determinants at the stalked pole and midcell are not sufficient to activate FlbE, suggesting that a distinct signal, perhaps initiation of a midcell division event, is required for FlbE activation. In this instance, the localization of FlbE to the stalked pole may serve as a way of sequestering FlbE from the activation signal at the midcell site. Alternatively, other factors such as a FlbD-specific phosphatase activity may be present at the stalked pole.

Chromosome partitioning or septum formation are two cell cycle events that could be utilized by bacterial signal transduction systems to govern temporal and spatial gene expression. This possibility is evident during *B. subtilis* sporulation, in which asymmetric septum formation is coupled to SpoIIE-directed activation of forespore-specific transcription (*16*). These events may also regulate the activity of the *C. crescentus* response regulator CtrA, which has a role in controlling both DNA replication and the transcription of early flagellar genes (*21*). We propose that the temporal and spatial activation of FlbD and FlbE, a two-component signal transduction system, is triggered by early cell division events. The coupling of FlbE and FlbD activation to cell division would serve to coordinate the assembly of the flagellum with the genesis of a daughter swarmer cell.

REFERENCES AND NOTES

- R. Horvitz and I. Herskowitz, *Cell* **68**, 237 (1992).
- Y. V. Brun, G. Marczynski, L. Shapiro, *Annu. Rev. Biochem.* **63**, 419 (1994); J. W. Gober and M. V. Marques, *Microbiol. Rev.* **59**, 31 (1995).
- J. W. Gober, R. Champer, S. Reuter, L. Shapiro, *Cell* **64**, 381 (1991); J. W. Gober and L. Shapiro, *Mol. Biol. Cell* **3**, 913 (1992).
- J. A. Wingrove, E. K. Mangan, J. W. Gober, *Genes Dev.* **7**, 1979 (1993).
- J. A. Wingrove and J. W. Gober, *ibid.* **8**, 1839 (1994).
6. G. Ramakrishnan and A. Newton, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2369 (19 A. Benson, J. Wu, A. Newton, *Res. Microbiol.* **145**, 420 (1994); A. K. Benson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4989 (1994); J. Wu, A. K. Benson, A. Newton, *J. Bacteriol.* **177**, 3241 (1995).
- J. S. Parkinson and E. C. Kofold, *Annu. Rev. Genet.* **26**, 71 (19 J. S. Parkinson, *Cell* **73**, 857 (19 L. A. Alex and M. I. Simon, *Trends Genet.* **10**, 133 (19 R. V. Swanson, L. A. Alex, M. I. Simon, *Trends Biochem. Sci.* **19**, 485 (1994).
8. G. Ramakirshnan, J.-L. Zhao, A. Newton, *J. Bacteriol.* **176**, 7587 (1994).
9. Overexpression and purification of FlbE. A Bam HI site was introduced by site-directed mutagenesis [T. A. Kunkel and J. D. Roberts, *Methods Enzymol.* **154**, 367 (1987)] at the initiation codon of FlbE. The resulting fragment was cloned into the Bam HI–Eco RI sites of pTrcHisA (Invitrogen) and introduced into the *Escherichia coli* strain YMC10 [K. Backman, Y. M. Chen, B. Magasanik, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3743 (1981)]. Protein was induced by the addition of 0.1 mM isopropyl-β-D thiogalactopyranoside to a 0.5-liter, mid–log phase culture, which was subsequently grown for 180 min at 37°C. Cells were harvested by centrifugation and were resuspended in buffer containing 20 mM tris-HCl (pH 8.0), 0.5 M NaCl (buffer A), and 5 mM imidazole. Cells were lysed by French pressure cell, and the supernatant was applied to a 2-ml nickel-Sepharose column. The column was first rinsed with 10 column volumes of buffer A containing 60 mM imidazole, followed by a 10-ml elution step with buffer A containing 1 M imidazole. The His-FlbE obtained was judged to be 95% pure by SDS–polyacrylamide gel electrophoresis (PAGE). Samples containing pure his-FlbE were pooled and dialyzed extensively against buffer containing 20 mM tris-HCl (pH 8.0), 0.1 M NaCl, 12.5 mM $MgCl_2$, and 5% glycerol. $FlbE^{H83A}$ was generated by changing His^{83} to an Ala through the use of a mutagenic polymerase chain reaction (PCR) primer containing an endogenous Ban II site 3′ to the histidine codon. The PCR product containing the mutated NH_2-terminal portion of FlbE was used as a cassette to replace the wild-type sequence with Sal I and Ban II sites. The mutant gene was placed into pTrcHisA and overexpressed and purified as described above. $FlbD^{D52E/S140F}$ was generated, overexpressed, and purified as described (*5*).
10. Phosphorylated FlbE was obtained by incubating 2 μM purified FlbE (final volume, 50 μl) in the presence of 50 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, Amersham) for 120 min at 30°C. Phosphorylated FlbE (5 μl) was then removed and added either to 30 μl of buffer containing 20 mM tris-HCl (pH 8.0), 0.1 M NaCl, and 12.5 mM $MgCl_2$ or to 25 μl of the same buffer containing pure FlbD (60 μM). As a control, 60 μM FlbD was incubated alone in the presence of 20 μCi of [γ-^{32}P]ATP. All reactions were allowed to proceed for 30 min at 30°C, after which time they were quenched by the addition of SDS-PAGE sample buffer. Phosphorylated products were separated by 15% SDS-PAGE and visualized by PhosphorImager analysis. Reactions with mutant $FlbE^{H83A}$ and $FlbD^{D52E/S140F}$ were done as described above.
- D. E. Koshland, *J. Am. Chem. Soc.* **74**, 2286 (19 D. E. Hultquist, *Biochim. Biophys. Acta* **153**, 329 (1968). Acid-base stability analysis was done as described by D. Burbulys, K. A. Trach, and J. A. Hoch [*Cell* **64**, 545 (1992)]. The phosphorylation assay was done as described above. After electrophoresis, the gel slices containing ^{32}P-FlbE were incubated in either 0.2 N HCl or 1.0 N NaOH for 45 min at 55°C.
12. To create a *flbE* deletion mutant (UC3003), we integrated a pJBZ-*sacB* vector [J. A. Wingrove, unpublished c H. P. Schweizer, *Mol. Microbiol.* **6**, 1195 (1992)] containing a 490–base pair (bp) internal fragment of the *flbE* gene into the *C. crescentus* genome by homologous recombination. Integrants were selected for resistance to kanamycin and judged nonmotile by microscopy. The disrupted strain was subcultured three successive times onto PYE plates containing 5% sucrose. Genomic DNA was isolated from sucrose-tolerant, nonmotile, kanamycin-sensitive colonies, digested with Eco RI, and then probed by Southern (DNA) hybridization with a 1150-bp Eco RI fragment containing the entire *flbE* gene. The *flbE* deletion strain (UC3003) was assayed for motility on PYE swarmer plates containing 0.3% agar. Complementation of the UC3003 phenotype could be accomplished by introducing a 796-bp Sal I–Eco RI fragment containing *flbE* on the plasmid pMR4, indicating that the deletion affected only *flbE* and not other genes within the *fliF* operon.
13. For assay of compartmentalized gene expression, the wild-type NA1000 strain or the *flbE* null strain (UC3003) harboring the *fliF-lacZ* plasmid cells was synchronized as described (*3*) and was allowed to proceed through the cell cycle. At the late predivisional stage, cells were pulsed for 10 min with 50 μCi of Trans ^{35}S-label (ICN, Irvine, CA), followed by the addition of cold methionine. Cells were allowed to divide, and subsequent progeny cells were re-isolated by Ludox density centrifugation. Proteins were immunoprecipitated, subjected to SDS-PAGE, and visualized by fluorography.
14. To assay subcellular distribution of FlbE, a version of FlbE containing a seven–amino acid M2 epitope at the COOH-terminal end of the protein was constructed as described (*4*). The stop codon was removed by introducing a Bam HI site by PCR. The PCR product was cloned into the Hind III–Bam HI sites of pJM21, creating an in-frame fusion with the seven–amino acid M2 epitope. The entire *flbE-M2* fusion gene was then removed on a Hind III–Spe I fragment and placed into the Hind III–Xba I sites of pMR4.
15. J. A. Wingrove and J. W. Gober, unpublished data.
16. F. Arigoni, K. Pogliano, C. D. Webb, P. Stragier, R. Losick, *Science* **270**, 637 (1995); L. Duncan, S. Alper, F. Arigoni, R. Losick, P. Stragier, *ibid.*, p. 641.
17. Immunofluorescence microscopy was performed on strains containing either FlbE-M2, MCP-M2, or β-Gal expressed from the *fliF* promoter, essentially as described [J. R. Maddock and L. Shapiro, *Science* **259**, 1717 (1993)]. Fluorescent membrane labeling was accomplished by adding DiA (Molecular Probes, OR) to a concentration of 20 μg/ml during the primary antibody incubation step.
- M. R. K. Alley, J. Maddock, L. Shapiro, *Genes Dev.* **6**, 825 (19 *Science* **259**, 1754 (1993).
19. The FlbE-LacZ protein fusion was constructed by creating an in-frame fusion at codon 54 of *flbE* and codon 8 of *lacZ*. The fusion contains the *flbE* upstream sequences through the putative input domain. The *flbE* sequence ends at the start of the glutamine-rich linker (Fig. 1). The *lacZ* portion of the fusion contains sequences up to amino acid residue 652 and is enzymatically inactive.
20. pJS14 is a multicopy plasmid with broad host range and is derived from pBBR1MCS [M. E. Kovach, R. W. Phillips, P. H. Elzer, R. M. I. Roop, K. M. Peterson, *Biotechniques* **16**, 800 (1994)].
- K. C. Quon, G. T. Marczynski, L. Shapiro, *Cell* **84**, 83 (1996).
22. J. A. Wingrove and J. W. Gober, data not shown.
23. Bacterial strains, plasmids, and growth conditions. *Caulobacter crescentus* strain NA1000 was used as a wild-type, synchronizable motile strain. SC1048 is a mutant strain with a Tn5 insertion in the *fliP* gene (*13*). LS1298 is a mutant strain containing a disruption in the *fliF* gene [U. Jenal and L. Shapiro, *EMBO J.* **15**, 2393 (1996)]. Construction of the *fliF-lacZ*, *flbG-lacZ*, *fljL-lacZ*, and *fljK-lacZ* transcription fusions are described elsewhere (*3, 4*). Unless otherwise noted, strains were grown either in PYE media [J. S Poindexter, *Bacteriol. Rev.* **28**, 231 (1964)] or minimal M2-glucose media [I. Contreras, L. Shapiro, S. Henry, *J. Bacteriol.* **135**, 1130 (1978)] at 31°C.
24. We thank U. Jenal for LS1298, M.R.K. Alley for the MCP-M2 construct, and J. Maddock for suggesting the use of DiA. We are also grateful to L. Shapiro, R. Losick, and members of our laboratory for helpful comments on the manuscript. J.A.W. was supported by USPHS predoctoral fellowship GM-07104 and a University of California Dissertation Year Fellowship. This work was supported by Public Health Service grant GM48417 from the National Institutes of Health and Junior Faculty Research Award JFRA-466 from the American Cancer Society (J.W.G.).

28 February 1996; accepted 21 August 1996