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; Marczynski 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., 1998) and in the cloned C. crescentus promoters under conditions where unphosphorylated CtrA failed to bind (Reisenauer et al., 1999).

In order for chromosome replication to occur, CtrA must bind to the replication origin. This requires periodic phosphorylation of CtrA: CtrA binds to the origin only when phosphorylated (Marczynski et al., 1999). 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) (Marczynski 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]
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**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/H11005 BamHI, P/H11005 PstI, Bg/H11005 BglII, Hp/H11005 HpaI and E/EcoRI 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 His<sub>6</sub>-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 μM) and CtrA (0.1–5 μM) 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 μM 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 μM 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 μM 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

<table>
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<tr>
<th>Binding site</th>
<th>Kd (µM)</th>
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<tr>
<td></td>
<td>CtrA</td>
</tr>
<tr>
<td>a</td>
<td>0.6</td>
</tr>
<tr>
<td>b</td>
<td>0.3</td>
</tr>
<tr>
<td>c</td>
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</tr>
<tr>
<td>d</td>
<td>0.2</td>
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<tr>
<td>e</td>
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(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. The 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 TTAAs, half-sites. This is investigated further in Figure 4 where half-sites occupancy is quantified at both TTAAs half-sites. Occupancy by unphosphorylated CtrA at site [c] was practically the same on both the 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 DNA fragments (Kd = 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 (Kd = 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 (Kd = 0.6 µM; Figure 4A). Therefore, CtrA binding to the left half of site [d] accounts for an ~2-fold difference in the binding Kd 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 II), 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 II) was used in our binding protocol. This Cori fragment was labelled at the EcoRI site (+452) and cleaved at the Psrl 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 IIIB). CtrA and CtrA–P could not bind site [a], and we could only place a lower limit on these Kd values (Table IIIB). 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 Kd 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 Kd (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 Kd 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 Kd 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 BglII site and cut with PstI. Increasing concentrations of CtrA (0.05–1 μM) and CtrA–P (0.05–1 μM) 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 BglII site and cut with PstI. CtrA and CtrA–P binding are compared. (C) Calculated Kd values of the CtrA-binding sites in both the wild-type context and mut-d and mut-e backgrounds.

Table II. Mutant Cori CtrA binding sites [ab]

<table>
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<tr>
<th>Mutant</th>
<th>Binding sites</th>
<th>Kd (μM)</th>
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<tr>
<td></td>
<td></td>
<td>CtrA</td>
</tr>
<tr>
<td>mut-d</td>
<td>d</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>e</td>
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</tr>
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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 BglII site (Figure 1). (A) Band intensities at site [d] (left and right) on the mut-d Cori DNA. (B) Band intensities at site [d] and wild-type Cori DNA. (C) Band intensities at site [c] in 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 Kd 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).

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 Kd 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.
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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 μM). 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 μM) 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 BamHI (position 998). Subsequent cleavage with AatII at Cori position 194 yields a fragment with all five binding sites intact, and cleavage with NarI 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 distant 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 XhoI, EcoRI and HpaI 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-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 Ps promoter

We tested the consequences of deleting CtrA-binding sites [a] and [b] on transcription from the Cori strong promoter (Ps). 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 in mRNA start region (Marczynski et al., 1995).
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 comparatively 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 phosphophore 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 swarm 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 \( \sigma \)-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 μM MBP–EnvZ was incubated with 1 μM CtrA and 0.4 mM ATP for 1 h at 37°C. To measure the percentage of CtrA phosphorylated in a reaction, 10 μCi of [γ-32P]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 32P 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
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.

Acknowledgements

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References


Harfocker, S.L., Bergstrom, L. and Inouye, M. (1995) Tandem binding of

Table III.  Bacterial strains and plasmids

<table>
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<tr>
<th>Strain/plasmid</th>
<th>Genotype or description</th>
<th>Reference/source</th>
</tr>
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<tr>
<td>E. coli</td>
<td>F minus, lon, ompT, hsdSry</td>
<td>Studier and Moffatt (1986)</td>
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<td>S17-1</td>
<td>E. coli 294::RP4-2(Tc::Mu) (Km::Tn7)</td>
<td>Simon et al. (1983)</td>
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<td>C. crescentus</td>
<td>ctaA401(Ts) crTA+ isogenic to LS1094</td>
<td>Quon et al. (1996)</td>
</tr>
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<td>LS1094</td>
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<td>Plasmids</td>
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<td>pTrc7.4</td>
<td>His6 N-terminal tagged CtrA protein</td>
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<td>pKlK290</td>
<td>MBP–EnvZ kinase protein fusion</td>
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<td>RK2-based lacZ transcription reporter</td>
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4Plasmids are ampicillin resistant, except pRK290lacZ plasmids, which are tetracycline resistant.

5His6 N-terminal: the six histidine polypeptide leader originates from pTrcHisA (Invitrogen), and adds 38 amino acids to the presumed start methionine of C. crescentus. (Novagen) was used to remove the enterokinase, as described in the J. Mol. Biol. (1996). Enterokinase cleavage removes the first 31 amino acids.

6Phosphorylation of 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.

References


Modular organization of replication control in *Caulobacter*


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