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Transcriptional Activation of the *Bacillus subtilis ackA* Promoter Requires Sequences Upstream of the CcpA Binding Site

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Carbon catabolite protein A (CcpA) is a global regulator of carbon metabolism in gram-positive bacteria, repressing transcription of genes for the utilization of secondary carbon sources in the presence of a readily metabolized carbon source and activating transcription of genes, such as *ackA* and *pta*, that are required for carbon excretion. The promoter region of the *Bacillus subtilis ackA* gene contains two catabolite responsive elements (*cre* sites), of which only the site closest to the promoter (*cre2*) binds CcpA to activate transcription. A region immediately upstream of the *cre2* site is also important for transcriptional activation. The required elements in this region were further defined by mutagenesis. CcpA binds to the *ackA* promoter region in gel shift assays even in the presence of mutations in the upstream element that block transcriptional activation, indicating that this region has a function other than promoting binding of CcpA.

Carbon catabolite repression in *Bacillus subtilis* and other gram-positive bacteria is controlled by a mechanism different from that in gram-negative bacteria. The key regulator is carbon catabolite protein A (CcpA), which represses the transcription of various genes encoding proteins involved with the utilization of secondary carbon sources (12). CcpA also activates the transcription of genes involved in carbon excretion. These genes include *pta* and *ackA*, which function together to convert acetyl coenzyme A to acetate for excretion into the growth medium (11, 22, 26).

The CcpA protein is a member of the LacI-GaIR family of transcriptional repressors (13). Members of this family contain an amino-terminal helix-turn-helix DNA binding domain and carboxy-terminal regions involved with effector recognition and oligomerization (28). The activity of CcpA is controlled by HPr or the HPr homologue Crh, both of which are phosphorylated by an ATP-dependent kinase during growth in glucose (8, 9, 23, 24). Mutations which block this signaling pathway cause loss of glucose repression of many target genes and loss of transcriptional activation of *ackA* and *pta* (1, 2, 22, 27). Activation of *ackA* expression during growth in glucose is dependent on the *cre2* CcpA binding site (centered at −56.5 relative to the transcription start site) and sequences upstream of *cre2* (11, 27). The molecular mechanism of transcriptional activation by CcpA is unknown, and the role of the region upstream of the CcpA binding site has not been characterized.

Deletion analysis of the *ackA* upstream region. Deletions of the region upstream of *cre2* were generated by PCR, and transcriptional fusions to *lacZ* were generated using the plasmid pFG328 (11) and inserted in single copy into the *B. subtilis* chromosome by recombination into an SPβ prophage. Expression of the *ackA-lacZ* fusions was monitored during growth in TSS medium (5) containing 1% Casamino Acids (Difco) in the presence or absence of glucose (1%). Deletion constructs up to and including ACKBAM11, which contains 28 bp of sequence upstream of *cre2*, exhibited normal induction of *ackA-lacZ* expression (Table 1). ACKBAM6, containing 23 bp upstream of *cre2*, exhibited a small decrease in activation. These results map the 5′ end of the sequence elements required for complete activation of the *ackA-lacZ* fusion in the presence of glucose to between 23 and 28 bp upstream of *cre2*.

Random mutagenesis. To identify the sequence elements required for activation, the 33 bp upstream of *cre2* were randomly mutagenized by amplifying this region using an oligonucleotide containing 6% non-wild-type bases (94% wild-type) at each position in the region. This frequency of doping is predicted to create a pool of oligonucleotides with 1 to 3 mutations per individual oligonucleotide with a ≤1% probability of the wild-type sequence (15). The oligonucleotide included 13 bp of wild-type *cre2* sequence at its 3′ end to ensure efficient annealing of primers containing mismatches at the 3′ end of the target sequence. The resulting pool of PCR fragments was inserted into the plasmid pFG328 to generate *ackA-lacZ* transcriptional fusions, and the plasmids were propagated

TABLE 1. Deletion analysis of the 5′ end of the *ackA* promoter region

ackA-lacZ fusion construct	*ackA* DNA	β-Galactosidase activity[a] for BR151MA	
		−Glucose	+Glucose
Wild type	*cre2* + 987 bp[b]	150	500
ACKBAM7	*cre2* + 33 bp	180	500
ACKBAM11	*cre2* + 28 bp	140	500
ACKBAM6	*cre2* + 23 bp	110	360
ACKKPN2	*cre2* + 11 bp	50	50

[a] Cells were grown in TSS medium (5) containing 1% Casamino Acids or 1% Casamino Acids plus glucose (1%). β-Galactosidase activities are expressed in Miller units (20) and indicate activity at 30 min prior to the time of entry of the culture into stationary phase. Growth experiments were performed at least two times and showed less than 10% variation.

[b] The wild-type fusion contains a 1.35-kb *Eco*RI fragment, including 987 bp upstream of *cre2*, inserted into the plasmid pFG328 (11). Deletion constructs represent a 5′ truncation of this fragment and were generated by PCR using the 1.35-kb fragment as a template. All constructs were verified by DNA sequencing.

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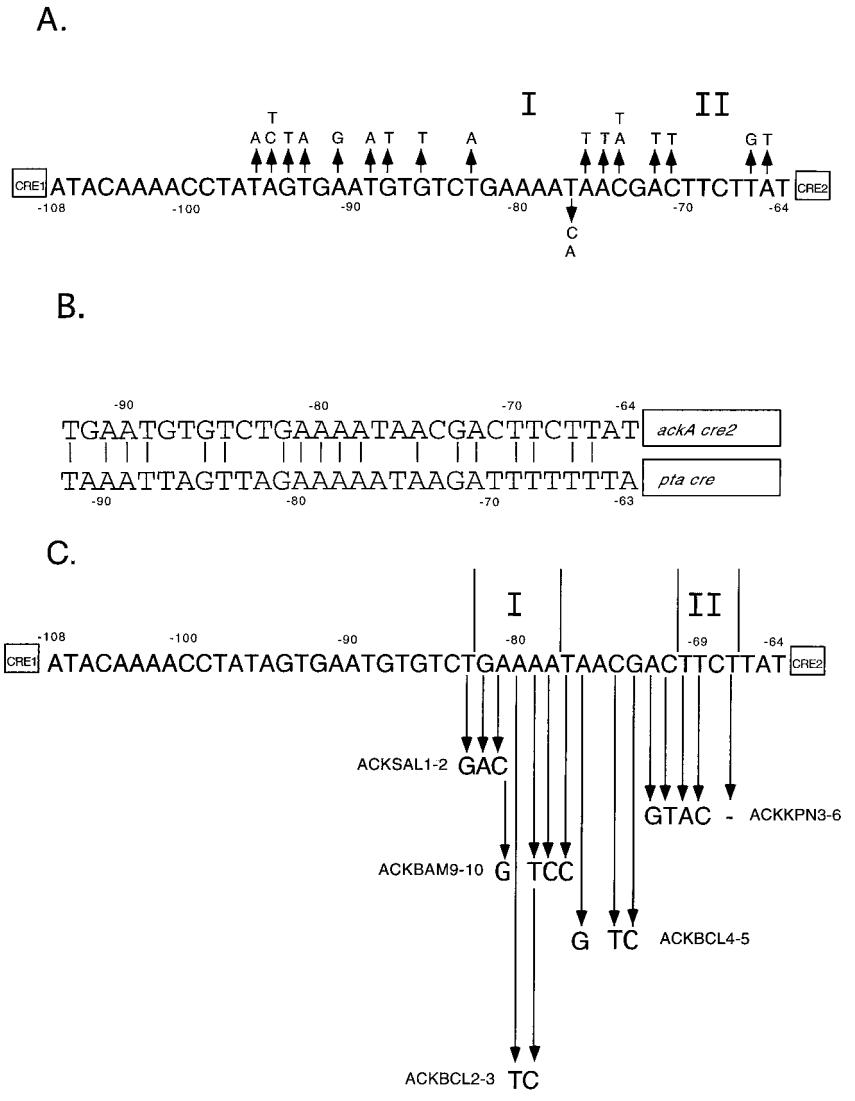

FIG. 1. Sequence of the *ackA* upstream region. Numbering is relative to the transcription start site. (A) Random mutagenesis of the region between nucleotides −96 and −64. Up arrows indicate mutations that allowed a significant degree of activation of the *ackA* promoter in the presence of glucose. Down arrows indicate mutations that did not allow significant activation. Two triple mutations were obtained: A(−95)C A(−91)G G(−86)T and G(−94)T A(−91)G T(−89)A. Four double mutations were obtained: A(−76)T A(−72)T, A(−95)T A(−72)T, A(−95)C C(−74)A, and T(−93)A C(−74)T. Eleven single mutations were obtained: A(−65)T, T(−66)G, C(−71)T, C(−74)A, A(−75)T, A(−76)T, T(−77)C, T(−77)A, T(−83)A, G(−88)T, and T(−96)A. (B) Alignment of sequences upstream of the *ackA* and *pta cre* sites. (C) Site-directed mutagenesis. Mutations were generated by PCR and introduced restriction sites at the positions shown. ACKKPN3-6 deleted a T residue at position −67. ACKBCL1-2T is the same as ACKBCL2-3 with the introduction of an additional T residue between positions −69 and −70.

as mixed pools and then introduced into the *B. subtilis* chromosome by recombination of the fusion into an SPβ prophage. Isolates that retained normal activation of *ackA-lacZ* transcription during growth on tryptose blood agar base (Difco) containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 μg/ml) and glucose (1%) were selected for further analysis, and the DNA sequence of the *ackA* upstream region was determined. Of the 34 candidates chosen, 17 were wild type, 2 had three mutations, 4 had two mutations, and 11 had one mutation. The results revealed two sequence elements in which no mutations appeared, the first between positions −82 and −77 (region I) and the second between positions −70 and −67 (region II) (Fig. 1A). These elements were presumed to be important for the activation of transcription.

The importance of these elements is also supported by conservation between the *ackA* and *pta* upstream regions (Fig. 1B), suggesting that transcriptional activation of *ackA* and that of *pta* operate by similar mechanisms. Interestingly, 7 of the 12 differences between the *ackA* and *pta* regions were found as single mutations in the random mutagenesis and therefore do not drastically affect expression of *ackA*. Four of the remaining five differences are at positions which were invariant in these experiments but are surrounded by positions which did change. The final difference between *ackA* and *pta* is within region II [C(−68)T], suggesting that T may be allowed at this position or that there are context-specific effects.

β-Galactosidase measurements for isolates with the mutations T(−96)A, T(−83)A, T(−77)A, C(−74)A, and

TABLE 2. Effect of upstream region mutations on *ackA-lacZ* expression

Mutation	β-Galactosidase activity[a] for:			
	BR151MA		BR151MACcp::Spc[b]	
	− Glucose	+ Glucose	− Glucose	+ Glucose
None	120	410	60	64
T(−96)A	140	400	NT[c]	NT
T(−83)A	180	430	NT	NT
T(−77)A	60	130	NT	NT
C(−74)A	240	860	160	160
T(−66)G	75	270	NT	NT
ACKBAM9–10	45	80	32	32
ACKKPN3–6	50	85	NT	NT
ACKBCL1–2T	50	98	NT	NT
ACKBCL2–3	52	130	41	41
ACKSAL1–2	35	75	NT	NT
ACKBCL4–5	45	98	NT	NT

[a] Cells were grown in TSS medium containing 1% Casamino Acids or 1% Casamino Acids plus glucose (1%). β-Galactosidase activities are expressed in Miller units (20) and indicate activity at 30 min prior to the time of entry of the culture into stationary phase. Growth experiments were performed at least two times and showed less than 10% variation.

[b] BR151MACcp::Spc contains a null allele of *ccpA* (10).

[c] NT, Not tested.

T(−66)G, each of which contained only a single mutation, are shown in Table 2. The activation ratio ranged from 2.5- to 3.6-fold, relative to 3.4-fold for the wild-type fusion, and the expression level varied from half to twice that of the wild-type fusion. The C(−74)A mutant exhibited a basal level and glucose-activated transcriptional level two-fold over the wild-type level. This fusion was confirmed to be in single copy (data not shown). To determine if this increase in expression was dependent on CcpA, the mutant fusion was introduced into a *ccpA* mutant strain. While no activation in the presence of glucose was observed, the basal activity of the fusion was still increased over that of the wild-type fusion, indicating that CcpA is required for activation but that some other factor is leading to the increase in basal activity.

Site-directed mutagenesis of the region upstream of *cre2*. Site-directed mutagenesis was performed on the sequence elements highlighted by the random mutagenesis (Fig. 1C). Each mutation drastically affected activation in the presence of glucose, confirming the importance of these sequence elements (Table 2). These constructs were generated in the context of the entire *ackA* upstream region, indicating that the presence of *cre1* does not obviate the requirement for these elements. The ACKBAM9-10 and ACKBCL2-3 mutant fusions were also introduced into BR151MACcpA::Spc, and the residual activation was found to require CcpA.

Binding of CcpA to the *ackA* upstream region. The sequence elements required for activation could function as a protein binding site, assist in the binding of CcpA to the *cre2* site, or confer a DNA conformation required for activation of the *ackA* promoter. To test the hypothesis that the region upstream of *ackA cre2* is important for CcpA binding, a gel mobility retardation assay was performed using wild-type and mutant DNA fragments. CcpA was purified to homogeneity by incorporating a six-histidine tag at the amino terminus followed by passage through a nickel-nitrilotriacetic acid (Ni-NTA) column, essentially as described for *Bacillus megaterium* CcpA (1). The His-tagged CcpA bound efficiently to a 163-bp wild-type *ackA cre2*-containing DNA fragment (Fig. 2a). It was previously shown that mutagenesis of *cre2* conferred a loss of transcriptional activation from the *ackA* promoter, most likely due to the lower affinity of CcpA for the mutant *cre2* site (27). It was determined that CcpA can still bind in vitro to the DNA fragment containing the mutant *cre2*, but with a significant reduction in affinity (data not shown). Since the sequence upstream of *cre2* might affect CcpA binding to *cre2*, binding of CcpA to the mutant DNA fragments was tested. As represented by the site-directed mutant ACKKPN3-6, CcpA was still able to bind the fragments in each site-directed mutant (Fig. 2a and data not shown). In a competition assay (Fig. 2b), ACKSAL1-2 and ACKBAM9-10 DNA fragments were able to sequester CcpA from the labeled wild-type DNA as efficiently as did unlabeled wild-type DNA. It therefore appears that the upstream elements are not required for CcpA binding.

The requirement for these DNA elements for transcriptional activation of *ackA* suggests that additional factors may be involved. It is known that phosphorylated HPr or Crh is required for activation in vivo (27); however, in other systems these proteins have been shown to affect the efficiency of binding of CcpA to *cre* sites without directly contacting the DNA themselves (6, 7). Another unknown protein may be binding to the region upstream of the *cre2* site. RNAP has been shown to respond to two bound activators to initiate transcription (14). For example, in an artificial promoter construct, λcI contacts the σ subunit of RNAP while cyclic AMP receptor protein (CRP) contacts the carboxy-terminal domain of the α subunit (α-CTD) of RNAP to stimulate transcriptional initiation (16). In the activation of the divergent *malEp* and *malKp* promoters, CRP effects a shift of the activator MalT to its functional sites in phase with the −10 region of the promoter to allow a favorable interaction with RNAP (25). For the *proP* P2 promoter, CRP (at −121.5) and Fis (at −41) act together to stimulate transcription initiation (19); each of these proteins individually makes contact with the α-CTD portion of RNAP. It is possible that some form of coactivation is occurring at the *ackA* promoter, which includes binding of CcpA to *cre2* and binding of some other factor to the region upstream of *cre2*. Alternatively, the upstream region may interact directly with RNAP, perhaps functioning as an UP element to contact the α-CTD; if so, activation must be dependent on CcpA binding, perhaps to reposition the upstream region to permit contact with RNAP. Proteins such as integration host factor have been shown to bend the DNA to allow optimal contact between the subunits of RNAP and a DNA sequence or another protein binding upstream of the promoter (21). UP elements, which like the *ackA* upstream region contain A- and T-rich sequences, have been identified upstream of *Escherichia coli* and *B. subtilis* promoters and have been shown to interact with α-CTD (3). This interaction between the UP element and the α subunit of RNAP may assist the binding of RNAP to the promoter and further steps of transcription initiation (4). CcpA could also bend the DNA to facilitate an interaction between RNAP and region I and/or region II.

Further study will be required to determine the function of the upstream sequence elements and the molecular mechanism of transcriptional activation. Since these elements are conserved in *pta*, it is likely that activation is similar for the two

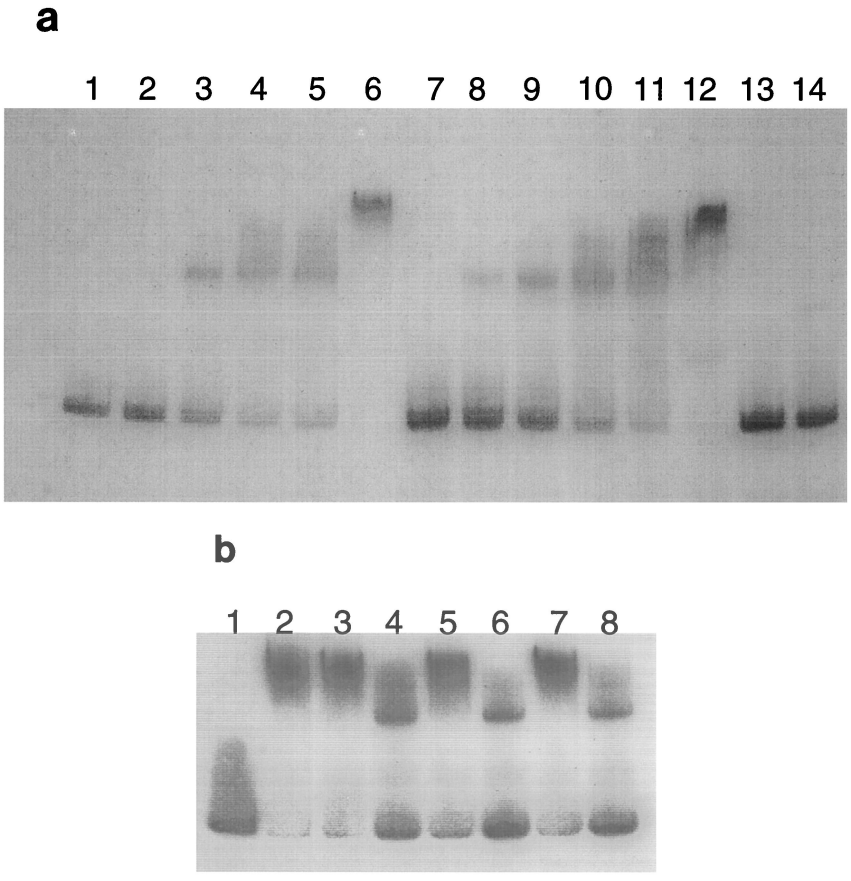

FIG. 2. (a) Binding of CcpA-His to an *ackA cre2*-containing DNA fragment. The *ackA* DNA (163 bp; positions −139 to +24 relative to the transcription start site) was generated by PCR, labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP, and purified with the Qiagen QIAquick PCR purification kit. Mutant DNA was generated in parallel to the wild-type fragment. The nonspecific DNA probe (169 bp) contained a portion of the *ccpA* coding region. The intact *ccpA* coding region was isolated by PCR and inserted into the plasmid pQE30 (Qiagen) to generate the amino-terminal six-histidine tag, introduced into *E. coli* M15(pREP4) (Qiagen), and expressed upon addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 2 mM. The His-tagged protein was purified using nickel-nitrilotriacetic acid spin columns (Qiagen), and imidazole (250 mM) was used to elute the protein. Probe DNA (1 ng) was incubated with CcpA in a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 50 mM KCl, 5% glycerol, 50 μg of bovine serum albumin/ml, and 0.05% igepal CA-630 (Sigma) in a 20-μl volume for 10 to 30 min at 37°C (18). Samples were mixed with nondenaturing sample buffer (40% sucrose, 0.25% bromphenol blue) and loaded directly onto a 5% polyacrylamide gel prepared in 6.7 mM Tris-HCl (pH 8.0)–1 mM EDTA–2.5% glycerol. Gels were run at 200 V for 1.5 h in a Protean II xi Cell (Bio-Rad) gel electrophoresis unit, dried, and exposed to X-ray film (Kodak) for autoradiography. Lanes 1 to 6, wild-type DNA (1 ng); lanes 7 to 12, ACKKPN3-6; lanes 13 and 14, CcpA9-10 (nonspecific sequence). Lanes 1, 7, and 13, no protein; lanes 2 and 8, 1 ng of CcpA-His (0.66 nM); lanes 3 and 9, 3 ng of CcpA-His (2.0 nM); lanes 4, 10, and 14, 7 ng of CcpA-His (4.6 nM); lanes 5 and 11, 10 ng of CcpA-His (6.6 nM); lanes 6 and 12, 20 ng of CcpA-His (13 nM). (b) Competition gel retardation assay. Unlabeled wild-type and mutant DNA was tested for the ability to sequester CcpA-His from the labeled wild-type probe (1 ng). The unlabeled DNA contained wild-type sequence (lanes 3 and 4), ACKSAL1-2 sequence (lanes 5 and 6), or ACKBAM9-10 sequence (lanes 7 and 8). Lanes 3, 5, and 7, 10 ng of unlabeled DNA; lanes 4, 6, and 8, 100 ng of unlabeled DNA; lanes 2 to 8, 50 ng of CcpA (33 nM); lane 1, no protein; lane 2, no competing DNA.

genes; this would provide coordinate expression consistent with cotranscription of these genes in *E. coli* (17).

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