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Transcriptional Repressor CcpN from *Bacillus subtilis* Compensates Asymmetric Contact Distribution by Cooperative Binding

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Carbon catabolite repression in *Bacillus subtilis* is carried out mainly by the major regulator CcpA. In contrast, sugar-dependent repression of three genes, *sr1* encoding a small untranslated RNA, and two genes, *gapB* and *pckA*, coding for gluconeogenic enzymes is mediated by the recently identified transcriptional repressor CcpN. Since previous DNase I footprinting yielded only basic information on the operator sequences of CcpN, chemical interference footprinting studies were performed for a precise contact mapping. Methylation interference, potassium permanganate and hydroxylamine footprinting were used to identify all contacted residues in both strands in the three operator sequences. Furthermore, ethylation interference experiments were performed to identify phosphate residues essential for CcpN binding. Here, we show that each operator has two binding sites for CcpN, one of which was always contacted more strongly than the other. The three sites that exhibited close contacts were very similar in sequence, with only a few slight variations, whereas the other three corresponding sites showed several deviations. Gel retardation assays with purified CcpN demonstrated that the differences in contact number and strength correlated well with significantly different K_D values for the corresponding single binding sites. However, quantitative DNase I footprinting of whole operator sequences revealed cooperative binding of CcpN that, apparently, compensated the asymmetric contact distribution. Based on these data, possible consequences for the repression mechanism of CcpN are discussed.

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Introduction

Although many bacteria, including *Bacillus subtilis*, are able to utilise a vast number of other nutrients,[1,2] glucose is their preferred carbon source.[3] Therefore, cells need to shut-down other catabolic pathways in the presence of glucose to maximise the energy yield.[4] This is accomplished by so-called catabolite repression. In *Escherichia coli*, catabolite repression is mediated by the central signalling molecule cAMP and its receptor protein CRP.[5,6] By contrast, *B. subtilis* does not encode a CRP homologue nor does it produce detectable amounts of cAMP under aerobic conditions.[7] Instead, catabolite repression in *B. subtilis* is carried out mainly by the concerted action of CcpA and HPr-Ser46-P, which can interact to form a transcriptional repressor or activator, regulating genes involved in carbon catabolism.[8] However, it has been shown recently that at least two genes, *gapB* and *pckA*, are down-regulated in the presence of glucose, independent of CcpA.[9,10] Instead, they are regulated by a novel transcriptional repressor found by transposon mutagenesis screening for derepression of *gapB* and, therefore, named CcpN (for control catabolite protein of gluconeogenic genes).[11] The *gapB* gene encodes the rare isotype B of glyceraldehyde-3-phosphate dehydrogenase, and its gene product catalyses the conversion of 1,3-bisphosphoglycerate to glyceraldehyde 3-phosphate, but only during gluconeogenesis.[9] The *pckA* gene codes for another enzyme required for the synthesis of glucose from

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Krebs cycle intermediates, PEP carboxykinase, which catalyses the conversion of oxaloacetate to phosphoenolpyruvate.[12]

The *ccpN* gene is cotranscribed with the *yqfL* gene, resulting in a bicistronic mRNA. It was shown that this operon is not autoregulated, but constitutively expressed under both glycolytic and gluconeogenic conditions.[11] Homologues of CcpN have been found in the genomes of other Bacilli, e.g. *B. halodurans*, *B. cereus*, *B. anthracis* and *Geobacillus stearothermophilus*, and in different Firmicutes.[11]

Recently, a third gene regulated by CcpN, *sr1*, has been discovered. This gene codes for a small untranslated RNA, SR1, which has been identified by a systematic search for small RNAs within intergenic regions of the *B. subtilis* genome.[13] *sr1* was expressed during gluconeogenesis, but repressed under glycolytic conditions. The *trans*-acting factor responsible for sugar-mediated repression was identified as CcpN.[13] Previous DNase I footprinting experiments for all three known CcpN operators indicated different locations of the binding regions relative to the transcription start site.

The aim of the present work was to investigate the interaction between CcpN and its operator regions in more detail using chemical interference footprinting. These experiments showed that contact strength varied greatly, depending on the sequence of a given site. Gel retardation assays with single binding sites confirmed these observations. However, quantitative DNase I footprinting experiments with DNA fragments of all three genes spanning the corresponding complete operator sequences indicated cooperative binding of CcpN. The possible impact of these results on the repression mechanism is discussed.

Results

Chemical interference footprinting experiments were performed with CcpN-His$_5$ (containing five additional C-terminal histidine residues) purified from an *E. coli* over-expression strain. Electrophoretic mobility shift assays (EMSAs) have verified that His-tagged CcpN shows the same binding properties as wild-type CcpN and Northern blots showed that it can exert the function of wild-type CcpN in a *ccpN* knockout strain (data not shown). All nucleotide numbers in the following paragraphs refer to the transcription start sites. The coding strand is always termed the top strand, and the non-coding strand is always termed the bottom strand.

Methylation interference

Methylation interference experiments were performed to determine guanine and adenine bases contacted by CcpN. DNA fragments were modified at purine residues by dimethyl sulphate before CcpN binding. Adenine is methylated at position N3 in the minor groove and guanine is methylated at position N7 in the major groove. Figure 1 shows the positions of the methyl groups interfering with CcpN binding. The top strand of the *sr1* operator exhibited interference at positions $G_{(-53)}$ and $G_{(-51)}$ in site I and, to a weaker extent, at $G_{(-21)}$ and $G_{(-19)}$ in site II. Adenine residues with a major contribution to CcpN binding were found only in site I at the top strand ($A_{(-48)}$ and $A_{(-46)}$), whereas in site II only less close contacts were detected. At the bottom strand, methylation of $G_{(-45)}$ in site I and $G_{(-17)}$ in site II interfered with CcpN binding. Only less close contacts to adenine residues have been found in the bottom strand: $A_{(-52)}$ and $A_{(-47)}$ in site I and $A_{(-20)}$ in site II were contacted by CcpN.

Since the contacts to guanine residues were in all cases closer than those to adenine, these results indicate that CcpN contacts the DNA mainly via the major groove with some auxiliary contacts in the minor groove. Furthermore, the contacts in site II were generally less close than those in site I.

In the *pckA* operator, only three contacts to guanine residues have been observed: $G_{(-38)}$ in site I and $G_{(-15)}$ in site II at the top strand as well as $G_{(-9)}$ in site II at the bottom strand. Binding site II was found to be contacted much more strongly than site I. The same was true for contacts to adenine. Whereas there were some significant contacts in binding site II ($A_{(-17)}$ and $A_{(-12)}$ at the top strand and $A_{(-11)}$ at the bottom strand), only one contacted adenine was detected in site I ($A_{(-36)}$ at the top strand). No significant contact was found in binding site I on the bottom strand.

The *gapB* operator showed a similarly asymmetric contact distribution, but here, contacts were concentrated in binding site I: Close contacts to guanine ($G_{(-17)}$ and $G_{(-15)}$ at the top strand and $G_{(-9)}$ on the bottom strand) were found, whereas in site II only one less closely contacted guanine ($G_{(+22)}$ on the bottom strand) was detected. The same contact distribution was found for adenine residues. Close and medium contacts were observed only in binding site I ($A_{(-19)}$ at the top strand and $A_{(-11)}$, $A_{(-8)}$ at the bottom strand). In binding site II, no close contact to adenine was observed.

Interestingly, contacts in the *sr1* operator were concentrated upstream of the −35 region and, to a lesser extent, in the spacer between −35 and −10, whereas almost no contact was observed directly within the −35 and −10 regions of p_{SR1}. In contrast, in both the *pckA* and *gapB* operator, close contacts were found only within the −10 region, whereas weak binding sites covered the −35 region and the region downstream from the transcription start site in the *pckA* and *gapB* operator, respectively. Moreover, the interference footprinting revealed that each of the three operators had two CcpN binding sites, although they appeared, due to the short spacer region, as one extended site in the previous DNase I footprints of the *pckA* operator.[11]

Potassium permanganate footprinting

Potassium permanganate footprinting was performed to determine contacts of CcpN to thymine

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