Cell cycle regulator phosphorylation stimulates two distinct modes of binding at a chromosome replication origin

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In *Caulobacter crescentus*, the global response regulator CtrA controls chromosome replication and determines the fate of two different cell progenies. Previous studies proposed that CtrA represses replication by binding to five sites, designated [a–e], in the replication origin. We show that phosphorylated CtrA binds sites [a–e] with 35- to 100-fold lower K_d values than unphosphorylated CtrA. CtrA phosphorylation stimulates two distinct modes of binding to the replication origin. Phosphorylation stimulates weak intrinsic protein–protein cooperation between half-sites and does not stimulate CtrA–P binding unless protein–DNA contacts are made at both half-sites. CtrA phosphorylation also stimulates cooperative binding between complete sites [a] and [b]. However, binding to each of the other CtrA-binding sites [c], [d] and [e] is completely independent and suggests a modular organization of replication control by CtrA. We therefore propose a model where a phosphorelay targets separate biochemical activities inside the replication origin through both cooperative and independent CtrA-binding sites.

Keywords: binding/cell cycle/CtrA/replication origin/response regulator

Introduction

The cell cycle in the Gram-negative bacterium *Caulobacter crescentus* is regulated by signal transduction pathways that appear to control cell fate and chromosome replication (Jacobs and Shapiro, 1998; Quon *et al.*, 1998). Cell division in *C. crescentus* produces two distinct cells, a swarmer progeny and a stalked progeny, which have different morphologies and cell cycle fates (Brun *et al.*, 1994; Gober and Marques, 1995; Wu *et al.*, 1998) (Figure 1). The swarmer cell has a polar flagellum and is the motile chemotactic progeny. However, the swarmer cell is non-replicating (Degnen and Newton, 1972; Marczyński *et al.*, 1990). In order for chromosome replication to initiate, the swarmer cell must differentiate into a stalked cell via flagellum detachment and stalk biogenesis. The stalked cell then initiates chromosome replication and asymmetric cell division, resulting in distinct stalked and swarmer cells (Jacobs and Shapiro, 1998).

Why chromosome replication occurs only in the stalked cell and not in the swarmer cell remains hypothetical. Recently, response regulators and sensor kinases were identified that seem to play essential roles in coordinating

cell cycle events in this bacterium (Ohta and Newton, 1996; Shapiro and Losick, 1997). One key control system is a phosphorelay involving the response regulator CtrA (cell cycle transcription regulator) (Quon *et al.*, 1996). CtrA phosphorylation is essential for cell viability (Domain *et al.*, 1997), and an essential histidine kinase CckA (cell cycle kinase) was recently identified that appears to phosphorylate CtrA, either directly or as part of a phosphorelay system (Jacobs *et al.*, 1999). CtrA was initially identified in a screen for cell cycle regulators of flagellar biogenesis (Quon *et al.*, 1996). However, CtrA is a global transcription regulator: it regulates the class II flagellar genes (*fliQ*), an essential DNA methyltransferase gene (*ccrM*) (Quon *et al.*, 1996) and cell division genes (Quon *et al.*, 1996; Quardokus *et al.*, 1997). CtrA also regulates transcription and probably other biochemical activities inside the chromosome replication origin (Quon *et al.*, 1998) (discussed below).

CtrA is present in swarmer cells (non-replicating progeny), and is degraded during the swarmer to stalked cell transition by regulated proteolysis (Domain *et al.*, 1997). CtrA is both replenished and phosphorylated at mid S-phase, and remains phosphorylated in the progeny swarmer cells (Domain *et al.*, 1997). Homologues of the *Escherichia coli* chaperone and ATP protease pair, ClpX,P, are required to degrade CtrA in the stalked cell (Jenal and Fuchs, 1998). However, CtrA is regulated primarily by phosphorylation (Reisenauer *et al.*, 1999). When CtrA protein degradation is blocked and CtrA protein is present in all cell types, periodic CtrA phosphorylation still drives an apparently normal cell cycle (Domain *et al.*, 1997).

CtrA is homologous to *E. coli* OmpR, a two-component response regulator. CtrA receives a phosphate on a key aspartate (position 51) inside the receiver domain (Quon *et al.*, 1996). This phosphorylation then alters the output protein domain, which in OmpR, and presumably also CtrA, binds DNA with a higher affinity. Recent work showed that CtrA–P binds to the *fliQ* and *ccrM* promoters under conditions where unphosphorylated CtrA failed to bind (Reisenauer *et al.*, 1999).

Earlier work demonstrated that unphosphorylated CtrA selectively binds 9mer motifs with the characteristic sequence (GTAA–N7–TTAA) (Quon *et al.*, 1998), which is present upstream of the *fliQ* gene (Quon *et al.*, 1996) and in the cloned *C. crescentus* replication origin (*Cori*) (Marczyński and Shapiro, 1992). The five 9mer motifs designated [a]–[e] are implicated in the regulation of chromosome replication (Figure 1). For example, CtrA-binding sites [a] and [b] overlap a strong transcription promoter (Ps) (Marczyński *et al.*, 1995), whose transcription coincides with chromosome replication.

Previous studies proposed that CtrA represses chromosome replication in the swarmer cell. One proposed mechanism involves CtrA binding to sites [a] and [b]

C. crescentus cell cycle and chromosome replication origin (*Cori*)

Fig. 1. The *C. crescentus* dimorphic cell cycle and the chromosome replication origin (*Cori*). The swarmer cell (Sw) differentiates into a stalked cell (St) where chromosome replication and asymmetric cell division take place. The shading indicates both the presence and the phosphorylation of the CtrA protein. The thick line in the *Cori* DNA map indicates the minimal sequences required for autonomous *Cori* plasmid replication. Pw and Ps designate the mRNA starts of the weak and strong promoters. B = *Bam*HI, P = *Pst*I, Bg = *Bgl*II, Hp = *Hpa*I and E = *Eco*RI restriction sites. The filled arrowhead designates the essential DnaA protein-binding site. The sequence of the indicated CtrA-binding sites [a]–[e] is shown in Table IA. Based on Quon *et al.* (1998).

(Figure 1). This would repress Ps transcription, which in turn would block chromosome replication (Quon *et al.*, 1998). Another proposed mechanism involves the interaction of CtrA and DnaA protein (Quon *et al.*, 1998). CtrA-binding site [e] is adjacent to an essential DnaA box, and protein footprint assays suggested that CtrA binding extends to this DnaA box (Quon *et al.*, 1998). Point mutations in this DnaA box abolish *Cori* plasmid replication (Marczynski and Shapiro, 1992), suggesting that CtrA binding could modulate DnaA binding or its other essential replication functions.

In this study, we performed *in vitro* footprinting assays to understand how CtrA and CtrA-P bind and interact inside the chromosome replication origin of *C. crescentus*. We examined CtrA and CtrA-P binding to all five CtrA-binding sites inside the wild-type and mutant forms of this replication origin. We observed that CtrA-P phosphorylation can stimulate both cooperative and independent binding, and we discuss the implications for the regulation of chromosome replication.

Results

Enhanced and altered binding of CtrA upon phosphorylation

To understand how CtrA interacts with each *Cori*-binding site, we first examined CtrA and CtrA-P binding in the wild-type *Cori* context. As reported previously, unphosphorylated CtrA selectively protects five DNA regions from DNase I cleavage (Figure 2A). The exact base pairs protected relative to the consensus TTAA-N7-TTAA on

both the top and bottom strands are shown in Table IB, which is derived from this and similar footprinting experiments. CtrA has different affinities for its five cognate binding sites in the wild-type context. CtrA has the lowest affinity for site [a], presumably due to the CTAA mismatch to the TTAA half-site consensus (Table I).

CtrA phosphorylation stimulates binding to *Cori*. CtrA was phosphorylated using maltose-binding protein (MBP)-EnvZ (the cognate histidine kinase for OmpR) (Reisenauer *et al.*, 1999). A CtrA aspartate to glutamate mutation at position 51 cannot be phosphorylated by EnvZ (Reisenauer *et al.*, 1999), and this residue appears to be the only site for phosphorylation (Domain *et al.*, 1997). Control experiments described in Materials and methods indicate that ~70% of the CtrA protein was phosphorylated in our footprint assays. Dissociation binding constants (K_d values) were obtained by titrating CtrA and CtrA-P through their half-maximal binding concentrations (Table IC). Phosphorylated CtrA bound these same five *Cori* sites, but with substantially higher affinities (Figure 2A). Depending on the binding site, phosphorylation stimulated CtrA-P binding between 35- and 100-fold within the wild-type *Cori* context (Table IC). The relative binding order [a] < [c, b, d, e] for CtrA changed upon phosphorylation to [a] < [c, d, e] < [b] for CtrA-P. Therefore, phosphorylation stimulates CtrA binding to site [b] to an exceptional degree. We will argue below that this is due to cooperative interactions between CtrA at sites [a] and [b].

CtrA-P has a footprint pattern that is distinct from unphosphorylated CtrA at all five *Cori* sites. This is best

Fig. 2. Phosphorylation of CtrA enhances binding to five CtrA-binding sites in the replication origin. Independent binding of distant CtrA-binding sites. DNA fragments used to illustrate the independent binding of CtrA-binding sites. Labelling of pGM1877 at the unique *Hind*III site and then digestion with *Xho*I to yield fragment (A) with all five CtrA-binding sites, representing the wild-type context. (B) Digestion with *Eco*RI gives a fragment with only [a], [b] and [c] sites. (C) An *Hpa*I-digested fragment with only binding sites [a] and [b] intact. Phosphorylated His₆-CtrA using MBP-EnvZ was used in a DNase I footprinting assay and the manner of binding was compared with the same concentration of unphosphorylated CtrA. The labelled fragments illustrated above were incubated with increasing concentrations of CtrA-P (0.002–0.02 μ M) and CtrA (0.1–5 μ M) as shown in the DNase I footprinting gels. The brackets represent the sites occupied by the protein with the designated letter adjacent. Fragment (A) was incubated with up to 1 μ M CtrA-P. The asterisk in footprint (A) illustrates the bands that are occupied by the protein in the phosphorylated form and spared in the unphosphorylated form.

observed for sites [a] and [b] in Figure 2A. Some bands (marked * in Figure 2A) remained only partially protected by unphosphorylated CtrA. However, these bands were completely protected by CtrA-P. In addition, unphosphorylated CtrA occupied site [b] completely, but only partially occupied site [a], and the 4 bp between [a] and [b] were not protected from DNase I cleavage, even at high concentrations of unphosphorylated CtrA (Figure 2A). On phosphorylation of CtrA, CtrA-P completely protects an ~40 bp region spanning sites [a, b], and there is no DNase I cleavage between [a] and [b]. These characteristics of CtrA-P binding indicate higher binding affinities, and also imply altered protein–DNA and protein–protein contacts, discussed further below.

Contact with both TTAA half-sites is required for high affinity CtrA-P binding

Sequence alignment of CtrA-binding sites in *Cori* suggests that they are composed of two TTAA ‘half-sites’ separated by exactly 7bp (Table IA and B). Previous results suggested that CtrA protein could bind to separate half-sites (Quon *et al.*, 1998). To investigate how CtrA phosphorylation might influence half-site binding, we employed mutations directed to half of site [d] and [e] (designated as mut-d and mut-e, respectively, in Table IA). Figure 3 presents a DNase I footprinting assay comparing both CtrA and

CtrA-P binding to mut-d. Unaltered sites [c] and [e] on the same end-labelled *Cori* DNA fragment served as internal binding controls. CtrA bound only to the unaltered half of site [d]. Interestingly, phosphorylation did not stimulate CtrA-P binding to this [d] half-site, while binding to whole-site [c] and [e] was clearly stimulated (Figure 3). Likewise, in a separate footprinting experiment, phosphorylation did not stimulate CtrA-P binding to the [e] half-site on a mut-e *Cori* DNA fragment (Figure 3). This implies that each site is composed of two half-sites, and that each half-site binds one molecule of CtrA or CtrA-P.

Most significantly, the data in Figure 3 also indicate that the whole site (TTAA-N7-TTAA) is required for phosphorylation-dependent binding. Phosphorylation only stimulates CtrA binding to whole sites but not to the half-sites. Presumably, phosphorylation-dependent binding requires protein–protein interactions between adjacent CtrA molecules. The approximate K_d values extracted from these footprinting experiments are also shown in Figure 3. The K_d values of 0.8 μ M at both mut-d and mut-e half-sites indicate very poor CtrA binding that is unchanged by phosphorylation. Note that the K_d values of 0.4 and 0.01 μ M for CtrA and CtrA-P, respectively, at the adjacent binding site [c] were the same as in a wild-type context (Table IC). This not only argues for the

Table I. *Cori* CtrA-binding sites

A			
CtrA binding sites			
a	+246-gTCCC	<u>TTAA</u> gATCTTT	<u>CTAA</u> CATAT
b	+267-ATATA	<u>TTAA</u> TAAgAAA	<u>TTAA</u> gATTg
c	+389-Tgggg	<u>TTAA</u> CgCTCTg	<u>TTAA</u> TCATg
d	+437-ACACC	<u>TTAA</u> TgAATTC	<u>TTAA</u> gTCCT
	A CAgg		Wt mut-d
e	+650-AgTgg	<u>TTAA</u> gCAACCg	<u>TTAA</u> CggAT
	C gCT		Wt mut-e

B			
h	xxxxx	x x x	xxxxx
h h	xxxxxx	xxxxxx	xxxxxx
hHHh	xxxxxx	xxxxxx	xxxxxx
	-----	-----	-----
	-----	-----	-----
hhHH	xxxxxx	xxxxxx	xxxxxx
hh	xxxxxx	xxxxxx	xxxxxx
HHh	xxxxxx	xxxxxx	xxxxxx
h	xxxx	x x	xxxx

C			
Binding site	K _d (μM)		
	CtrA	CtrA-P	
a	0.6	0.015	40x
b	0.3	0.003	100x
c	0.45	0.01	45x
d	0.2	0.006	35x
e	0.2	0.006	35x

(A) DNA sequence, positions and the half-site consensus (underlined). Site-directed mutations, mut-d and mut-e, are shown below the wild-type *Cori* DNA sequence. (B) DNase I protection patterns compiled from sites [a], [b] and [c] on the top strand, and sites [a], [b], [c] and [e] on the bottom strand. xX indicates CtrA-dependent protection, hH indicates His-CtrA-dependent enhanced DNase I cleavage. (C) Calculated dissociation constant values of CtrA and CtrA-P for the five CtrA-binding sites in *Cori*. These values were extracted from similar footprint assays to those in Figure 2. The gels were analysed by PhosphorImaging and the IQuant program (Molecular Dynamics), and the band intensities were normalized to the no protein controls.

consistency of our phosphorylation and binding analyses, but it also argues against long-range cooperative CtrA binding between sites [c] and [d], which will be discussed further below.

Phosphorylation stimulates weak intrinsic CtrA protein-protein binding

The preceding results indicated that phosphorylation does not stimulate CtrA-P binding unless protein-DNA contacts are made simultaneously at both TTAA half-sites. This is investigated further in Figure 4 where half-site occupancy is quantified on both mut-d and wild-type *Cori* DNA fragments. Occupancy by unphosphorylated CtrA at site [c] was practically the same on both the mut-d and wild-type *Cori* fragments ($K_d = 0.6$ and 0.65 μM; Figure 4C). At site [d], on the wild-type *Cori* fragment, unphosphorylated CtrA simultaneously bound both left and right half-sites ($K_d = 0.25$ μM; Figure 4B). At site [d], on the mut-d *Cori* fragment, unphosphorylated CtrA bound only the right half-site but with lower affinity ($K_d =$

0.6 μM; Figure 4A). Therefore, CtrA binding to the left half of site [d] accounts for an ~2-fold difference in the binding K_d at the right half of site [d].

The cartoons in Figure 4 provide the simplest interpretation. Apparently, unphosphorylated monomer CtrA protein binds left and right half-sites and makes weak protein-protein contacts that promote cooperative DNA binding. Phosphorylation must stimulate this protein-protein binding, since it clearly does not stimulate direct protein-DNA half-site binding (Figure 3).

Cooperative binding between CtrA-binding sites [a] and [b] is enhanced by CtrA phosphorylation

Cooperative CtrA binding between sites [a] and [b] was suggested by their proximity (Table IIA), and by their threshold and simultaneous occupancy with increased CtrA-P concentration (Figure 2A). To test directly for cooperative CtrA binding and the effects of phosphorylation, a *Cori* fragment with CtrA-binding sites [a] and [b] separated by an extra 14 bp (Table IIA) was used in our binding protocol. This *Cori* fragment was labelled at the *EcoRI* site (+452) and cleaved at the *PstI* site (+1), and therefore contained complete sites [a], [b] and [c]. The +14 bp separation dramatically reduced CtrA binding to both sites [a] and [b], but not to site [c] (Figure 5, Table IIB). CtrA and CtrA-P could not bind site [a], and we could only place a lower limit on these K_d values (Table IIB). Both CtrA and CtrA-P bound site [b], but with significantly lower binding affinities compared with the wild-type *Cori* context (Table I). Note that binding to site [c] was the same in both the +14ab and the wild-type *Cori* contexts. Therefore, lower binding affinity to [b] is due solely to the altered +14 spacing.

Unphosphorylated CtrA bound site [b], on the +14ab *Cori* fragment, with a 2.3-fold higher K_d compared with the wild-type context (Table IIC). CtrA-P binding to site [b] was more affected. CtrA-P bound site [b] with a 10-fold higher K_d (Table IIC). The enhanced binding of CtrA to site [b], on phosphorylation of the protein, is only 23-fold in the +14ab context, compared with 100-fold enhanced binding observed in the wild-type context (Table IIC). This demonstrates that both CtrA and CtrA-P require the wild-type 4 bp proximity between sites [a] and [b] for maximal binding and that phosphorylation stimulates weak intrinsic cooperative binding between both sites.

CtrA binding to a *Ps* promoter mutant that deregulates both transcription and replication

A -14 bp deletion in CtrA-binding sites [a] and [b] (Table II) reduces transcription from the strong promoter, and alters the cell cycle timing of both *Ps* transcription and *Cori* plasmid replication (Marczynski *et al.*, 1995). This suggested that CtrA-binding sites [a] and [b] are significant for cell cycle control. The -14 bp deletion retains the left half of site [a] and most of site [b]. We assayed CtrA binding to the -14ab deletion in a parallel series of experiments to those performed above for +14ab. Unphosphorylated CtrA did not bind to -14ab (data not shown) and phosphorylated CtrA-P produced one altered footprint at [b'] with a K_d value of 0.3 μM. CtrA binding to site [c] served as an internal control and was not affected in this mutation (Figure 5). Since the K_d at site

Fig. 3. Half-site binding by CtrA and CtrA-P. (A) DNase I protection footprint experiment using the mut-e fragment. pGM2082 was labelled at a *Bgl*II site and cut with *Pst*I. Increasing concentrations of CtrA (0.05–1 μ M) and CtrA-P (0.05–1 μ M) were added as illustrated by the triangle. (B) Binding of CtrA to site [d] in the mut-d background illustrated by a DNase I footprint experiment, using pGM1964 labelled at a *Bgl*II site and cut with *Pst*I. CtrA and CtrA-P binding are compared. (C) Calculated K_d values of the CtrA-binding sites in both the wild-type context and mut-d and mut-e backgrounds.

Fig. 4. Intrinsic cooperative binding of unphosphorylated CtrA protein. The DNase I footprint protocol described in Materials and methods employed His-CtrA protein and *Cori* wild-type or *Cori* mut-d DNA 5'-³²P-end-labelled at the *Bgl*III site (Figure 1). (A) Band intensities at site [d] (left and right) on the mut-d *Cori* DNA. (B) Band intensities at site [d] (left and right) on the wild-type *Cori* DNA. (C) Band intensities at site [c] on the mut-d and wild-type *Cori* DNA. The cartoons interpret CtrA binding, as described in the text. The oval and square shapes represent the receiver and DNA-binding domains of CtrA protein. The table presents approximate K_d values extracted from these data.

[b'] is 100-fold higher than at site [b] in the wild-type context, it seems unlikely that CtrA-P can bind to and regulate the -14ab promoter *in vivo*, and this most probably accounts for the absence of repression in swarmer cells (Marczynski *et al.*, 1995).

Table II. Mutant *Cori* CtrA binding sites [ab]

Independent CtrA binding to sites [ab], [c], [d] and [e]

Although we demonstrated that CtrA binding to adjacent sites [a] and [b] is highly cooperative, we find no evidence for cooperative binding between distant sites and [c], [d] and [e]. For example, in both the -14ab and +14ab mutations, poor binding of CtrA and CtrA-P to mutant sites [ab] did not affect binding to site [c], and its K_d values were identical to those in the wild-type context containing all five binding sites (compare Figures 4 and 5 with Table IC). This demonstrates the absence of long-range cooperativity between sites [ab] and [c]. Likewise, the binding of CtrA-binding sites [e] and [c] was unaffected in the half-site mutation (mut-d). These observations suggest independent CtrA binding to distant binding sites.

Fig. 5. Cooperative binding of CtrA-binding sites [a] and [b]. (A) Binding of CtrA-P in the +14ab mutation. DNase I footprinting assay employing +14ab with different concentrations of CtrA-P (0.02–0.6 μ M). Binding of CtrA to site [a] is not evident, and the binding to site [b] was markedly diminished. Binding of CtrA-P to site [c] was not affected in this mutation. Refer to Table II for dissociation constant values. (B) Binding of CtrA-P in the -14ab mutation. Increasing concentrations of CtrA-P (0.02–0.6 μ M) are used in a DNase I footprinting assay. CtrA-P binding to the [b'] site is markedly diminished. The binding to site [c] is not affected in this mutation. Refer to Table II for dissociation constant values.

To investigate cooperative binding to *Cori* further, CtrA-P binding to a single site [e] was compared with CtrA binding to site [e] in a wild-type *Cori* context containing all five binding sites. A *Cori* fragment was 5' end-labelled at *Bam*HI (position 998). Subsequent cleavage with *Aat*II at *Cori* position 194 yields a fragment with all five binding sites intact, and cleavage with *Nar*I at position 621 yields a fragment with only CtrA-binding site [e]. Interestingly, CtrA-P binding to a single site is enhanced when compared with the binding of site [e] in the wild-type context. The K_d values were 5 and 1.5 nM, respectively. Since the CtrA protein is in ~100-fold molar excess over the labelled DNA fragment, binding to site [e] should not be influenced by a relatively small change in the number of binding sites. Although this deserves further investigation, this observation suggests that distal CtrA-binding sites might communicate and compete with site [e] for CtrA protein. However, we clearly do not see cooperative binding between site [e] and other *Cori* sites.

To characterize cooperative versus independent CtrA binding further, *Cori* DNA was end labelled and digested to remove CtrA-binding sites progressively. As presented in Figure 2, digestion with *Xho*I, *Eco*RI and *Hpa*I yields three fragments having the same end-labelled specific activity but missing one or more CtrA-binding sites. DNase I footprinting assays employed these *Cori* fragments and the same CtrA protein concentrations (Figure 2). The presence of binding sites [d] and [e] did not affect CtrA and CtrA-P binding to sites [a], [b] and [c] (Figure 2). The binding affinity and character of binding of CtrA and CtrA-P to all three DNA fragments was indistinguishable (Figure 2). To confirm this conclusion, the K_d values for each of the binding sites for each fragment were calculated, and these K_d values did not differ from those in the wild-type context (Table IC). This suggests that distant CtrA-

Fig. 6. *In vivo* transcription from the *Cori* strong (P_s) promoter, and the effects of deleted CtrA-binding sites and *ctrA* mutant backgrounds. (A) Structure and sequence at the vector and *Cori* junctions in the pRK290 *lacZ* reporter plasmids (Table III). The arrowhead marks the P_s +1 mRNA start site (Marczynski *et al.*, 1995). (B) *lacZ* expression from exponentially grown PYE cultures supplemented with 1.5 μ g/ml tetracycline to maintain the respective plasmids. The isogenic *ctrA* wild-type and loss-of-function *ctrA401* *C. crescentus* strains are described in Table III. (C) Cartoon interpretation of phosphorylated CtrA binding to sites [a] and [b] in pGM976, and to sites [a'] and [b] in pGM1027. The lateral arrows between CtrA protein molecules imply cooperative binding.

binding sites are bound independently and without any significant long-range cooperative interactions.

The CtrA protein used in our assays (Figures 2–4) was histidine tagged at the N-terminus. This 34 amino acid tag facilitates the purification of the protein. To determine if such a tag affects CtrA binding, the histidine tag was removed by cleavage with recombinant enterokinase. The three experiments presented in Figure 2 were repeated using CtrA and CtrA-P without the histidine tag. The binding affinities of CtrA and CtrA-P for the five binding sites with and without the histidine tag were identical (data not shown). Therefore, the N-terminal CtrA histidine tag is neutral in our assays. The histidine tag does not influence CtrA-P phosphorylation by EnvZ, cooperative binding between CtrA-P half-sites or cooperative binding between CtrA-P at complete sites [a] and [b].

Cooperative *in vivo* CtrA binding implied by transcription from the P_s promoter

We tested the consequences of deleting CtrA-binding sites [a] and [b] on transcription from the *Cori* strong promoter (P_s). Sites [a] and [b] completely overlap this promoter and repress transcription in the swarmer cells (Marczynski *et al.*, 1995). When *lacZ* expression from plasmid pGM976 (Figure 6A) was compared in wild-type versus isogenic *ctrA401* mutant *C. crescentus* cells, transcription increased ~5-fold (Figure 6B), indicating that *ctrA401* is a loss-of-function allele and that wild-type CtrA plays a major role

Fig. 7. Model for CtrA and CtrA-P binding to one site (left and right half-sites) as described in the text.

in repressing this promoter (Quon *et al.*, 1998). We used the ratio between *lacZ* expression in *ctrA401* and isogenic *ctrA* (wild-type) cells as an *in vivo* measure of Ps promoter repression by CtrA (Quon *et al.*, 1996).

In agreement with our *in vitro* cooperative binding data, we observed that a small deletion (pGM1027) and a large deletion (pGM1028) both abolish transcription repression (Figure 6B). The comparably low expression ratios (1.6 and 1.4) argue that CtrA does not bind and block Ps transcription effectively either in pGM1027 (Figure 6A), where only the left half-site of [a] is deleted, or in pGM1028 (Figure 6A), where site [a] is completely deleted. Note that pGM976, pGM1027 and pGM1028 produce progressively less β -galactosidase in both genetic backgrounds, presumably because these downstream deletions alter the mRNA stability and promoter sequence.

The cartoon in Figure 6C interprets CtrA repression at the wild-type and pGM1027 promoters in view of our *in vivo* transcription and *in vitro* binding data. We observed that half-site mutations block strong CtrA-P binding to the remaining half-site (Figure 3), and high-affinity CtrA-P binding to site [b] requires cooperative binding with site [a] (Table II, Figure 5). Therefore, deleting half of site [a] in pGM1027 will remove two interdependent and cooperative binding interactions as effectively as deleting the whole [a] site in pGM1028.

Discussion

Our experiments addressed how the response regulator CtrA binds and interacts at the *Cori* of *C. crescentus*. CtrA is homologous to the well-studied response regulator in *E. coli* OmpR (Quon *et al.*, 1996). These phosphorelay proteins have a characteristic N-terminal receiver domain and a C-terminal DNA-binding domain (Hoch and Silhavy, 1995). We employed both phosphorylated and unphosphorylated forms of the CtrA protein with wild-type and mutant *Cori* DNA fragments in our DNase I footprinting assays. This analysis allows us to draw conclusions relevant to the regulation of chromosome replication in *C. crescentus* and to the function of response regulator proteins in other systems.

Our model (Figure 7) summarizes protein and DNA interactions at one CtrA-binding site. CtrA shows weak binding to one half-site (Figure 7A) that is unchanged by phosphorylation (Figure 7B). Unphosphorylated CtrA shows weak but cooperative binding between two half-sites (Figure 7C). Strong binding is only observed when

phosphorylated CtrA-P binds and cooperates between two half-sites (Figure 7D). This model is based primarily on our data in Figures 3 and 4. The 40-fold enhanced binding by phosphorylated CtrA-P to site [c] is contrasted by completely unaltered CtrA-P binding to the isolated TTAA half-sites at mut-d and mut-e (Figure 3). The ~2-fold enhanced binding between two adjacent unphosphorylated CtrA proteins implies weak but specific protein contacts (Figure 4). The lateral arrows in our model are drawn to imply that phosphorylation strengthens these protein-protein contacts (Figure 7D). However, the particular amino acid contacts, as well as their location on CtrA, are completely speculative. Since the histidine tag at the N-terminus did not influence cooperative CtrA binding, this suggests that protein-protein interactions do not occur at the extreme N-terminus.

We stress two interesting features of our one-site binding model (Figure 7). First, phosphorylation stimulates weak intrinsic CtrA binding to DNA and to the adjacent partner CtrA protein. Secondly, in order to stimulate strong binding, at least two synergistic signals must enter the DNA-binding domain of CtrA: a signal from the phosphorylated receiver domain and a signal from the partner CtrA protein (Figure 7D). That half-site binding is completely unaltered by CtrA phosphorylation (Figure 3) indicates DNA participation and suggests that CtrA-P dimers do not form in solution. It is also likely that OmpR only forms dimers upon binding to DNA (Harlocker *et al.*, 1995). Therefore, weak target DNA contacts may be a third required signal that activates the strong binding state. In this hypothetical view, protein phosphorylation is not the one dominant signal, but the last in a series of essential signals that together trigger strong DNA binding.

Interestingly, cooperative CtrA binding to complete sites [a] and [b] resembles cooperative binding between half-sites. Both cases exhibit an intrinsic (unphosphorylated CtrA) cooperative binding such that the K_d is lowered ~2-fold by the proximity of the partner protein(s). Also, both cooperative binding interactions are enhanced upon CtrA-P phosphorylation. At site [b], the CtrA-P K_d is lowered 10-fold by wild-type proximity to site [a]. The CtrA-P K_d is also lowered 23-fold by half-site interactions at [b], separated from interactions with site [a] (Table IIC). Combining both cooperative binding interactions, the CtrA-P K_d dramatically falls 230-fold at site [b] in the wild-type *Cori* context. These multiple interactions are implied in the cartoon in Figure 6C. At site [a], intrinsic binding is very low, presumably due to the CTAA half-site variation. Cooperative binding is absolutely essential for CtrA binding at site [a]. We were unable to detect binding to site [a] in the +14ab mutation, and CtrA binding to site [a] requires wild-type proximity to site [b].

In vivo transcription studies from the *Cori* strong promoter (Ps) also support our cooperative binding model (Figure 6C). For example, deleting the left TTAA half-site of [a] in pGM1027 (Figure 6A) appears to unravel the repressed state imposed by CtrA, as revealed by the relative *lacZ* expression in cells containing the wild-type *ctrA* versus the *ctrA401* loss-of-function allele (Figure 6B). The Ps promoter is repressed selectively in swarmer cells, and it was proposed that Ps transcription restricts *Cori* replication to the stalked cells (Marczynski *et al.*, 1995). The -14ab deletion (Table IIA) deregulates both Ps

transcription and *Cori* plasmid replication. Ps transcription from the -14ab mutant occurs in both swarmer and stalked cells, and -14ab *Cori* plasmid replication occurs promiscuously throughout the cell cycle (Marczynski *et al.*, 1995). We therefore also studied CtrA binding to the -14ab deletion that retained half of site [a] and most of site [b] (site [b'], Table IIA). Only phosphorylated CtrA-P bound only to site [b'], but with a 100-fold higher K_d than to site [b] in the wild-type *Cori* context (Table IC), and with a 10-fold higher K_d than to site [b] in the +14ab context (Table IIB). This is an impressive decrease in binding affinity since site [b'], like site [a], only differs by 1 bp from the apparent TTAA-N7-TTAA consensus (Table IA and B). These results also indicate that one weak CtrA-binding site is not sufficient to mediate cell type-specific and cell cycle control.

Other response regulators also bind DNA cooperatively. OmpR binding to three adjacent sites is both hierarchical and cooperative, and produces both positive and negative regulation of the *OmpF* promoter (Huang and Igo, 1996). Removing one OmpR site decreases the binding of OmpR to the adjacent sites (Huang *et al.*, 1994b). Half-site mutation studies, employing the gel mobility shift assay, suggest that OmpR requires both half-sites for stable OmpR-DNA complex formation (Huang and Igo, 1996). Our half-site mutation studies (Figure 3), employing footprint assays, demonstrate a similar requirement for a stable CtrA-DNA complex. NtrC is another well studied response regulator (Magasanik, 1993). NtrC binding to one site is not enhanced by phosphorylation of the protein. However, NtrC phosphorylation is essential for cooperative binding between two adjacent sites (Weiss *et al.*, 1991) and transcription activation of the *glnA* promoter (Klose *et al.*, 1993; Wyman *et al.*, 1997). Apparently, phosphorylation induces oligomerization of NtrC-P at two adjacent regulatory sites. Oligomerized NtrC-P makes subsequent contacts with the σ -54 RNA polymerase at the promoter with the aid of DNA looping induced by IHF protein (Wyman *et al.*, 1997). Unlike NtrC, CtrA and OmpR binding to one site is enhanced upon phosphorylation. Like NtrC, phosphorylation also stimulates CtrA-P binding between adjacent sites [a] and [b], a property also shared by OmpR.

We also observed independent CtrA binding inside *Cori*. While CtrA binds cooperatively to adjacent sites [a] and [b], CtrA binds independently to distant sites [c], [d] and [e]. As described above in our results, the data in Figures 2-6 demonstrate that CtrA and CtrA-P binding affinities for these sites are not reduced when neighbouring sites are eliminated. For example, CtrA and CtrA-P binding to sites [a] and [b] are completely unaltered by a progressive deletion of sites [c], [d] and [e] (Figure 2). Independent binding probably results from the ~100, ~50 and ~200 bp distant spacing between the [ab], [c], [d] and [e] sites, respectively (Figure 1).

This independent CtrA binding implies a modular organization of *Cori*. While sites [a] and [b] regulate the Ps promoter (Quon *et al.*, 1998; Figures 1 and 6), the function(s) of sites [c], [d] and [e] remains speculative. Site [c] overlaps a binding site for the *E. coli* IHF protein (R. Siam and G.T. Marczynski, unpublished). Since *E. coli* and *C. crescentus* IHF proteins appear to be interchangeable (Gober and Shapiro, 1990), this suggests that CtrA may

regulate *Cori* DNA bending by binding at site [c]. IHF and other histone-like proteins assist the DnaA initiator protein in unwinding the *E. coli* replication origin (Hwang and Kornberg, 1992). Mutations at site [d] elevate *Cori* plasmid copy number (Quon *et al.*, 1998) and, apparently, CtrA represses replication by binding at site [d]. Therefore, CtrA-binding sites [a], [b] and [d] are all involved in repressing replication. Considering the position of the IHF-binding site, between sites [ab] and [d], CtrA repression of Ps may also use a DNA looping mechanism similar to OmpR at *OmpF* (Huang *et al.*, 1994a). Site [e] is only 4 bp away from an essential DnaA box (Marczynski and Shapiro, 1992). DnaA protein is essential for the initiation of chromosome replication in *E. coli* (Messer and Weigel, 1997) and *C. crescentus* (B. Gorbatyuk and G.T. Marczynski, unpublished). Therefore, CtrA may also regulate DnaA protein activity and the initiation of chromosome replication by binding at site [e].

Based on our one-site model (Figure 7), we propose that CtrA phosphorylation and cooperative binding between two half-sites is sufficient to discriminate between specific targets inside *Cori* and accidental targets on the genome. The same argument would apply to transcription promoters that apparently use only one CtrA-binding site (Reisenauer *et al.*, 1999). The cell cycle variations of CtrA protein abundance and the variations in CtrA-P phosphorylation presumably switch *Cori* between CtrA-bound and unbound states. Additional cooperative interactions at CtrA-binding sites [a] and [b] probably reflect a special requirement to repress the *Cori* Ps promoter completely in swarmer cells.

CtrA is the first example of a response regulator protein that binds a replication origin and controls chromosome replication (Quon *et al.*, 1998). Previous work presented primarily *in vivo* experiments, and the influence of CtrA phosphorylation was not analysed. Our binding data provide the first biochemical insights on how regulation might occur through CtrA binding coupled to cell cycle phosphorylation.

Materials and methods

Bacterial strains, protein purification and phosphorylation

Strains and plasmids used in this study are listed in Table III. Plasmids for *in vivo* transcription studies were mobilized to *C. crescentus* from *E. coli* S17-1 by conjugation (Simon *et al.*, 1983). *Caulobacter crescentus* cells were grown exponentially at 30°C (OD ~0.5), and β -galactosidase assays were performed (Marczynski *et al.*, 1995). Histidine-tagged CtrA fusion protein was overexpressed in *E. coli* BL21 (Novagen) from pTRC7.4, and purified as described (Quon *et al.*, 1996). MBP-EnvZ was purified from *E. coli* BL21 containing plasmid pKJH5 as described (Huang and Igo, 1996). CtrA was phosphorylated using purified MBP-EnvZ as described (Reisenauer *et al.*, 1999), except that 1 μ M MBP-EnvZ was incubated with 1 μ M CtrA and 0.4 mM ATP for 1 h at 37°C. To measure the percentage of CtrA phosphorylated in a reaction, 10 μ Ci of [γ - 32 P]dATP (Amersham) was added to this standard kinase reaction. The labelled CtrA-P was subjected to SDS-PAGE without boiling, and the intensity of the phosphorylated CtrA bands was measured by PhosphorImaging (Molecular Dynamics), alongside 32 P standards (data not shown).

DNase I footprinting experiments

DNase I protection assays (Galas and Schmitz, 1978) were performed as described (Quon *et al.*, 1998). Both salmon sperm DNA (Sigma) and poly(dI-dC) (Pharmacia Biotech) were employed as non-specific DNA with no detectable differences. The DNA fragments employed were end labelled and prepared as described (Quon *et al.*, 1998). Quantitatively

Table III. Bacterial strains and plasmids

Strain/plasmid	Genotype or description	Reference/source
<i>E.coli</i>		
BL21	F minus, <i>lon</i> , <i>ompT</i> , <i>hsdS_B</i>	Studier and Moffatt (1986)
S17-1	<i>E.coli</i> 294::RP4-2(Tc::Mu) (Km::Tn7)	Simon <i>et al.</i> (1983)
<i>C.rescens</i>		
LS1094	<i>ctrA</i> 401(Ts)	Quon <i>et al.</i> (1996)
LS1089	<i>ctrA</i> + isogenic to LS1094	Quon <i>et al.</i> (1996)
Plasmids ^a		
pTrc7.4	His ₆ N-terminal tagged CtrA protein ^b	Quon <i>et al.</i> (1996)
pKJH5	MBP–EnvZ kinase protein fusion	Huang and Igo (1996)
pRKlac290	RK2-based <i>lacZ</i> transcription reporter	Gober and Shapiro (1992)
pGM976	pRKlac290 <i>Cori BamHI</i> to Ps::lacZ ^c	this work, Figure 6
pGM1027	pRKlac290 <i>Cori BamHI</i> to Ps::lacZ	this work, Figure 6
pGM1028	pRKlac290 <i>Cori BamHI</i> to Ps::lacZ	this work, Figure 6
pBluescript II	SK(+) or KS(+) cloning vector	Stratagene
pGM1610	<i>Cori HindIII</i> to <i>BamHI</i> at +714 in pKS(+) ^d	this work
pGM1877	<i>Cori HindIII</i> at +214 to <i>XhoI</i> in pSK(+)	this work
pGM2080	–14ab <i>Cori HindIII</i> to <i>XhoI</i> in pKS(+)	this work, Figure 5
pGM2081	+14ab <i>Cori HindIII</i> to <i>XhoI</i> in pKS(+)	this work, Figure 5
pGM2082	Mut-e <i>Cori BamHI</i> to <i>BamHI</i> in pKS(+) ^e	Marczynski <i>et al.</i> (1995), Figure 3
pGM1964	Mut-d <i>Cori BamHI</i> to <i>BamHI</i> in pKS(+) ^e	Quon <i>et al.</i> (1998), Figure 3

^aPlasmids are ampicillin resistant, except pRK290lacZ plasmids, which are tetracycline resistant.

^bHis₆ N-terminal: the six histidine polypeptide leader originates from pTrcHisA (Invitrogen), and adds 38 amino acids to the presumed start methionine of CtrA (Quon *et al.*, 1996). Enterokinase cleavage removes the first 31 amino acids.

^cPs: strong promoter overlapping CtrA-binding sites [a] and [b]; *Cori* DNA end points are shown in Figure 6, and were placed as indicated upstream from the promoterless *lacZ* gene.

^d*Cori* DNA positions relative to the +1 *PstI* site are indicated for the artificial restriction sites at +714 and +214. *Cori* DNA is wild type, except at the mutations –14ab, +14ab, mut-e and mut-d shown in Figure 3.

^eThis pBluescript KS(+) vector lacks an *EcoRI* site in the multicloning site.

phosphorylated CtrA–P protein (70% phosphorylation) was also used in the specific DNase I footprinting experiments presented in Figure 2 and Table I. CtrA–P has a half-life of 1 h under our assay conditions (data not shown). The lengthy half-life of CtrA–P allowed us confidently to perform the binding assays that take 10 min. In the experiment employing non-histidine-tagged CtrA, the Enterokinase Cleavage Capture Kit (Novagen) was used to remove the enterokinase, as described in the Novagen catalogue.

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