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Transcriptional Activation of the *Bacillus subtilis ackA* Gene Requires Sequences Upstream of the Promoter

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Transcriptional activation of the *Bacillus subtilis ackA* gene, encoding acetate kinase, was previously shown to require catabolite control protein A (CcpA) and sequences upstream of the *ackA* promoter. CcpA, which is responsible for catabolite repression of a number of secondary carbon source utilization genes in *B. subtilis* and other gram-positive bacteria, recognizes a *cis*-acting consensus sequence, designated *cre* (catabolite response element), generally located within or downstream of the promoter of the repressed gene. Two sites resembling this sequence are centered at positions −116.5 and −56.5 of the *ackA* promoter and have been termed *cre1* and *cre2*, respectively. Synthesis of acetate kinase, which is involved in the conversion of acetyl coenzyme A to acetate, is induced when cells are grown in the presence of an easily metabolized carbon source such as glucose. In this study, *cre2*, the site closer to the promoter, and the region upstream of *cre2* were shown to be indispensable for CcpA-dependent transcriptional activation of *ackA*, whereas *cre1* was not required. In addition, insertion of 5 bp between *cre2* and the promoter disrupted activation, while 10 bp was tolerated, suggesting face-of-the-helix dependence of the position of *cre2* and/or upstream sequences. DNase footprinting experiments demonstrated binding of CcpA in vitro to *cre2* but not *cre1*, consistent with the genetic data. Activation of *ackA* transcription was blocked in a *ptsH1*/*crh* double mutant, suggesting involvement of this pathway in CcpA-mediated transcriptional activation.

The mechanism of carbon catabolite regulation in gram-positive bacteria appears to be fundamentally different from that employed by enteric gram-negative bacteria. The absence of detectable amounts of cyclic AMP under normal growth conditions in *Bacillus subtilis* has long indicated that the regulation of secondary carbon source utilization genes in *B. subtilis* is different from that of *Escherichia coli*, which relies on cyclic AMP as an effector of the CAP/CRP protein (see references 17 and 25 for a review). Unlike the positive regulatory mechanism found in *E. coli*, carbon catabolite regulation in gram-positive bacteria appears to be mediated by transcriptional repression, requiring *trans*-acting CcpA (catabolite control protein A), a member of the LacI-GalR family of bacterial regulatory proteins (16), and a *cis*-acting consensus sequence, designated *cre* (20, 51). The *cis*-acting site, first identified in the regulation of the gene for α-amylase (*amyE*) and termed *amyO*, is a palindromic sequence structurally similar to the *E. coli lac* and *gal* operators (38, 51). Elements resembling the consensus sequence have been found in other secondary carbon source utilization genes, including *acsA* and *acu* (12), *hut* (52), *gnt* (33), *xyl* (26), *bgl* (27), *lev* (29), and *mmg* (1). Catabolite repression of these genes is relieved upon mutation of the consensus sequences or expression in a *ccpA* mutant.

CcpA homologs have been identified in a number of other gram-positive organisms, including *B. megaterium* (21), *Staphylococcus xylosus* (8), *Clostridium acetobutylicum* (4), and *Lactobacillus casei* (35). Western analysis suggests that proteins with related antigenic determinants are widely distributed in gram-positive bacteria (28), although it has not been demonstrated that these proteins are functional homologs of CcpA. The *cre* element has also been found in a large number of genes in a variety of gram-positive organisms (20), although the significance of most of these sequences has not been tested. A second *B. subtilis* gene related to *ccpA* has recently been identified (2); this gene, designated *ccpB*, appears to play a role in catabolite repression under low-oxygen growth conditions.

CcpA is constitutively expressed in *B. subtilis* (1a, 17, 34) and is therefore likely to require a signalling pathway to control its activity. The details of this pathway are not completely clear. CcpA has been shown to bind in vitro to the HPr protein of the phosphoenolpyruvate-dependent phosphotransferase system when HPr is phosphorylated at Ser-46, and this interaction affects the DNA binding activity of CcpA (5, 11, 22). The ATP-dependent phosphorylation of Hpr at Ser-46 is catalyzed by the *ptsK*-encoded kinase, activity of which is stimulated by fructose 1,6-bisphosphate (41). A mutant form of HPr containing an alanine substitution at Ser-46 (*ptsH1*) results in the complete or partial loss of catabolite repression of several genes that are subject to control by CcpA (3, 6, 29, 42); however, the *ptsH1* mutation has no effect on repression of *amyE* (50), suggesting at least one other mechanism for CcpA activation. Crh, an HPr-like protein that can only be phosphorylated at Ser-46, has recently been identified in *B. subtilis* (10) and may provide an alternate pathway for control of CcpA activity.

Along with its role as a transcriptional repressor, CcpA is also required for the activation of at least two carbon excretion pathways, acetoin biosynthesis (43) and acetate production (13). Acetyl coenzyme A (acetyl-CoA) and acetate are interconverted by two separate pathways in *B. subtilis*. Phosphotransacetylase, the product of the *pta* gene, catalyzes the conversion of acetyl-CoA to the intermediate acetyl phosphate (39), while conversion of acetyl phosphate to acetate is cata-

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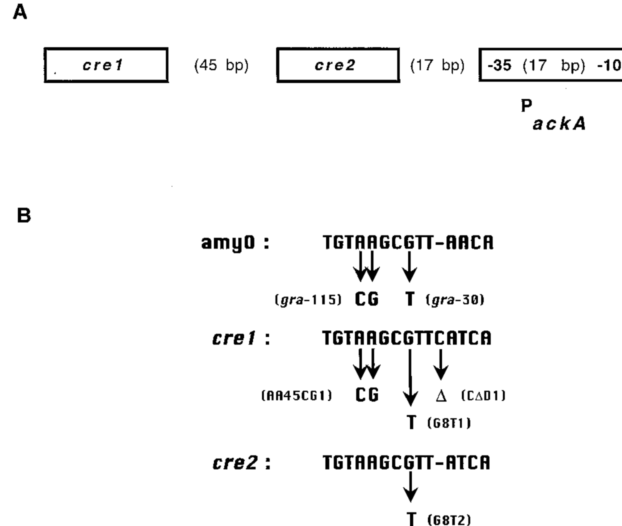


FIG. 1. (A) Structure of the *ackA* promoter region. The positions of *cre1* and *cre2* upstream of the *ackA* promoter are shown. (B) Mutations in *cre1* and *cre2*. The G-to-T substitutions in *cre1* and *cre2* are labeled G8T1 and G8T2, respectively. Mutation of the upstream region of *cre1* is labeled AA45CG1. Δ indicates the base that was deleted in *cre1* to generate a sequence identical to *cre2*. The *amyO* consensus sequence is included for comparison. *gra*-115 and *gra*-30 indicate mutations which eliminate repression at *amyO* (51).

lyzed by acetate kinase, encoded by *ackA* (13). Utilization of acetate by its conversion back to acetyl-CoA requires the *acsA* gene product, acetyl-CoA synthetase (14). The *acsA* gene contains a *cre* site downstream of the transcription start site, and transcription is repressed by CcpA in the presence of excess glucose (12, 14). In contrast to its effect on *acsA* transcription, CcpA activates transcription of *ackA* during growth in the presence of excess glucose (13).

Activation of *ackA* transcription requires sequences upstream of the *ackA* promoter (13). Two *cre* sites, designated *cre1* and *cre2*, are centered at positions −116.5 and −56.5 relative to the transcription start site of *ackA* (Fig. 1). These sites differ from *amyO* by a single base substitution of an A to a T; this substitution at *amyO* has no effect on repression (51). In addition, *cre1* contains a one-base insertion. While *cre* sequences are generally positioned within or downstream of the promoter in genes that are repressed by CcpA, *cre1* and *cre2* are located upstream of the *ackA* promoter. In this study, the roles of these sites in the CcpA-dependent activation of *ackA* transcription were investigated. Mutational analyses demonstrated that *cre2* and the region immediately upstream of this site are required for transcriptional activation and that the position of *cre2* relative to the −35 region of the *ackA* promoter is important for function.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Plasmids were propagated in *E. coli* DH5α grown in LB medium (32). *B. subtilis* was grown in tryptose blood agar base (Difco), 2XYT, or TSS medium (9) with NH_4Cl as the nitrogen source and 1% Casamino Acids as the carbon source, in the presence or absence of 1% glucose. All growth was at 37°C. Antibiotics (Sigma) were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 5 μg/ml for selection and 0.1 μg/ml for induction; erythromycin, 1 μg/ml for selection and 0.1 μg/ml for induction; lincomycin, 25 μg/ml; kanamycin, 5 μg/ml; spectinomycin, 200 μg/ml. Amino acids were added to TSS medium at 50 μg/ml as required for auxotrophic strains. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at 40 μg/ml as an indicator of β-galactosidase activity.

Genetic techniques. Restriction endonucleases were purchased from New England BioLabs or Promega and used as described by the manufacturer. Preparation of single-stranded M13 template DNA and double-stranded plasmid DNA and dideoxynucleotide sequencing (Sequenase; United States Biochemicals) were performed by using standard techniques. Oligonucleotide primers were purchased from Cruachem and are shown in Table 2. PCR was carried out on a Perkin-Elmer DNA Thermal Cycler 480 using a Gene-Amp kit (Perkin-Elmer Cetus). *B. subtilis* transformation was carried out as described previously (15).

***ackA* deletion analysis and operator mutations.** Mutations in the region upstream of *ackA* were generated by PCR-mediated oligonucleotide-directed mutagenesis. All PCRs were performed on a 1.35-kb *Eco*RI fragment which contained the *ackA* promoter and 1 kb of DNA upstream of the promoter. The 1.35-kb fragment was inserted into the *Eco*RI site of plasmid pGEM7Zf(+) (Promega). The *Eco*RI site in pGEM7Zf(+) is flanked by a *Kpn*I restriction site on the 5′ end and an *Xba*I restriction site on the 3′ end. Truncation of the region upstream of *ackA* used preexisting restriction sites or the creation of new sites by PCR. The 3′ end of each deletion fragment made use of the same *Xba*I site and primer M13Sal. Wild-type activity was based on expression from the 1.35-kb *ackA* fragment.

The G8T1 mutation in *cre1* was generated by PCR using the 1.35-kb *Eco*RI fragment as the template, with primers M13Rev and TH449 (5′ fragment) and M13Sal and TH448 (3′ fragment) in separate reactions. The products were digested with *Kpn*I plus *Hin*dIII and *Hin*dIII plus *Xba*I, respectively, and inserted together into plasmid pFG328 digested with *Kpn*I and *Xba*I to regenerate the 1.35-kb fragment with a *Hin*dIII site at *cre1*. The G8T2 mutation in *cre2* was generated in a similar manner by using primers M13Rev and AckO2 (5′ fragment) and primers M13Sal and AckO1 (3′ fragment). The AA45CG1 mutation in *cre1* was generated by mismatched PCR mutagenesis (45). Primers AA45CG1, containing the mutation, and M13Sal were used in one reaction to generate the downstream portion of the *ackA* fragment, and primers Ack4 and M13Rev were used to generate an overlapping upstream fragment. The PCR fragments were gel purified, mixed in equimolar concentrations, denatured, and annealed to form heteroduplex molecules, and the 3′ ends of the heteroduplex molecules were extended in a second PCR using primers M13Rev and M13Sal. The resulting fragment was then cloned by using *Kpn*I and *Xba*I. The CΔD1 mutation was generated in a similar manner by using primer CΔD1 in place of AA45CG1.

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source or reference
B. subtilis		
BR151MA	*lys-3 trpC2*	12
BR151MACcp::spc	*lys-3 trpC2 ccpA*::Tn*917*lacΔ(*lacZ-erm*)::*spc*	12
ZB307A	SPβ*c2del2*::Tn*917*::pSK10Δ6	54
ZB449	*trpC2 pheA1 abrB703* (SPβ cured)	36
168	*trpC2*	Laboratory stock
MIV1	*trpC2 ptsH1*	Transformation of 168 with DNA of SA003 (6)
168*crh*	*trpC2 crh*::*aphA3*	Transformation of 168 with DNA of QB7096 (I. Martin-Verstraete)
MIV1*crh*	*trpC2 ptsH1 crh*::*aphA3*	Transformation of MIV1 with DNA of QB7096
E. coli DH5α	φ80d*lacZ*Δ*M15 endA1 recA1 hsdR17* (r_k^- m_k^+) *thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169	Bethesda Research Laboratories

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence	Use
AckBam1	5′-TTTATGGATCCAGTTGAC-3′	5′ deletion
AckKpn1	5′-GGAGAGGGTACCAAAGCGCCGGGGC-3′	5′ deletion
AckBam2	5′-CGAATTGGATCCAGCTTTG-3′	5′ deletion
AckBam3	5′-GCGTTCATGGATCCAAAACCTATAGTG-3′	5′ deletion
AckKpn2	5′-GTGTCTGAAGGTACCGACTTCTTATTG-3′	5′ deletion
M13Sal	5′-CGACGTTGTAAGTCGACGGCCACTG-3′	5′ pGEM7Zf(+) polylinker
M13Rev	5′-GATAACAATTTCACACAGGA-3′	3′ pGEM7Zf(+) polylinker
AckO1	5′-TTCTTATTGTAAGCTTTATCAATAC-3′	*cre2* G8T2
AckO2	5′-GTATTGATAAAGCTTACAAT-3′	*cre2* G8T2
TH448	5′-CAAATTGTAAGCTTTCATCAATACAAAACC-3′	*cre1* G8T1
TH449	5′-ATTGATGAAAGCTTACAATTTGCGG-3′	*cre1* G8T1
AA45CG1	5′-AATTGTCGGCGTTCATCAATAC-3′	*cre1* AA45CG1
Ack4	5′-CGCTCCTTTATACTCTG-3′	*cre1* AA45CG1
AckBRV	5′-CGCGGATCCTCAACTTGCGTATTG-3′	Linker insertion
AckBam5	5′-CGCGGATCCTTGAAAAGCCGACATG-3′	Linker insertion
AckBS	5′-CGCGGATCCAGGCCTTGAAAAGCCGACATG-3′	Linker insertion
CΔD1	5′-AGCGTTATCAATACAAAAC-3′	CΔD1 mutation

Insertions of 5 and 10 bp between the *ackA* promoter and *cre2* were obtained by using oligonucleotide primers with *Bam*HI sites. Two separate amplification reactions were set up for each insertion. PCR of the upstream fragment for the two insertions with primers AckBRV and M13Rev and digestion with *Kpn*I and *Bam*HI produced a 1-kb fragment that contained the two *cre* sites with a *Bam*HI site at the 3′ end. Amplification of the downstream fragments with primers M13Sal and AckBam5 or AckBS for the 5- or 10-bp insertions, respectively, and digestion with *Bam*HI and *Xba*I resulted in two 350-bp fragments with a *Bam*HI site just upstream of the −35 region of the promoter. Ligation of the AckBRV fragment with the AckBam5 or AckBS fragment resulted in a 5- or 10-bp insertion, respectively, between the *ackA* promoter and *cre2*. All mutations were verified by DNA sequencing.

Construction of *lacZ* fusions. *ackA-lacZ* transcriptional fusions were generated by cloning the sequenced *ackA* fragment into *E. coli* plasmid pFG328 (13) by using *Kpn*I or *Bam*HI at the 5′ end and *Xba*I at the 3′ end. The fusions were incorporated into specialized transducing phage SPβ by homologous recombination between the plasmid and the prophage contained in strain ZB307A. The resulting phage were purified by passage through strain ZB449 (SPβ cured) and used to transduce isogenic wild-type and CcpA$^-$ strains (12).

β-Galactosidase measurements. Strains carrying the *ackA-lacZ* transcriptional fusions were grown in TSS medium in the presence or absence of 1% glucose and maintained in early exponential growth by serial dilutions for adaptation to the growth medium. Samples were then taken at 30-min intervals until 2 h past entry into stationary phase (T_0). β-Galactosidase assays were performed as described by Miller (32), by using toluene permeabilization of the cells. All growth experiments were repeated at least twice and showed less than 10% variation.

Acetate production. Cells were grown in TSS medium with 1% Casamino Acids in the presence or absence of glucose (0.25%). Samples were harvested at 30 min prior to T_0, and culture supernatants were assayed for acetate by using a kit purchased from Boehringer Mannheim.

DNase footprinting. A DNA fragment containing the *ackA* promoter region between positions −195 and +18 was subcloned into plasmid pUC18 and labeled at the 3′ ends with [α-^{32}P]dATP by using Klenow fragment (Promega). The labelled DNA was gel purified and recovered by electroelution as described previously (23). Purification of the CcpA protein and DNase footprinting were carried out as described by Kim et al. (24).

RESULTS

Deletion analysis. Deletion of the entire region upstream of the *ackA* promoter eliminates transcriptional activation in the presence of glucose (13). This region contains two sequences resembling *amyO*, the sequence in the *amyE* promoter region where the CcpA protein binds to repress transcription during growth in glucose (23, 24, 51). These sequences, designated *cre1* and *cre2* (Fig. 1), were considered likely targets for CcpA-dependent transcriptional activation of *ackA*. To precisely identify the sequences required for transcriptional activation, a more detailed deletion analysis was performed. Transcriptional fusions to *lacZ* using deletion fragments extending from 3 bp to 1 kb upstream of the −35 region of the *ackA* promoter (Fig. 2) were introduced in single copy into the *B. subtilis* chromosome by using specialized transducing phage SPβ. Expression of the fusions was monitored in TSS medium containing 1% Casamino Acids in the presence (induced) or absence (uninduced) of 1% glucose.

Deletion of the region upstream of *cre1* had no effect on induction of *ackA-lacZ* expression (Fig. 2). The shortest deletion fragment, AckHincII (13), made use of a *Hinc*II restriction site just upstream of the −35 region of the *ackA* promoter. This mutant was previously shown to exhibit reduced basal activity and no induction by growth in the presence of glucose. Based on these results, two additional deletion mutants were generated by using sites in the region separating the two *cre* sites. AckKpn2, which included sequences 11 bp upstream of *cre2*, resulted in loss of transcriptional activation of *ackA-lacZ* in the presence of glucose. AckBam3, which contained *cre2*

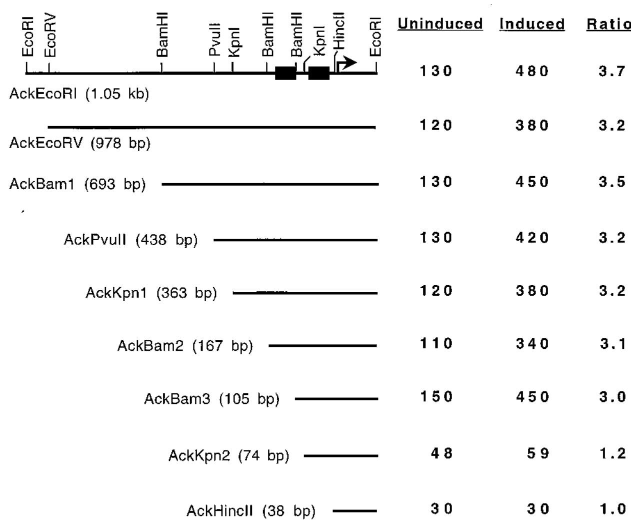


FIG. 2. Deletion analysis of the region upstream of the *ackA* promoter. Each number in parentheses indicates the distance of the deletion end point from the transcription start point (arrow). The two black boxes indicate the positions of the *cre* sites. Cells were grown in TSS medium containing 1% Casamino Acids (uninduced) or TSS medium containing 1% Casamino Acids and 1% glucose (induced). β-Galactosidase activity is expressed in Miller units (32). Values are shown for samples taken 30 min prior to entry into stationary phase. Results for AckHincII were previously reported (13). The ratio indicates the level of induction during growth in the presence of glucose.

along with the entire region separating the two sites (42 bp upstream of *cre2*), retained full transcriptional activation. This region encompasses 70 bp upstream of the −35 region of the *ackA* promoter. These results indicated that sequences between *cre1* and the AckKpn2 deletion end point are necessary for transcriptional activation of *ackA* in the presence of glucose.

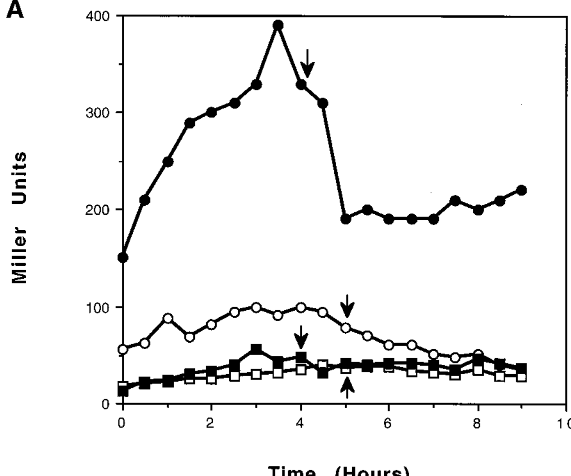
Mutation of Ccp boxes. The results of the deletion analysis indicated that *cre2* is not sufficient for transcriptional activation and that *cre1* is not required. To specifically test the role of these elements, substitutions were made which corresponded to mutations known to eliminate repression at *amyO* (51; Fig. 1B). As shown in Fig. 3A, the wild-type *ackA-lacZ* fusion reaches maximal induction toward the end of exponential growth. A single base substitution in *cre2*, termed G8T2, eliminated transcriptional activation of *ackA* in the presence of glucose. The level of activity was less than the basal activity of the wild-type fusion and was comparable to the activity obtained with the deletion of both elements (Fig. 2). This indicates that *cre2* is necessary for transcriptional activation and also plays a role in basal expression in the absence of glucose.

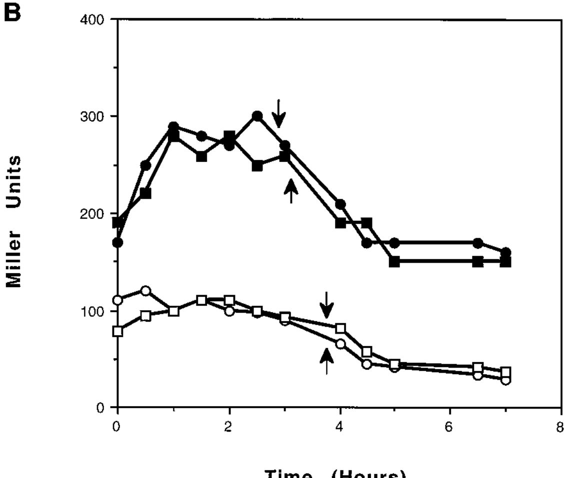
Two different mutations in *cre1* were tested. The G8T1 mutation, which was identical to the mutation made in *cre2*, had no effect on *ackA-lacZ* expression (Fig. 3B), in contrast to the drastic effect of this substitution in *cre2*. These substitutions targeted the downstream region of the elements. To test the possibility that only a portion of *cre1* is necessary, a second mutation in the upstream portion of *cre1*, termed AA45CG1, was generated. This mutation also had no effect on transcriptional activation of *ackA* (data not shown). These results, in conjunction with the deletion analysis, demonstrate that *cre2*, but not *cre1*, is required for transcriptional activation of *ackA*.

cre1 differs from *cre2* by the presence of an extra cytosine 5 bp from the 3′ end (Fig. 1B). A deletion of this extra base, termed CΔD1, was tested to determine if the presence of a second *cre2* sequence would increase transcriptional activation of *ackA*. This mutation resulted in reduction of both the basal and induced levels of expression but had no effect on the induction ratio (Fig. 3C). The presence of a "perfect" element at the position of *cre1* apparently interferes with the normal function of *cre2*, possibly by sequestering the CcpA protein in a nonfavorable interaction.

Linker insertions. The center of *cre2* is located 21.5 bp upstream of the −35 region of the *ackA* promoter. To determine whether the position of the *cre* site is important in transcriptional activation of *ackA*, insertions of 5 and 10 bp were made between *cre2* and the −35 region of the *ackA* promoter. The 5-bp insertion, which positioned *cre2* on the opposite face of the DNA helix from its normal position, eliminated glucose induction and decreased expression approximately 10-fold (Fig. 4). Insertion of 10 bp, which is predicted to position *cre2* on the same face of the DNA helix as in the wild type but 10 bp further upstream, resulted in normal expression and glucose activation. These results demonstrate a face-of-the-helix dependence between the location of *cre2*, and/or upstream sequences, and the *ackA* promoter.

Expression of *ackA* in a CcpA mutant. The reduction in basal expression caused by mutations of the *ackA* upstream region could be due to loss of normal CcpA-dependent activation during growth in the absence of glucose or to other factors. As previously shown (13), mutation of *ccpA* resulted in reduced expression of *ackA* and loss of activation during growth in glucose (Table 3). Mutation of *cre2* or deletion of the region just upstream of *cre2* reduced expression twofold more than the CcpA mutation alone, in both the wild-type and CcpA mutant strains; this suggests that while most of the reduction in





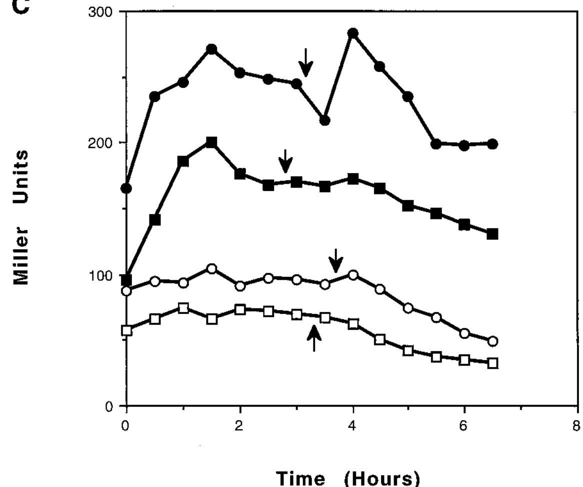


FIG. 3. Effect of mutations in *cre* sites on expression of *ackA-lacZ* transcriptional fusions. Cells were grown in TSS medium containing 1% Casamino Acids (open symbols) or TSS medium containing 1% Casamino Acids and 1% glucose (filled symbols). β-Galactosidase activity is expressed in Miller units (32). The vertical arrows indicate time of entry of the culture into stationary phase. (A) Effect of the G8T2 *cre2* mutant (squares) compared to the wild-type fusion (circles). (B) Effect of the G8T1 *cre1* mutant (squares) compared to the wild-type fusion (circles). (C) Effect of the CΔD1 mutation in *cre1* (squares) compared to the wild-type fusion (circles).

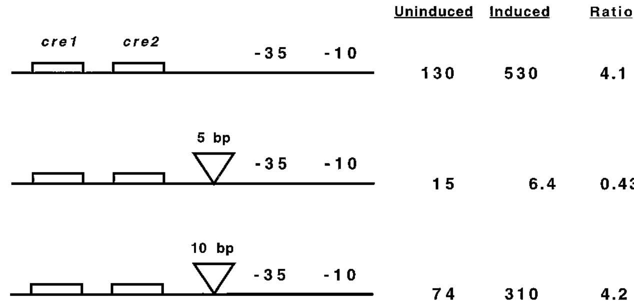


FIG. 4. Effect of linker insertions between *cre2* and the *ackA* promoter. The −35 and −10 regions of the *ackA* promoter are indicated. Cells were grown in TSS medium with 1% Casamino Acids as the carbon source (uninduced) or in TSS medium with 1% Casamino Acids and 1% glucose (induced). Values shown are for samples taken 30 min prior to entry into stationary phase and indicate β-galactosidase activity expressed in Miller units (32). The ratio reflects the level of induction during growth in the presence of glucose.

basal expression of these mutants is likely to be due to loss of interaction with CcpA, there may be additional factors. The insertion of 5 bp between *cre2* and the −35 region of the promoter dramatically reduced basal expression. The reduction is apparently not due to improper positioning of CcpA on the wrong face of the DNA helix, since it was also observed in the CcpA mutant. Since sequences upstream of *cre2* are also required for activation, this reduction in basal activation could be caused by effects of this upstream region on regulatory events other than CcpA binding.

DNase footprinting. Binding of the CcpA protein to the *ackA* promoter region was directly tested by in vitro DNase footprinting (Fig. 5). Protection of *cre2* was readily observed, with an affinity comparable to that of *amyO* (24); in contrast, little if any protection of *cre1* was detected. These results are consistent with the genetic data indicating that *cre2* is the critical element for CcpA-dependent activation. In addition, binding of CcpA to the *ackA cre2* element in vitro was not dependent on any other protein.

Role of HPr/Crh in *ackA* transcriptional activation. Phosphorylation of the HPr and Crh proteins has been suggested to play a key role in CcpA-mediated repression of carbon source utilization genes (6, 10). The effects of the *ptsH1* mutation, which specifically blocks ATP-dependent phosphorylation of HPrSer-46, and a *crh* null mutation on *ackA* transcription were therefore tested (Table 4). While neither single mutation had any effect, the double mutant exhibited a phenotype similar to that of a CcpA mutant. As a control, the effect of the *ptsH1* mutation on glucose repression of gluconate kinase activity was tested; the results were identical to those previously reported (6), confirming that the mutation is intact (data not shown). These results suggest that CcpA-mediated activation of *ackA* requires either HPrSer-46-P or Crh-P.

Acetate production. The effect of *ackA* transcriptional activation on production of acetate was tested by measurements of acetate accumulation in the culture supernatant. *B. subtilis* excretes large amounts of acetate during growth in media containing glucose (48). Acetate production is dependent on acetate kinase, and growth of an *ackA* null mutant is inhibited by addition of glucose to the medium (13). Both wild-type and CcpA mutant strains produced very low levels of acetate (0.04 g/liter) during growth in TSS medium in the absence of glucose; addition of 0.25% glucose resulted in acetate accumulation to 0.84 g/liter in the wild-type strain and to 0.35 g/liter in the CcpA mutant 30 min prior to T_0. Normal acetate production is therefore apparently dependent on CcpA-directed activation of *ackA* transcription.

DISCUSSION

The *B. subtilis ackA* gene encoding acetate kinase is involved in the production of acetate from acetyl phosphate during growth in media containing large amounts of glucose. Transcription of *ackA* is induced during growth in glucose. This induction was previously shown to require CcpA and sequences upstream of the promoter, which include two *cre*-like sequences (13). In this study, the role of these two sites in the CcpA-dependent transcriptional activation of *ackA* was investigated.

Deletion analysis of the region upstream of the *ackA* promoter localized sequences necessary for transcriptional activation to 107 bp upstream of the transcription start site. Mutation of *cre2*, which is centered 56.5 bp upstream of the transcription start site, eliminated transcriptional activation and identified *cre2* as a key regulatory sequence. *cre1*, which is centered at −116.5, is outside of the region identified by deletion analysis as being sufficient for transcriptional activation. The presence of a sequence identical to *cre2*, except for a single additional base, six helical turns upstream and with no apparent function is somewhat surprising. *cre1* may be necessary under growth conditions other than those employed in this study and may assume a role similar to that suggested for two CcpA auxiliary sequences in the *xyl* operon (11). *cre1* could also be required for regulation by a different regulatory factor, such as CcpB, which is apparently active under conditions of low aeration (2); the effect of a *ccpB* mutation on *ackA* expression has not been tested. The genetic data on the relative effects of *cre1* and *cre2* are consistent with DNase I footprinting analyses of CcpA binding to the *ackA* promoter region in vitro; CcpA bound to *cre2* with much higher affinity than to *cre1*.

The position of the CcpA binding site at −56.5 is interme-

TABLE 3. Expression of *ackA-lacZ* fusions in a *ccpA* mutant

ackA-lacZ fusion	Mutation	β-Galactosidase activity[a]			
		BR151MA		BR151MACcp::spc	
		Uninduced	Induced	Uninduced	Induced
AckEcoRI	None (wild type)	75	280	32	39
G8T2	*cre2*	26	39	19	25
AckKpn2	Δ-74	23	31	18	17
LS	5-bp insertion	6.4	3.4	4.8	3.7

[a] Cells were grown in TSS medium containing 1% Casamino Acids (uninduced) or 1% Casamino Acids and 1% glucose (induced). β-Galactosidase activities are expressed in Miller units (32) and indicate activity at 30 min prior to entry of the culture into stationary phase (T_0).

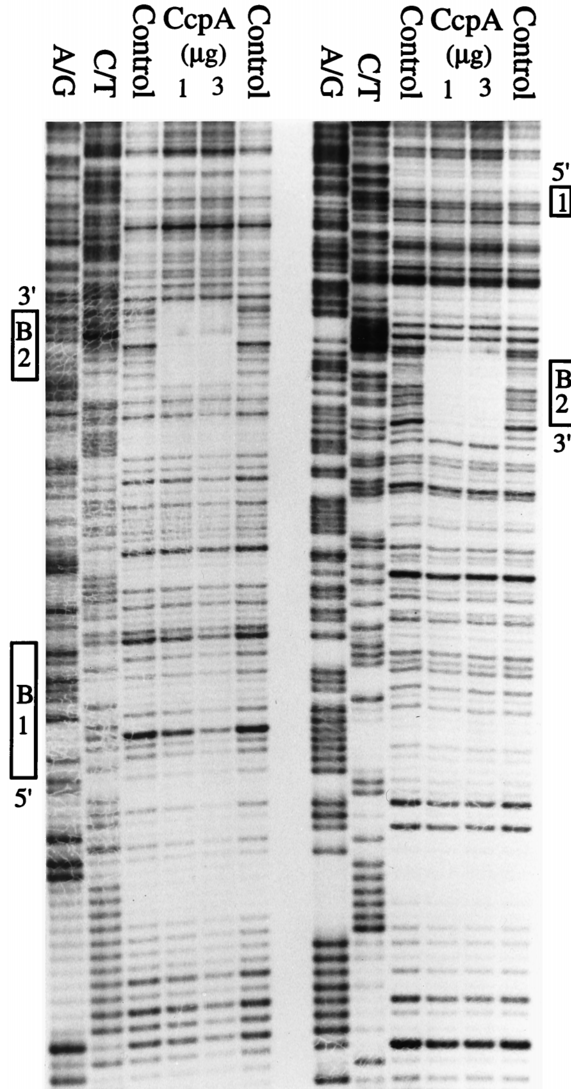


FIG. 5. DNase footprinting of the *ackA* promoter region. End-labeled *ackA* DNA (0.5 nM) was incubated with purified CcpA protein in TGED buffer (10 mM Tris-HCl [pH 7.4], 1 mM dithiothreitol, 50 mM KCl, 5% glycerol, 2 μg of bovine serum albumin) with 2 μg of poly(dI-dC)-poly(dI-dC) as competitor DNA for 15 min at room temperature. DNase I footprinting was carried out as previously described (24), and digested DNA was resolved on a 6% DNA sequencing gel. A/G and C/T are DNA sequencing ladders; the control contained no CcpA protein. Strand polarities and *cre* sites (B1, *cre1*; B2, *cre2*) are labeled.

diate between the standard position of class I activators (−61.5 or farther upstream, in 10-bp increments) and class II activators (−41.5); the best-studied example is the *E. coli* CAP (CRP) protein (9, 25). The *E. coli* Cra protein, a CcpA homolog, also acts as both a repressor and an activator of target genes (40, 46); its binding site for activation of the *ppsA* gene is centered at −45.5 (37). A variety of molecular mechanisms for transcriptional activation have been reported (18, 19). The most common mechanisms involve interactions with the C-terminal domain of the α subunit (α-CTD) of RNA polymerase (RNAP), for class I activators, and interactions with the σ subunit, for class II activators, or both. The only transcriptional

TABLE 4. Expression of *ackA-lacZ* fusion in *ptsH1*/*crh* mutant strains

Strain	β-Galactosidase activity[a]		Ratio
	Uninduced	Induced	
Wild type	92	330	3.6
ptsH1	120	360	3.0
crh::*aphA3*	53	250	4.7
ptsH1/*crh*::*aphA3*	38	40	1.1

[a] Cells were grown in TSS medium containing 1% Casamino Acids (uninduced) or 1% Casamino Acids and 1% glucose (induced). β-Galactosidase activities are expressed in Miller units (32) and indicate activity at 30 min prior to entry of the culture into stationary phase (T_0).

activator of this type characterized in detail in *B. subtilis* is the phage ϕ29 p4 protein, which binds at −82 and interacts with α-CTD (30, 31).

Transcriptional activation of *ackA* was lost with a 5-bp insertion between *cre2* and the promoter and was reestablished with a 10-bp insertion, demonstrating a position effect suggestive of face-of-the-helix dependence. A similar effect has been demonstrated for class I activators in *E. coli* where the position of the binding site relative to the transcription start site can vary as long as the sites for activator and RNAP binding remain on the same face of the DNA helix (7, 49). CcpA could activate transcription via a direct interaction with RNAP or could be required for binding or correct positioning of a second factor which, in turn, interacts with RNAP. Alternatively, the insertion of 5 bp could disrupt interactions between RNAP and sequences upstream of *cre2* or factors bound to these sequences. The similarity of the phenotype of the *ptsH1*/*crh* mutant to that of the CcpA mutant suggests that these proteins play a role in the control of CcpA activity, as has been proposed for genes repressed by CcpA. The reduced levels of β-galactosidase activity observed with the 5-bp insertion compared to that of the wild-type fusion expressed in a CcpA (or *ptsH1*/*crh*) mutant, or the G8T2 fusion expressed in the wild type versus a CcpA mutant strain, suggest that elements in addition to CcpA and HPr/Crh are involved. Transcriptional activation dependent on multiple proteins has been reported for a number of systems, including the *E. coli ansB*, *malK*, and *nir* promoters, and can employ a variety of mechanisms (19, 44, 47, 53). It will be of great interest to characterize the role of the region upstream of *cre2* and the molecular mechanism of transcriptional activation.

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