Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 1443–1448, February 1998
Biochemistry

An essential, multicomponent signal transduction pathway required for cell cycle regulation in *Caulobacter*

JIANGUO WU*, NORIKO OHTA*, AND AUSTIN NEWTON†

Department of Molecular Biology, Princeton University, Princeton, NJ 08544

Communicated by Thomas E. Shenk, Princeton University, December 18, 1997 (received for review October 7, 1997)

ABSTRACT **Cell differentiation and division in *Caulobacter crescentus* are regulated by a signal transduction pathway mediated by the histidine kinase DivJ and the essential response regulator DivK. Here we report genetic and biochemical evidence that the DivJ and DivK proteins function to control the activity of CtrA, a response regulator required for multiple cell cycle events, including flagellum biosynthesis, DNA replication, and cell division. Temperature-sensitive *sokA* (suppressor of *divK*) alleles were isolated as extragenic suppressors of a cold-sensitive *divK* mutation and mapped to the C terminus of the CtrA protein. The *sokA* alleles also suppress the lethal phenotype of a *divK* gene disruption and the cold-sensitive cell division phenotype of *divJ* mutants. The relationship between these signal transduction components and their target was further defined by demonstrating that the purified DivJ kinase phosphorylates CtrA, as well as DivK. Our studies also showed that phospho-CtrA activates transcription *in vitro* from the class II flagellar genes and that their promoters are recognized by the principal *C. crescentus* sigma factor σ^{73}. We propose that an essential signal transduction pathway mediated by DivJ, DivK, and CtrA coordinates cell cycle and developmental events in *C. crescentus* by regulating the level of CtrA phosphorylation and transcription from σ^{73}-dependent class II gene promoters. Our results suggest that an unidentified phosphotransfer protein or kinase (X) is responsible for phosphoryl group transfer to CtrA in the proposed DivJ $\Rightarrow$ DivK $\Rightarrow$ X $\Rightarrow$ CtrA phosphorelay pathway.**

Bacterial two-component signal transduction systems control a wide array of physiological processes in response to a variety of environmental conditions. These systems typically contain a sensor kinase, which is autophosphorylated on a conserved histidine residue, and a cognate response regulator containing a conserved aspartate residue to which the phosphoryl group is transferred (1, 2). This same protein family functions in multistep phosphorelay pathways (3). Recent results have provided evidence that sensor kinases and response regulators also play essential roles in the coordination of cell cycle and developmental events in the aquatic bacterium *Caulobacter crescentus* (4). The histidine kinase DivJ (5) and response regulator DivK (6) have been implicated in a signal transduction pathway required for cell division initiation. Another response regulator, CtrA, has been identified as a transcription factor responsible for the expression of multiple cell cycle-regulated genes (7). Our results now indicate that DivJ and DivK proteins function as part of a multicomponent signal transduction pathway to control the transcriptional activity of CtrA during the cell cycle.

Members of two-component signal transduction pathways regulating cell cycle events in *C. crescentus* were originally identified in a pseudoreversion analysis of *pleC*, a pleiotropic developmental gene required for motility and polar morphogenesis (8). Several of the *pleC* suppressors displayed conditional cell division defects (9) and were mapped to *divJ* (5), which, like *pleC* (10), encodes a histidine protein kinase, and to *divK* (6), which encodes the first essential response regulator identified in bacteria. DivK is a 125-residue polypeptide that belongs to the subfamily of single-domain response regulators that includes the chemotactic protein CheY of *Salmonella typhimurium* (11) and the sporulation protein SpoOF of *Bacillus subtilis* (12). Although DivJ and DivK appear to play central roles in regulating initiation of cell division in *C. crescentus* (6), the PleC kinase may be more directly involved in regulating motility, chemotaxis, and stalk formation (13). Isolation of *pleC* suppressors mapping to *divJ* and *divK* is consistent with a tight interconnection of cell cycle and developmental regulation in *C. crescentus*, as indicated originally by an analysis of developmental defects in conditional cell division cycle mutants (14, 15).

CtrA is also an essential response regulator required for cell cycle regulation in *C. crescentus* (7). It functions *in vivo* as a global regulator controlling transcription of class II flagellar genes, as well as the DNA methylase gene *ccrM* (16) and *hemE*, whose expression is associated with cell type-specific initiation of chromosome replication in the stalked cell (17). Promoters of these cell cycle-regulated genes contain a conserved CtrA-binding site (7), or "CtrA-Box", and an unusual consensus sequence originally thought to be recognized by a novel sigma factor (18, 19).

Here we present genetic and biochemical evidence that the DivJ and DivK proteins function to control CtrA activity in the transcription of cell cycle-regulated genes. Moreover, our *in vitro* transcriptional analysis of class II flagellar genes indicates that CtrA-regulated promoters are recognized by the principal *C. crescentus* sigma factor, σ^{73}. These results strongly support a model in which DivJ, DivK, and CtrA, probably in conjunction with an unidentified transphosphorylase or kinase, function in an essential signal transduction pathway to regulate the activity of σ^{73} in the transcription of cell cycle and developmental genes.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media. *C. crescentus* strains were derived from wild-type strain CB15 (ATCC19089) and grown in either PYE medium (20) or minimal M2-glucose medium (20) supplemented with antibiotics as indicated. The cold-sensitive (cs) *divK341*, *divJ331*, and *divJ332* alleles have been described (9). *Escherichia coli* strain DH5α was used for propagating plasmids and cultured at 37°C in ML medium (6)

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: RNAP, RNA polymerase; E, core RNA polymerase; cs, cold sensitive; ts, temperature sensitive.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF021339).

*J.W. and N.O. contributed equally to this work.

†To whom reprint requests should be addressed. e-mail: anewton@molecular.princeton.edu.

supplemented with ampicillin (50 μg/ml), tetracycline (15 μg/ml), or kanamycin (50 μg/ml) as required.

Isolation of Suppressors of *divK* Mutations and a Linked Tn5 Insertion. Colonies of cs *divK* mutants grown on PYE plates at the permissive temperature (37°C) were stabbed in 0.35% agar swarm plates (20), and revertants were isolated from flares formed after incubation for 3–4 days at the nonpermissive temperature (24°C). Mot$^+$ cells were purified and examined at 24°C and 37°C on swarm plates to identify temperature-sensitive (ts) revertants. Two revertants containing the ts *sokA301* (PC3241) and *sokA302* (PC3242) mutations, which were isolated from 300 revertants of cs strain PC4743 (*divK356*), displayed a filamentous phenotype at 37°C. None of the 1,000 revertants of cs strains PC4744 (*divK357*) and PC4746 (*divK358*) displayed a ts phenotype.

Tn*5* insertion *zzz471*::Tn*5*, which is *ca*. 15% linked to the *sokA301* mutation, was isolated as described previously (21) and displayed the same genetic linkage to *sokA302* by transduction. This Tn*5* insertion was used to construct the isogenic *sokA* strains PC3247 (*sokA301*) and PC3248 (*sokA302*) in wild-type strain CB15.

Complementation and Cloning of *sokA301* and *sokA302*. Cosmids from a pLAFR1–7 library containing random inserts of *C. crescentus* DNA (5) were isolated that complemented the ts *sokA301* mutation of strain PC3247 at 37°C. DNA fragments from the cosmid were subcloned in plasmid pRK2L1 (22) or pRK2L10, a derivative of pRK290 (23) that contains the polylinker sites from lambda phage tg131 (Amersham). DNA fragments containing the wild-type *ctrA* gene (7) and the *sokA301* and *sokA302* alleles of *ctrA* were cloned by PCR amplification of DNA from strain CB15 and the two mutant strains, respectively. DNA-sequencing reactions were carried out on double-stranded DNA cloned in pBluescript IIKS+ by using the Sequenase 2.0 sequencing kit with 7-deaza-dGTP (United States Biochemical).

Protein Purification. The C-terminal fragments of histidine kinases DivJ (DivJ′) and PleC (PleC′) and the full-length response regulator DivK were purified by using published procedures (6). Purification of the principal *C. crescentus* sigma factor, σ^{73}, and the RNA polymerase (RNAP) core has also been described (24). Native CtrA protein was overproduced and purified from isopropyl β-D-thiogalactopyranoside (IPTG)-induced *E. coli* strain BL21 (DE3) carrying the *ctrA* gene fused to the initiation codon of the T7 gene 10 in the T7–7 vector (25). Inclusion bodies formed by the CtrA protein were solubilized with 6 M guanidine-HCl. The protein was greater than 95% pure, as judged by Coomassie blue staining of SDS/PAGE gels after renaturation and purification by chromatography on DEAE-cellulose and heparin-agarose columns.

Phosphorylation Assays. *In vitro* phosphorylation assays have been described (6). Histidine kinases (1 μM) were incubated at 25°C for 15 min in phosphorylation buffer containing 5 μCi [γ-^{32}P]ATP followed by the addition of the response regulator(s) (10 μM). The phosphotransfer reaction was carried out at 25°C for 20 min. ^{32}P-labeled proteins were assayed by electrophoresis on 12.5% SDS/PAGE gels and visualized by autoradiography.

***In Vitro* Transcription Assays.** The formation of open complexes at promoters was measured in run-off transcription assays (26) by using as DNA templates either the 290-bp *Bam*HI-*Hin*dIII fragment from plasmid pJW001 containing the *fliF* promoter (27) or a 315-bp *Bam*HI-*Hin*dIII PCR fragment from plasmid pJW002 containing the *fliL* promoter (28). RNAP holoenzyme was reconstituted by addition of purified σ^{73} protein to purified *C. crescentus* RNAP core and incubation at 4°C for 10 min (24). CtrA (10 μM), FlbD (10 μM), and DivJ (1 μM) proteins were added as indicated. RNA products were analyzed by 7 M urea/PAGE with end-labeled *Sau*3A fragments of pUC18 as size markers.

The accession number AF021339 has been assigned for the partial *ctrA* sequence described here.

RESULTS

Isolation of Suppressors of *divK* Cell Division Mutations. To identify downstream components in the signal transduction pathway mediated by the response regulator DivK we carried out a pseudoreversion analysis of conditional *divK* strains PC4743, PC4744, and PC4746 (*Materials and Methods*). Because these cell division mutants have a severe cs phenotype and exhibit extensive filamentation at the nonpermissive temperature (9), they form tight swarms on 0.35% agar plates at 24°C, as shown for the *divK356* mutant (Fig. 1*A*). We isolated a total of 1,300 independent revertants from flares on swarm plates at 24°C that were Mot$^+$, Div$^+$. The revertants were then screened for Mot$^-$, Div$^-$ defects at 37°C. Two such ts mutants, *sokA301* strain PC3241 (Fig. 1*B*) and *sokA302* strain PC3242 (data not shown), were isolated.

To map the *sokA301* mutation, we isolated the linked Tn*5* insertion *zzz471*::Tn*5* and showed that it is also linked by transduction to the *sokA302* mutation. The Tn5 insertion is unlinked to the *divK* locus, however, indicating that the *sokA* alleles are outside suppressors. We confirmed this conclusion by showing that the *sokA301* and *sokA302* mutations conferred a ts swarm defect (Fig. 1 *D* and *E*) in the wild-type, strain CB15 background and that they also suppressed the cs cell division phenotype of the well characterized *divK341* allele (ref. 6; data not shown).

Electron micrographs of the *sokA301*, *divK*$^+$ strain PC3247 showed that it forms long, curled filaments *ca*. 12 hr after shifting from 24°C to 37°C (Fig. 1*F*) and arrests growth upon longer incubation at 37°C. Cultures of the *sokA301* mutant grown at the permissive temperatures of 24°C, by contrast, contained mostly single cells and short filaments of two to three cell lengths. The cells were flagellated, highly motile, and, like supermotile *pleD* mutants (29), failed to form stalks at 24°C (Fig. 1*F*). The isogenic *sokA302* strain PC3248 dis-

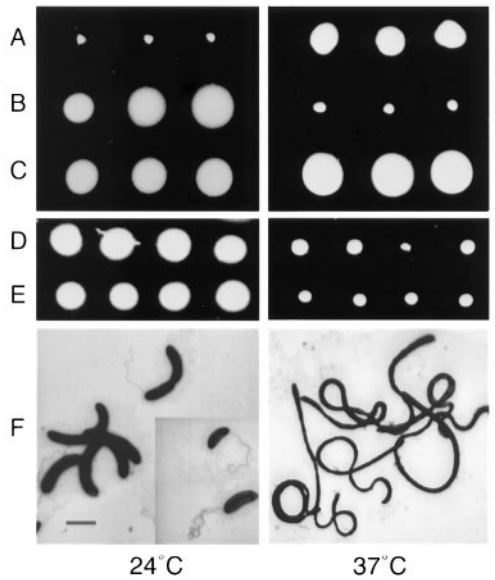

FIG. 1. Phenotype of *sokA* suppressor strains. (*A–E*) Single colonies were stabbed in swarm plates and incubated at 24°C (*Left*) or 37°C (*Right*) for *ca*. 40 hr. (*A*) PC4743 (*divK356*); (*B*) PC3241 (*divK356*, *sokA301*); (*C*) CB15 (wild type); (*D*) PC3247 (*sokA301*, *zzz-471*::Tn*5*); (*E*) PC3248 (*sokA302*, *zzz-471*::Tn*5*). (*F*) Electron micrographs of *sokA301* mutant cells grown in PYE medium at 24°C and shifted to the nonpermissive temperature of 37°C for 12 hr. Cells were stained with 0.5% uranylacetate and were examined by transmission electron microscopy (JEOL JEM-100C). Bar in *E* represents 1 μ.

played similar phenotypes at 24°C and 37°C (data not shown), but it reverted frequently at 37°C.

***sokA* Mutations Map to *ctrA*.** We isolated cosmid clones that complement the ts cell division defect of the *sokA301* allele at 37°C (*Materials and Methods*). Fragments of the 20-kb DNA insert from cosmid pNOR506 were subcloned to generate plasmid pNOR549 containing a 9-kb *Sst*Ia-*Sst*Ib fragment and plasmid pNOR546 containing an overlapping 8.5-kb *Eco*RI-*Sst*Ib fragment (Fig. 2). Because only plasmid pNOR549 rescued the *sokA301* mutation, we surmised that the 482-bp *Sst*Ia-*Eco*RI fragment was responsible for the observed marker rescue in the *rec*$^+$ background (Fig. 2). The 5′ portion of this DNA fragment was found to encode a partial ORF of 73 residues, which is 100% identical to the corresponding C-terminal amino acid sequence of CtrA (7). We obtained direct evidence that the suppressor mutation maps to *ctrA* by complementing the ts growth and division phenotypes of the *sokA301* allele in a *rec*$^-$ strain with a 1,154-bp *Hin*dIII-*Nco*I fragment cloned in plasmid pNOR552, which contains only the *ctrA* coding and 5′ regulatory sequences (Fig. 2).

To locate the suppressor mutation, a 700-bp fragment containing the entire *ctrA* coding sequence was cloned from suppressor strains PC3247 and PC3248 by PCR amplification. DNA sequence analysis of these clones demonstrated that both *sokA* mutant genes contain the identical A-to-G change at nucleotide 637 of the *ctrA* coding sequence, which corresponds to a T213A change in the CtrA protein (7). The *ctrA* sequence from the *sokA*302 mutant strain also contains a G-to-T change at nucleotide 107. This second mutation may contribute to the unstable phenotype of the allele at 37°C, but the *sokA*302 mutation has not been examined further.

***sokA301* Suppresses the Lethality of a *divK* Disruption and Cell Division Defects of *divJ* Mutations.** The *divK* gene is essential and can be disrupted only in cells that carry two copies of the *divK*$^+$ allele (6). If the essential function of DivK results solely from its regulation of CtrA, it should be possible to disrupt *divK* in strains containing a *sokA* mutation. We confirmed this prediction by transducing the *sokA301* strain PC3247 with a phage ϕCr30 lysate prepared on the *divK*::*aacC1*/*divK*$^+$ merodiploid strain PC4781 and recovering drug-resistant recombinants carrying the Gm^R marker of the *divK*::*aacC1* disruption. Gm^R recombinants were also recovered in crosses with the *divK*$^+$/*divK*$^+$ strain PC1123, which carries a second copy of *divK* on a plasmid. No drug-resistant recombinants with the *divK*::*aacC1* disruption were recovered, however, in wild-type strain CB15 that carried only the vector plasmid pRK2L1 (data not shown).

If the sensor kinase DivJ is involved in the regulation of DivK, as indicated by previous genetic and biochemical studies (5, 6), we expected that *divK* suppressors mapping to *ctrA* should also suppress the cell division defects of *divJ* mutations. The *sokA301* allele was transduced into *divJ332* strain PC4212 (Fig. 3 *E* and *F*) and, as a positive control, into the *divK341* strain PC4160 (Fig. 3 *A* and *B*). The *sokA301* mutation suppressed the cell division defect of the *divJ* strain (Fig. 3*G*) as well as the *divK* strain at 24°C (Fig. 3*C*). The recombinant strains displayed extensive filamentation at 37°C, however, indicating the presence of the *sokA301* allele (Fig. 3 *D* and *H*). In similar experiments, the *sokA301* allele also suppressed the cell division defect of the *divJ331* allele (data not shown). These results indicate that CtrA activity is regulated by both DivJ and DivK.

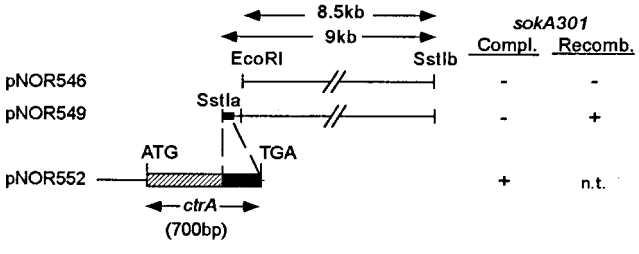

FIG. 2. Cloning and mapping of the *sokA301* allele to *ctrA*. pNOR546 and pNOR549 contain subcloned fragments from complementing cosmid clone pNOR506. Plasmid pNOR552 contains the 450-bp 5′ regulatory sequence and the coding sequence of *ctrA* (7). Complementation (Compl.) was assayed at 37°C in the *rec*$^-$ strain by the formation of uniform large swarms in soft agar (+) and a Div$^+$ phenotype by light microscopy. Recombination (Recomb.) was determined by the appearance of flares around the tight swarm at 37°C in the *rec*$^+$ strain (+), but not in the *rec*$^-$ strain (−).

The DivJ Kinase Phosphorylates CtrA *in Vitro*. We examined the ability of the C-terminal kinase domains, DivJ′ and PleC′, to phosphorylate the CtrA protein *in vitro* by using purified components. DivJ′ and PleC′ were autophosphorylated in the presence of [γ-^{32}P] ATP (Fig. 4, lanes 1 and 5), and the phosphate was efficiently transferred to the response regulator DivK (Fig. 4, lanes 2, 3, 6, and 7), as shown previously (6). More importantly for the present studies, DivJ′, but not PleC′, phosphorylated the purified CtrA protein under the same assay conditions (*cf*. Fig. 4, lanes 4 and 8). These results are consistent with the genetic results described in the previous section and support a model in which DivJ, DivK, and CtrA mediate a common signal transduction pathway, as considered below (see Fig. 7).

We also noted that DivJ′ was more efficient in the phosphorylation of DivK (Fig. 4, lane 2) than CtrA (Fig. 4, lane 4). Consistent with this result is the observation that in a reaction containing DivK and CtrA, DivJ′ selectively phosphorylated

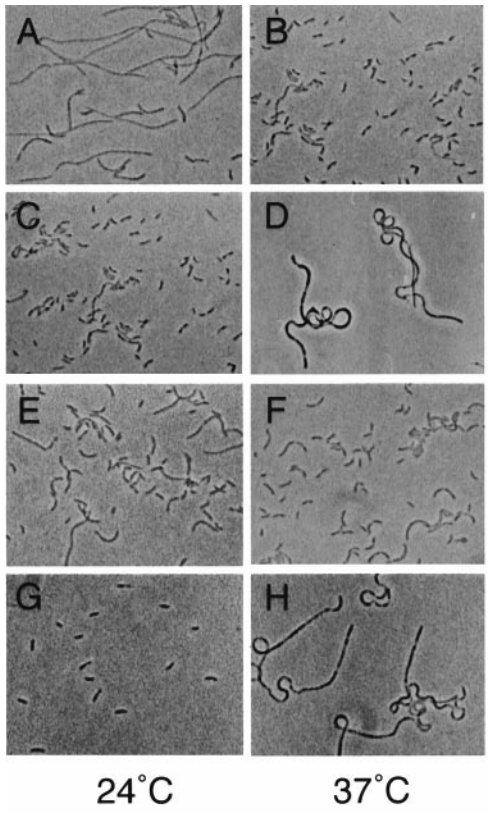

FIG. 3. Suppression of cs divK and *divJ* alleles by *sokA301*. Overnight cultures were grown in PYE medium, diluted 25-fold in duplicate, and incubated for *ca*. 12 hr at either 24°C (*Left*) or 37°C (*Right*). Culture samples were applied to a slide coated with 0.1% polylysine and examined by phase-contrast microscope at 630-fold magnification. (*A* and *B*) PC4160 (*divK341*); (*C* and *D*) PC3251 (*divK341*, *sokA301*, *zzz-471*::Tn*5*); (*E* and *F*) PC4212 (*divJ332*, *aux*::Tn*5-132*); and (*G* and *H*) (*divJ332*, *sokA301*, *aux*::Tn*5-132*, *zzz-471*::Tn*5*).

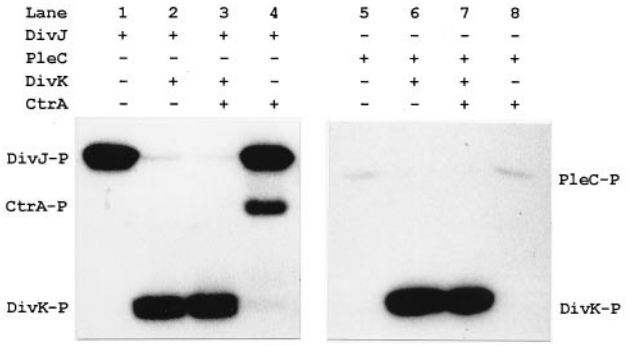

FIG. 4. Phosphorylation of response regulators DivK and CtrA by histidine kinases DivJ and PleC. All experiments were carried out with C-terminal fragments DivJ′ and PleC′ and the full-length DivK and CtrA proteins as described in *Materials and Methods*. The presence of each protein, including DivJ′ (lanes 1–4) and PleC′ (lanes 5–8), is indicated by +. Results of autophosphorylation and phosphotransfer were visualized by autoradiography.

DivK (Fig. 4, lane 3). The failure to detect phospho-CtrA in this last reaction strongly suggests that DivK cannot phosphorylate CtrA directly (see *Discussion*).

Phospho-CtrA Regulates *in Vitro* Transcription from Class II Flagellar Gene Promoters by Eσ^{73}. Several genes regulated by CtrA *in vivo*, including the class II flagellar genes, contain promoters with a conserved direct repeat recognized by the CtrA protein (ref. 7; Fig. 5). The unusual −35, −10 architecture of class II promoters has suggested that they are recognized by a novel sigma factor (18, 19). To characterize these promoters we examined transcription from class II flagellar genes by using RNAP holoenzymes reconstituted from purified *C. crescentus* components (24). In these experiments, we assayed for run-off transcripts from DNA fragments containing either the *fliL* or *fliF* promoter (*Materials and Methods*). Unexpectedly, the *fliL* promoter was recognized by Eσ^{73} RNAP, which contains the principal σ-factor, and produced a run-off transcript of 88 nt (Fig. 6*A*, lane 2), the size predicted from the *in vivo* transcription initiation start site (28). Holoenzymes containing purified σ^{32} (26) or σ^{54} (30) did not recognize the *fliL* promoter, however (data not shown). Importantly, transcription from the *fliL* promoter was stimulated by the addition of CtrA in the presence of DivJ and ATP (Fig. 6*A*, lane 3), conditions shown above to generate phospho-CtrA (Fig. 4). Unphosphorylated CtrA, by contrast, inhibited transcription from the *fliL* promoter (Fig. 6*A*, lane 4). Control experiments demonstrated that DivJ in the absence of CtrA did not affect the rate of transcription (data not shown). These results indicate that the *fliL* promoter is recognized by the Eσ^{73} holoenzyme and regulated *in vitro* by CtrA.

Gene	−35	Spacer	−10	+1
fliF	TATAAACGCCTCGTTTACCTTGTACTG		GGTAAATC	CTGCCT
fliL	GCAAAACACATCGTTAACCATGCTTCGC		GCATGAGT	ACGGGTA
fliQ	CCCTAACGCCCTGTTAACCATATTTCGT		CCATCTTC	GAGCC
rpoN	CGTTCCGCGACCCGTTAACCAGAACCGC		TCTTCAAT	GGCGT
divK	GATTAGCGCGGGCTTAAGGATATCCGT		GCGATCAG	GTTCTGAC
ccrM	GGTTAACGGCCCGCTAACCACGTCTC		TCAACACC	GGATTTA
hemE	TCTTAATTTCTTATTAATATATGTTA		GAAAGATC	TTAA
Consensus	TTAACGS N4 TTAAC	N8-10	GCWWNATC	5-7 N
CtrA-Box	TTAAC N6 TTAAC			
σ⁷³ Consensus	TTGACgS	N10-14	GCtANAWC	5-7 N

FIG. 5. Alignment of CtrA-Boxes and σ^{73}-dependent class II promoters. The −10, −35 regions, based on the σ^{73}-dependnet consensus (40), and the highly conserved sequences within the spacer region are boxed. Nucleotides that are identical to the consensus sequences are underlined. Transcription start sites are shown in boldface. The two direct repeats of the conserved CtrA binding sequence (7) lie within conserved −35 and spacer regions, respectively, and are indicated by arrows at the top of the figure. Promoter sequences shown correspond to *fliF* (27), *fliL* (42), *fliQ* (43), *rpoN* (30), *hemE* (44), *ccrM* (45), and *divK* (A. K. Benson and A.N., unpublished data). N, any nucleotides; S, G or C; W, A or T.

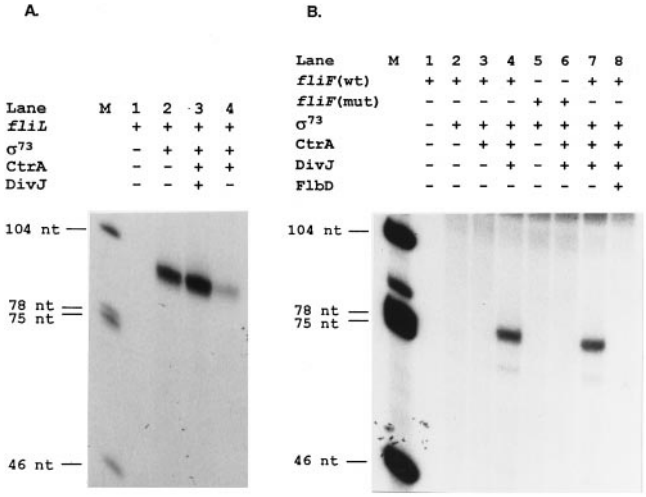

FIG. 6. Phospho-CtrA regulates transcription initiation from class II *fliL* and *fliF* flagellar promoters by RNAP containing σ^{73}. (*A*) *In vitro* run-off transcription assays from the *fliL* promoter (42) by using purified *C. crescentus* RNAP core and sigma factor σ^{73} (24). Reaction contained components as indicated by + (present) or − (absent). Final concentrations of proteins were 10 μM for CtrA and 1 μM for DivJ′. (*B*) Transcription initiation from the wild-type *fliF* promoter and a mutant promoter (see *Results*) was determined by the core RNAP alone or the reconstituted Eσ^{73}. CtrA, FlbD, and DivJ were added to the reactions as indicated by +. The transcripts were run on 7 M urea/PAGE gels with the size markers (M) and visualized by autoradiography.

More conclusive results were obtained from a transcription analysis of the *fliF* promoter, whose activation is required for synthesis of early flagellar components and two regulatory proteins required for transcription of class III and IV flagellar genes (reviewed in ref. 31). The *fliF* promoter was not recognized either by the RNAP core alone (Fig. 6*B*, lane 1), the reconstituted Eσ^{73} holoenzyme (Fig. 6*B*, lane 2), or Eσ^{73} with purified CtrA (Fig. 6*B*, lane 3). The *fliF* template was recognized by Eσ^{73} holoenzyme only under conditions permitting phosphorylation of the CtrA protein: it was transcribed in reaction mixtures containing DivJ and CtrA (Fig. 6*B*, lane 4), but not DivJ alone (data not shown). The 76-nt run-off transcript produced was exactly the size predicted from the previously determined *in vivo* transcription start site (27).

We confirmed the specificity of *fliF* promoter recognition by using a mutant DNA template (pUC18SV4#4) with a deletion of the TA residues at −24, −25 of the *fliF* promoter. This mutation, which removes two residues within the 3′ TTTAC element of the *fliF* CtrA-Box (Fig. 5), is known to abolish transcription from *fliF* promoter *in vivo* (27). The mutant template was not recognized by the reconstituted Eσ^{73} holoenzyme either in the absence (Fig. 6*B*, lane 5) or presence of phospho-CtrA (Fig. 6*B*, lane 6). We also showed that this promoter is repressed *in vitro* by the addition of the transcription regulator FlbD (compare lanes 7 and 8, Fig. 6), which binds to the *ftr4* sequence within the *fliF* promoter and negatively autoregulates transcription *in vivo* (27). Similar results have been reported by using a partially purified RNAP holoenzyme preparation (33). These results demonstrate that the class II *fliF* promoter is recognized by σ^{73} and that its activation is regulated *in vitro* by phosphorylated CtrA and FlbD. Consistent with a role of DivJ and DivK in the regulation of CtrA activity are the results of an *in vivo* analysis of *fliF-lacZ*

and *fliL-lacZ* fusions in *divJ331* and *divK341* mutant strains. Expression of the two transcription fusions was reduced 3- to 4-fold after the cs strains had been shifted from 37°C to the nonpermissive temperature of 24°C (data not shown).

DISCUSSION

Analysis of conditional cell division cycle mutants originally provided evidence for the close coupling of developmental events to cell cycle progression in *C. crescentus* (14, 15). The work presented here defines an essential signal transduction pathway that can account for the coordinate regulation of cell cycle and developmental events. The sensor protein kinase DivJ (5) and the essential response regulator DivK (6) had been shown to regulate initiation of the cell division cycle. Our genetic and biochemical analyses now indicate that these proteins control the activity of the transcription regulator CtrA (7). We propose a model in which DivJ, DivK, and CtrA, probably in conjunction with an unidentified phosphotransfer protein or kinase, mediate an essential signal transduction pathway that regulates the level of CtrA phosphorylation during the cell cycle. We also provide *in vitro* evidence that phospho-CtrA controls transcription from σ^{73} promoters of cell cycle-dependent class II flagellar genes.

Organization of DivJ, DivK, and CtrA in a Common Signal Transduction Pathway. The two *sokA* alleles isolated as suppressors of the conditional cell division defect of a *divK* mutation map to the *ctrA* gene and contain a T213A change in the CtrA protein. The T residue is highly conserved in the OmpR subfamily of transcriptional regulators, of which CtrA is a member. This conserved T residue in OmpR maps on the three-dimensional structure of the cytoplasmic domain in the β strand (β6, ref. 34, or β5, ref. 35), which is near the α3 DNA recognition helix, and one mutation at this site has been shown to affect OmpR-DNA binding (36). We have not investigated the mechanism of SokA suppression, but the location of the *sokA* allele suggests that it could affect DNA–protein binding and possibly interaction of the mutant regulator with RNA polymerase. Independent of the mechanism, the isolation of these suppressors provides strong genetic evidence that CtrA functions downstream of DivK and that CtrA is regulated directly or indirectly by DivK. The ability of the *sokA301* allele to suppress the lethal phenotype of the *divK* disruption mutation (see *Results*) also indicates that *divK* is essential because of its role as an upstream regulator of CtrA, which in turn controls expression of essential cell cycle genes like *ccrM* (16).

Results of genetic experiments also support the conclusion that DivK is a downstream target of DivJ. Overexpression of *divK* confers a severe filamentous phenotype in *C. crescentus* and causes cell lysis, but this effect can be reversed by increasing *divJ* kinase gene expression in the same cells (N.O. and A.N., unpublished results). These results are consistent with the phosphorylation of DivK by the DivJ kinase *in vivo*, as well as *in vitro* (Fig. 4).

The catalytic domain of PleC also acts as a DivK kinase *in vitro*, as shown in Fig. 4 and noted previously (6). Although both PleC and DivJ are thought to function through DivK *in vivo* (6), PleC appears to function primarily in the regulation of cell motility and to play no direct role in cell division. Thus, disruption mutations in *pleC* produce viable, nonmotile cells that divide normally under most growth conditions (13). How can one response regulator, like DivK, differentially mediate the DivJ and PleC activities? We speculate that these two kinases function at different times in the cell cycle, with DivJ acting early in the initiation of cell division (5) and PleC functioning late in the cell cycle to turn on flagellum rotation (6, 37).

Although DivJ also phosphorylates CtrA (Fig. 4), CtrA is unlikely to be the immediate target of DivJ in this pathway. In addition to the relatively inefficient phosphorylation of CtrA by this histidine kinase, DivK did not catalyze phosphotransfer to CtrA *in vitro* (Fig. 4). Given the H1 ⇒ D1 ⇒ H2 ⇒ D2 architecture of multicomponent signal transduction pathways (3), where H2 is typically the histidine residue of a phosphotransferase, phospho-DivK (D1) would not be expected to phosphorylate CtrA (D2) directly. Thus, it seems probable that an additional, unidentified component or components (X) is responsible for transferring phosphoryl groups to CtrA in a DivJ ⇒ DivK ⇒ X ⇒ CtrA phosphorelay pathway (Fig. 7).

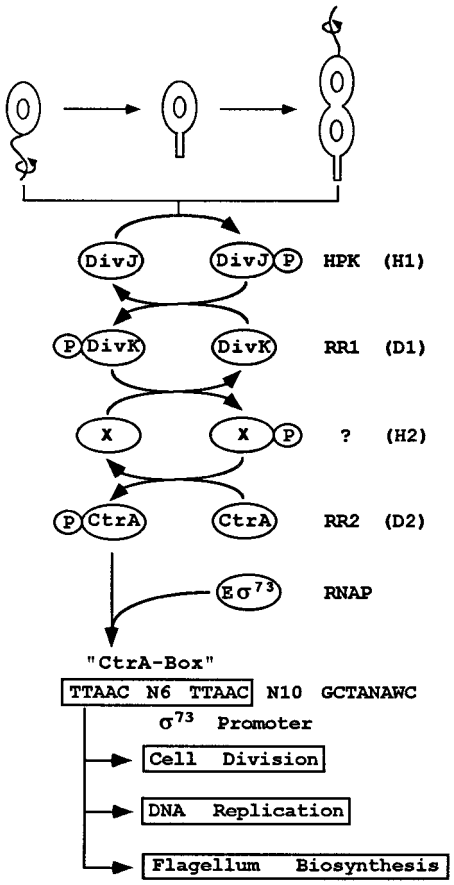

FIG. 7. Model for an essential, multicomponent signal transduction pathway in the regulation of cell division and development in *C. crescentus*. The *C. crescentus* cell cycle is diagrammed at the top of the figure. As drawn, the signal transduction pathway is initiated by unidentified cues provided during cell cycle progression to regulate the activity of DivJ and possibly other kinases. Cell cycle cues may also modulate the pathway by controlling the activity of phosphoprotein phosphatase. DivJ and DivK are visualized as mediating phosphotransfer to CtrA via an unidentified component (X). Phospho-CtrA regulates transcription from class II flagellar gene promoters and presumably from other cell cycle-regulated genes with a similar σ^{73} promoter architecture, including those involved in DNA replication (see *hemE* and *ccrM* in Fig. 5) and possibly cell division.

This provisional working model is similar in outline to the phosphorelay (KinA, KinB ⇒ SpoOF ⇒ SpoOB ⇒ SpoOA) established originally by Hoch and coworkers for the regulation of sporulation in *Bacillus subtilis* (reviewed in ref. 38). As in this *B. subtilis* phosphorelay, multiple sensor kinases and phosphoprotein phosphatases (38) could also regulate the signal transduction pathway in *C. crescentus*. Of particular interest in this regard is *divL,* another gene identified in the *pleC* pseudoreversion analysis whose function is required for initiation of cell division (9). The translated *divL* product is now known to contain sequence motifs conserved in the histidine protein kinase superfamily. The DivL protein is also a candidate for the phosphotransfer protein X postulated in Fig. 7, although preliminary results indicate that its presump-

tive C-terminal catalytic domain does not catalyze phosphate group transfer from phospho-DivK to CtrA (J.W. and A.N., unpublished data). The precise role of DivL in signal transduction will be of great interest given the observation that the conditional cell division defect of *divL* mutations can be suppressed by the *sokA301* allele (N.O. and A.N., unpublished data).

Regulation of σ^{73}-Dependent Class II Promoters by CtrA. The *in vitro* transcription assays indicate that the class II promoters of flagellar genes *fliF* and *fliL* are recognized by the principal sigma factor σ^{73} (39) and require phospho-CtrA for activation (Fig. 6). We have confirmed that the phosphorylated form of CtrA activates transcription from the *fliF* and *fliL* promoters in experiments by using phosphoramidate. This low-molecular-weight phosphoryl group donor (32) fully substituted for DivJ in activating CtrA in *in vitro* transcription assays (J. W. and A.N., unpublished results).

DNA sequence analysis suggests that other CtrA-dependent genes may also be transcribed from σ^{73}-dependent promoters. Alignment of these cell cycle-regulated promoters reveals some conservation of the σ^{73} consensus sequence at −35, but less similarity at −10 (Fig. 5). In addition, the −10 and −35 spacing of the developmentally regulated promoters is 17 to 19 bp, compared with the 10- to 14-bp spacing of σ^{73}-dependent *C. crescentus* housekeeping promoters (40). Fig. 5 shows that the 5′ TTAAC of the conserved CtrA-Box (TTAAC N6 TTAAC) overlaps the putative −35 element of the class II promoters and that the 3′ TTAAC element lies within spacer region. The CtrA-Box is not found in the σ^{73}-dependent housekeeping promoters, however (40). This unusual class II promoter architecture, including the conserved −35 element and the novel −10, −35 spacing, may account for the regulation of these σ^{73}-dependent promoters by CtrA.

Conclusion. Our results strongly support a model in which DivJ, DivK, CtrA, and almost certainly additional proteins, are members of an essential, multicomponent signal transduction pathway that integrates cell cycle cues and possibly environmental signals to coordinate cell cycle and developmental events in *Caulobacter* (Fig. 7). CtrA has recently been shown to undergo cell cycle-regulated phosphorylation (41), and it seems likely that the signal transduction pathway described here plays a central role in this regulation. Challenging questions for the future will be to identify other players in this pathway and the sensory inputs to which it responds during the course of the cell cycle.

We thank Ann Stock for helpful discussions of the OmpR protein structure. We are grateful to Heping Jiang and Benjamin Lee for invaluable assistance in the pseudoreversion analysis of *divK* strains and to Joseph Goodhouse for help with electron microscopy. Genetic experiments described here were supported in part by Research Grant VM-46 from the American Cancer Society; biochemical work on *in vitro* transcription was supported in part by Public Health Service Grant GM22299 from the National Institutes of Health to A.N.

1. Hoch, J. A. & Silhavy, T. J. (1995) *Two-Component Signal Transduction* (Am. Soc. Microbiol., Washington, DC).
2. Parkinson, J. S. (1993) *Cell* **73,** 857–871.
3. Appleby, J. L., Parkingson, J. S. & Bourret, R. B. (1996) *Cell* **86,** 845–848.
4. Ohta, N. & Newton, A. (1996) *Trends Microbiol.* **4,** 326–332.
5. Ohta, N., Lane, T., Ninfa, E. G., Sommer, J. M. & Newton, A. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10297–10301.
6. Hecht, G. B., Lane, T., Ohta, N., Sommer, J. N. & Newton, A. (1995) *EMBO J.* **14,** 3915–3924.
7. Quon, K. C., Marczynski, G. T. & Shapiro, L. (1996) *Cell* **84,** 83–93.
8. Ely, B., Croft, R. H. & Gerardot, C. J. (1984) *Genetics* **108,** 523–532.
9. Sommer, J. M. & Newton, A. (1991) *Genetics* **129,** 623–630.
10. Wang, S. P., Sharma, P. L., Schoenlein, P. V. & Ely, B. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 630–634.
11. Stock, A., Koshland, D. E., Jr., & Stock, J. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 7989–7993.
12. Trach, K. A., Chapman, J. W., Piggot, P., leCoq, D. & Hoch, J. A. (1988) *J. Bacteriol.* **170,** 4194–4208.
13. Burton, G., Hecht, G. B. & Newton, A. (1997) *J. Bacteriol.* **179,** 5849–5853.
14. Sheffery, M. & Newton, A. (1981) *Cell* **24,** 49–57.
15. Huguenel, E. D. & Newton, A. (1982) *Differentiation* **21,** 71–78.
16. Stephens, C., Reisenhauer, A., Wright, R. & Shapiro, L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1210–1214.
17. Marczynski, G. & Shapiro, L. (1992) *J. Mol. Biol.* **226,** 959–977.
18. Gober, J. W. & Marques, M. V. (1995) *Microbiol. Rev.* **59,** 31–47.
19. Brun, Y. V., Marczynski, G. & Shapiro, L. (1994) *Annu. Rev. Biochem.* **63,** 419–450.
20. Ely, B. (1991) *Methods Enzymol.* **204,** 372–384.
21. Ohta, N., Masurekar, M. & Newton, A. (1990) *J. Bacteriol.* **172,** 7027–7034.
22. Mullin, D. A. & Newton, A. (1989) *J. Bacteriol.* **171,** 3218–3227.
23. Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7347–7351.
24. Wu, J., Ohta, N., Benson, A. K., Ninfa, A. J. & Newton, A. (1997) *J. Biol. Chem.* **272,** 21558–21564.
25. Tabor, S. & Richardson, C. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1074–1078.
26. Wu, J. & Newton, A. (1996) *J. Bacteriol.* **178,** 2094–2101.
27. VanWay, S. M., Newton, A., Mullin, A. H. & Mullin, D. A. (1993) *J. Bacteriol.* **175,** 367–376.
28. Yu, J. & Shapiro, L. (1992) *J. Bacteriol.* **174,** 3327–3338.
29. Hecht, G. B. & Newton, A. (1995) *J. Bacteriol.* **177,** 6223–6229.
30. Anderson, D. K., Ohta, N., Wu, J. & Newton, A. (1995) *Mol. Gen. Genet.* **246,** 697–706.
31. Wu, J. & Newton, A. (1997) *Mol. Microbiol.* **24,** 233–240.
32. Lukat, G. S., McCleary, W. R., Stock, A. M. & Stock, J. B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 718–722.
33. Benson, A. K., Ramakrishnan, G., Ohta, N., Feng, J., Ninfa, A. & Newton, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4989–4993.
34. Martinez-Hackert, E. & Stock, A. M. (1997) *J. Mol. Biol.* **269,** 301–312.
35. Kondo, H., Nakagawa, A., Nishihira, J., Nishimura, Y., Mizuno, T. & Tanaka, I. (1996) *Nat. Struct. Biol.* **4(1),** 28–31.
36. Kato, N., Tsuzuki, M., Aiba, H. & T., M. (1995) *Mol. Gen. Genet.* **248,** 399–406.
37. Sommer, J. M. & Newton, A. (1989) *J. Bacteriol.* **171,** 392–401.
38. Hoch, J. A. (1995) in *Two-Component Signal Transduction*, eds. Hoch, J. A. & Silhavy, T. J. (Am. Soc. Microbiol., Washington, DC), pp. 129–144.
39. Malakooti, J. & Ely, B. (1995) *J. Bacteriol.* **177,** 6854–6860.
40. Malakooti, J., Wang, S. P. & Ely, B. (1995) *J. Bacteriol.* **177,** 4372–4376.
41. Domian, I. J., Quon, K. C. & Shapiro, L. (1997) *Cell* **90,** 415–424.
42. Stephens, C. M. & Shapiro, L. (1993) *Mol. Microbiol.* **9,** 1169–1179.
43. Zhuang, W. Y. & Shapiro, L. (1995) *J. Bacteriol.* **177,** 343–356.
44. Marczynski, G. T., Lentine, K. & Shapiro, L. (1995) *Genes Dev.* **9,** 1543–1557.
45. Stephens, C. M., Zweiger, G. & Shapiro, L. (1995) *J. Bacteriol.* **177,** 1662–1669.