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Cyclic AMP Receptor Protein-Dependent Activation of the *Escherichia coli acs*P2 Promoter by a Synergistic Class III Mechanism

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The cyclic AMP receptor protein (CRP) activates transcription of the *Escherichia coli acs* gene, which encodes an acetate-scavenging enzyme required for fitness during periods of carbon starvation. Two promoters direct transcription of *acs*, the distal *acs*P1 and the proximal *acs*P2. In this study, we demonstrated that *acs*P2 can function as the major promoter and showed by in vitro studies that CRP facilitates transcription by "focusing" RNA polymerase to *acs*P2. We proposed that CRP activates transcription from *acs*P2 by a synergistic class III mechanism. Consistent with this proposal, we showed that CRP binds two sites, CRP I and CRP II. Induction of *acs* expression absolutely required CRP I, while optimal expression required both CRP I and CRP II. The locations of these DNA sites for CRP (centered at positions −69.5 and −122.5, respectively) suggest that CRP interacts with RNA polymerase through class I interactions. In support of this hypothesis, we demonstrated that *acs* transcription requires the surfaces of CRP and the C-terminal domain of the α subunit of RNA polymerase holoenzyme (α-CTD), which is known to participate in class I interactions: activating region 1 of CRP and the 287, 265, and 261 determinants of the α-CTD. Other surface-exposed residues in the α-CTD contributed to *acs* transcription, suggesting that the α-CTD may interact with at least one protein other than CRP.

The *Escherichia coli* cyclic AMP (cAMP) receptor protein (CRP; also known as the catabolite activator protein) activates transcription from more than 100 promoters. When bound to its allosteric effector, cAMP, the CRP homodimer binds specific DNA sites near target promoters, enhancing the binding of RNA polymerase holoenzyme (RNAP) and facilitating the initiation of transcription. Simple CRP activation operates through two related mechanisms, designated class I and class II. Both mechanisms depend upon specific interactions between CRP and RNAP. At class I promoters, a CRP dimer binds to DNA at a site centered near position −61.5, −71.5, −82.5, or −92.5. CRP bound at any of these positions uses a defined surface (activating region 1 [AR1]) in the downstream subunit of CRP to contact a specific surface determinant, 287, of the C-terminal domain of the α subunit of RNA polymerase (α-CTD). Two additional α-CTD determinants contribute to class I activation: the 261 determinant, proposed to interact with the σ subunit of RNAP, and the 265 determinant, known to interact with DNA, especially A+T-rich sequences, most notably the UP element.

Class I interactions appear to recruit RNAP to promoter DNA by increasing the binding constant for closed-complex formation. At class II promoters, a CRP dimer binds to DNA at a site centered near position −41.5. When bound at this position, CRP uses AR1 of the upstream subunit of CRP to contact the 287 determinant of the α-CTD and a second surface (AR2) to contact the 162 to 165 determinant of the N-terminal domain of α. The α-CTD 265 determinant also contributes by interacting with DNA. Class II interactions appear to increase closed-complex formation and the rate of isomerization to the open complex. A variation of CRP-dependent activation, designated class III, utilizes two or more CRP dimers or a combination of CRP and other activators to achieve maximal transcription activation (reviewed in references 5 and 10).

Acetyl-coenzyme A synthetase (Acs; acetate:coenzyme A ligase [AMP forming]; EC 6.2.1.1) irreversibly catalyzes the conversion of acetate to acetyl-coenzyme A through an enzyme-bound acetyladenylate intermediate (2). This activity permits cells to use acetate, a two-carbon compound, as a source of both energy (via the tricarboxylic acid cycle) and biosynthetic subunits (via the glyoxylate shunt) (3). *Escherichia coli* cells require this high-affinity acetate-scavenging enzyme (3, 15) to compete successfully during periods of carbon starvation (A. J. Wolfe, unpublished data). Thus, the induction of Acs represents a survival response for cells entering a nutrient-poor environment.

Previously, we reported that cells control Acs activity primarily at the level of transcription initiation, inducing transcription during exponential phase, achieving maximal transcription during the transition to stationary phase, and partially repressing that transcription following entry into stationary phase. Transcription occurs from two σ^{70}-dependent promoters: the distal promoter *acs*P1 (P1) and the proximal promoter *acs*P2 (P2) (Fig. 1) (4, 13, 14). Transcription depends upon CRP, the anaerobically triggered transcription activator FNR, the glyoxylate shunt repressor IclR and its activator FadR, and several enzymes involved in acetate metabolism. While multiple factors influence transcription, CRP appears to function directly as the critical transcription factor (4, 13).

This study demonstrated that P2 can function as the primary

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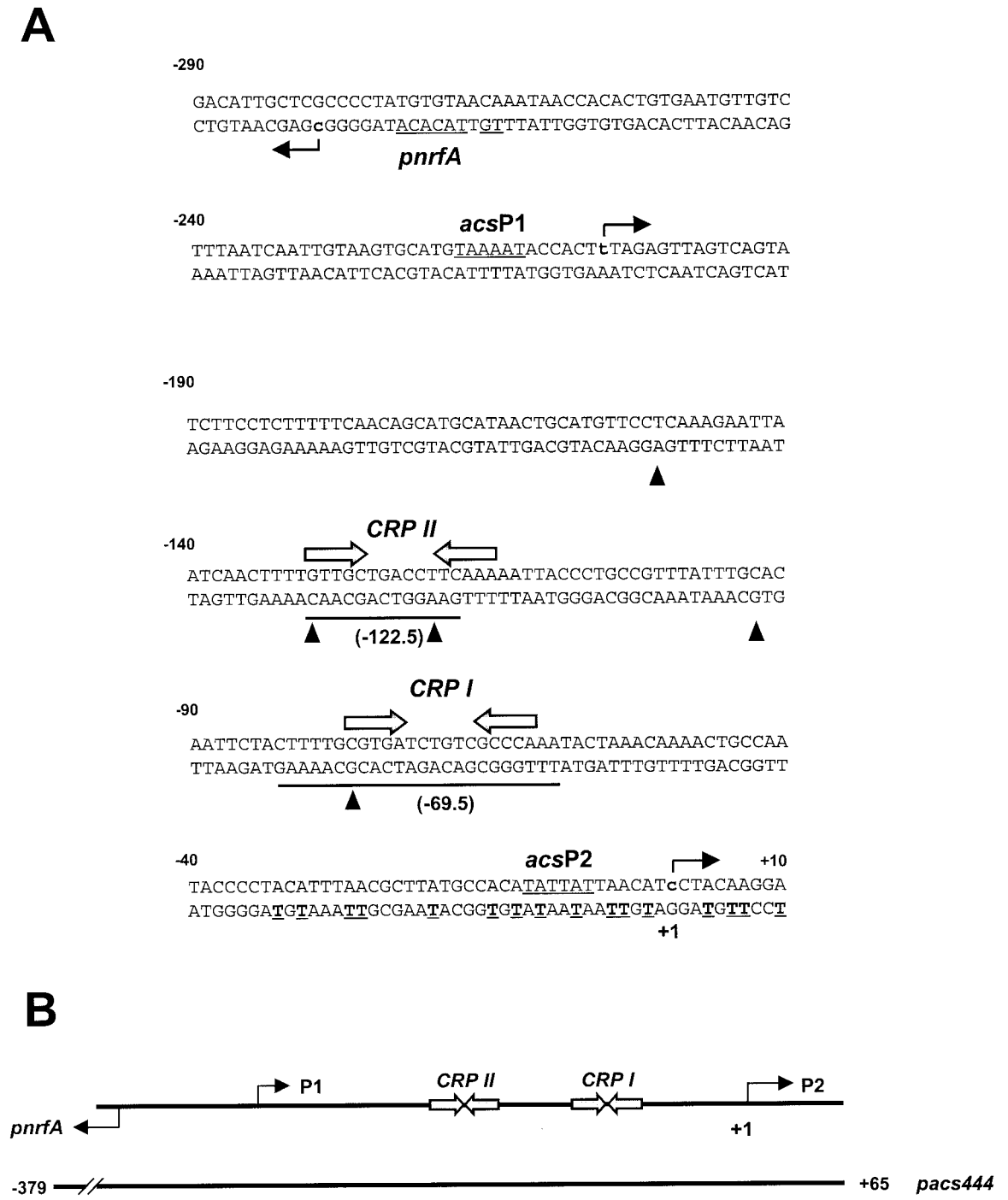

FIG. 1. Organization of the *acs* promoter region. (A) The sequence of the *acs* promoter region from positions −290 to +10 relative to the *acs*P2 transcription start site (+1). The *acs*P1, *acs*P2, and *pnrfA* −10 elements are underlined, and the predicted start points of transcription for *acs*P1, *acs*P2, and *pnrfA* are designated in bold lowercase letters, with the direction of transcription indicated by a horizontal arrow. Inverted arrows specify the location of DNA sites for CRP binding (CRP I and CRP II), while underlining shows the extent of protection afforded by CRP in DNase I footprint experiments. Triangles indicate hypersensitive sites within the relevant strand. The cleavage sites produced by potassium permanganate footprint analysis are in bold and underlined. (B) Schematic of the region, showing the locations of the +1 sites associated with each promoter, each binding site, and the extent of the *pacs444* fragment used for DNase I footprint experiments (Fig. 4).

acs promoter and that its activation in vitro and in vivo requires only CRP, which binds tandem DNA sites (centered at positions −69.5 and −122.5). *acs* activation required the proximal DNA site for the CRP site, involved the distal DNA binding site for CRP, required AR1 of CRP, and involved the 287, 261, and 265 determinants of the α-CTD. On the basis of these observations, we propose that P2 is a synergistic class III promoter that consists of tandem DNA sites for CRP, each lo-

TABLE 1. Strains, promoters, plasmids, and phages used in this study

Strain, promoter, plasmid, or phage	Relevant characteristics	Source or reference
Strains		
AJW678	*thi-1 thr-1*(Am) *leuB6 metF159*(Am) *rpsL1136* Δ*lacX74*	13
AJW1941	AJW678 λCB12	This study
AJW1942	AJW678 λCB13	This study
AJW1945	AJW678 λCB16	This study
AJW1969	AJW678 λCB20	This study
AJW1970	AJW678 λCB21	This study
AJW2162	AJW678 λCB21a	This study
AJW2163	AJW1941 *crp*::Km	This study
AJW2175	AJW678 λCB36	This study
AJW2181	AJW1969 *crp*::Km	This study
AJW2182	AJW2175 *crp*::Km	This study
CB369	MG1655 *crp*::Km	C. Bausch
DH5α	*lacZ*Δ*M15 recA*	Bethesda Research Laboratories
P90C	*ara* Δ(*pro-lac*) *thi*	22
Promoters		
pacs444	Fragment carrying *acs* sequences from −379 to +65	This study
Plasmids		
pRS415	*bla lacZ*$^+$ (transcriptional fusion vector)	22
pCB33	pRS415 *pacs444*	This study
pCB34	pCB33 derivative A214G/A215G	This study
pCB35	pCB33 derivative C16G/T10C	This study
pCB37	pCB33 derivative C16G/A11C	This study
pCB45	pCB33 derivative T76A	This study
pCB46	pCB33 derivative G77C	This study
pCB47	pCB33 derivative G76C	This study
pCB50	pCB33 derivative +A	This study
pDCRP	pBR322 derivative carrying *crp* gene	24
pDCRP/H159L	pDCRP derivative carrying *crpH159L* allele	24
pDCRP/K101E	pDCRP derivative carrying *crpK101E* allele	24
pDCRP/H159L, K52N	pDCRP derivative carrying *crpH159L,K52N* allele	24
pDU9	pDCRP derivative with *crp* deleted	24
pREII (and derivatives)	Plasmid carrying *rpoA* gene (and alanine substitution derivatives)	8
pGEM t	pUC19-derived TA cloning vector	Promega
pCB26 (and derivatives)	pGEM-t carrying −379 *acs* through the end of the *acs* open reading frame; template for all site-directed mutants	This study
pSR	pBR322 derivative containing transcription terminator	12
pSR*acs444*	pSR *pacs444*	This study
Phages		
λCB12	pCB33-based phage	This study
λCB13	λCB12 derivative A214G/A215G	This study
λCB14	λCB12 derivative C16G/T10C	This study
λCB15	λCB12 derivative T76A	This study
λCB16	λCB12 derivative C16G/A11C	This study
λCB20	λCB12 derivative G77C	This study
λCB21	λCB12 derivative G76C	This study
λCB21a	λCB12 derivative +A	This study
λCB28	λCB12 derivative T31C	This study
λCB36	λCB12 derivative G126C	This study

cated at a class I position. To our knowledge, this represents the first report of a native promoter with this particular architecture (23). Intriguingly, several α-CTD residues not within the 287, 265, and 261 determinants also affected *acs* transcription, suggesting that the α-CTD may interact with at least one factor other than CRP.

MATERIALS AND METHODS

Chemicals and biological reagents. Chemical reagents were purchased from Fisher Scientific (Pittsburgh, Pa.) and Sigma Chemical Company (St. Louis, Mo.). β-Galactosidase assay reagents were purchased from Pierce Biochemicals (Rockford, Ill.). Restriction endonucleases and modifying enzymes were purchased from Promega Corp. (Madison, Wis.), New England Biolabs (Beverly, Mass.), or Gibco-BRL (Gaithersburg, Md.). Purified *Escherichia coli* RNAP was purchased from Epicentre (Madison, Wis.). Primers were purchased from Integrated DNA Technologies (Ames, Iowa). Medium components were made by Difco.

Bacterial strains, plasmids, and bacteriophage. All strains used in this study were derivatives of *E. coli* K-12 and are listed in Table 1.

The plasmids and bacteriophage used in this study are also listed in Table 1. For cloning and transformation, plasmids were prepared with the Wizard miniprep kit (Promega), following the protocol provided. Large-scale DNA preparations for electrophoretic mobility shift assays and footprint analyses were purified over cesium chloride gradients, as described before (18). All restriction digestions and ligations were performed according to standard methods (18).

To construct the β-galactosidase fusion plasmids pCB33 and its mutant derivatives, 444-bp fragments were PCR amplified from pCB26 and its mutant derivatives with primers 2927 and 2926. These PCR products were subcloned into pGEM-T and verified by sequencing. Promoter fragments were excised from the resultant plasmids with *Eco*RI and *Bam*HI restriction enzymes and subcloned into pRS415. To construct strains that carry single-copy *acs*::*lacZ* fusions, β-ga-

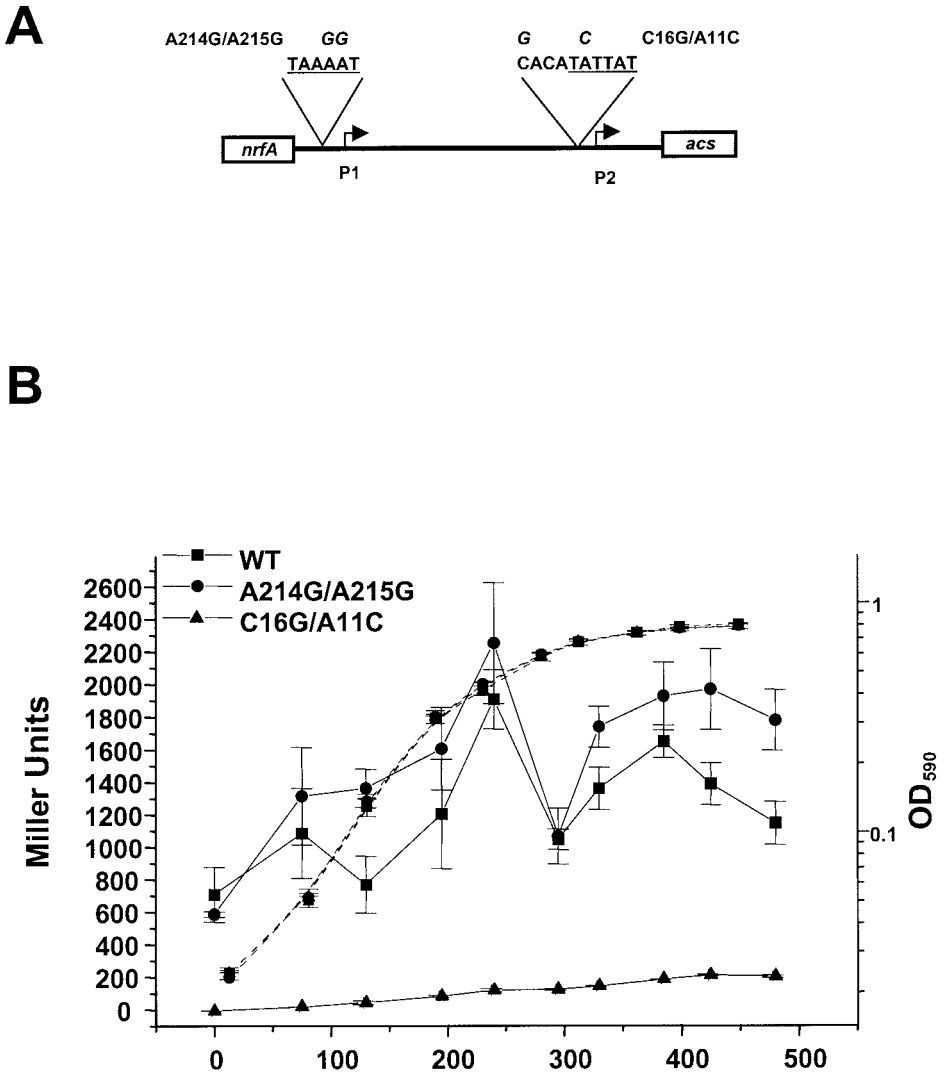

FIG. 2. Evidence that P2 is the major *acs* promoter. (A) Schematic of the *acs* promoter region, showing the mutant alleles. The sequences of the P1 and P2 −10 elements are underlined. The italic letters above the sequences indicate site-directed mutations. (B) Optical density at 590 nm (OD_{590}, dashed lines) and β-galactosidase activity (solid lines) of *acs*::*lacZ* transcriptional fusions, either wild type (WT) or mutant for P1 (A214G/A215G) or P2 (C16G/A11C). Wild-type strain AJW678 was lysogenized with a hybrid λ that carried either the wild-type *acs*::*lacZ* fusion (λCB12) or one of its mutant derivatives (λCB13 or λCB16). The resultant lysogens were grown in TB, samples were harvested during a growth curve, the β-galactosidase activity was determined, and the activity was expressed as a Miller units. Each value represents the mean ± standard error of the mean (SEM) of at least three independent measurements.

lactosidase fusion plasmids were converted to hybrid λ phage, as described (22). pSR derivatives were generated by PCR amplification from pCB26 with 2926 and 2927*Hin*dIII (AAGCTTCTTGTCATAGGGGCTTC). This PCR product was digested with *EcoR1* and *Hin*dIII and subcloned into pSR. The Δ*crp*::Km allele was transferred from strain CB369 by generalized transduction with P1kc (21).

PCRs. All PCRs were performed in a 50-μl reaction volume. The reactions contained 1× PCR buffer (Promega), 3 mm $MgCl_2$, 20 nM each primer, 0.2 mM deoxynucleoside triphosphate mix, and 1 U of *Taq* polymerase. Reactions were subjected to one cycle of 95°C for 5 min and then 30 cycles 95°C for denaturing, 68°C for annealing, and 72°C for extension. Site-directed mutations were generated either by megaprimer PCR or the Gene Editor site-directed mutagenesis kit (Promega), following the protocol provided. Megaprimers were produced by standard PCRs with the 5′ primer 2926 (GGATCCGTTGGGTCTGCGATG TTG) and a 3′ mutagenic primer (either ttgcgtCatctgtcgccca, ttgcgAgatctgtcgcca, ttgcCtgatctgtcgcca, or ttgcgtgatctAgtcgccca). Megaprimers were gel purified and quantified by spectrophotometry. A second PCR was carried out with the megaprimer and the 3′ primer 2927 (GAATTCCTTGTCATAGGGGCTTC). The resultant PCR products were subcloned into pGEM-t, and the successful incorporation of mutations was verified by sequencing.

Medium and growth conditions. For strain constructions, cells were grown in Luria broth (LB; 1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, and 0.5% [wt/vol] sodium chloride). For β-galactosidase assays, cells were grown at 37°C in tryptone broth (TB; 1% [wt/vol] tryptone and 0.5% [wt/vol] sodium chloride). The medium was supplemented with antibiotics or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), as needed.

Promoter activity assays. β-Galactosidase activity was determined quantitatively with the Y-PER β-galactosidase assay kit (Pierce Biochemical). The values expressed are the mean and standard error of the mean of three independent measurements. Each experiment was repeated two to five times.

Protein purification. CRP was purified as described previously (13).

DNase I and potassium permanganate footprint experiments. All footprint experiments were performed on α-^{32}P-end-labeled *Aat*II-*Hin*dIII fragments containing the *acs-nrfA* intergenic region, as described previously (19). For DNase I footprinting experiments, each reaction (20 μl) contained a final concentration

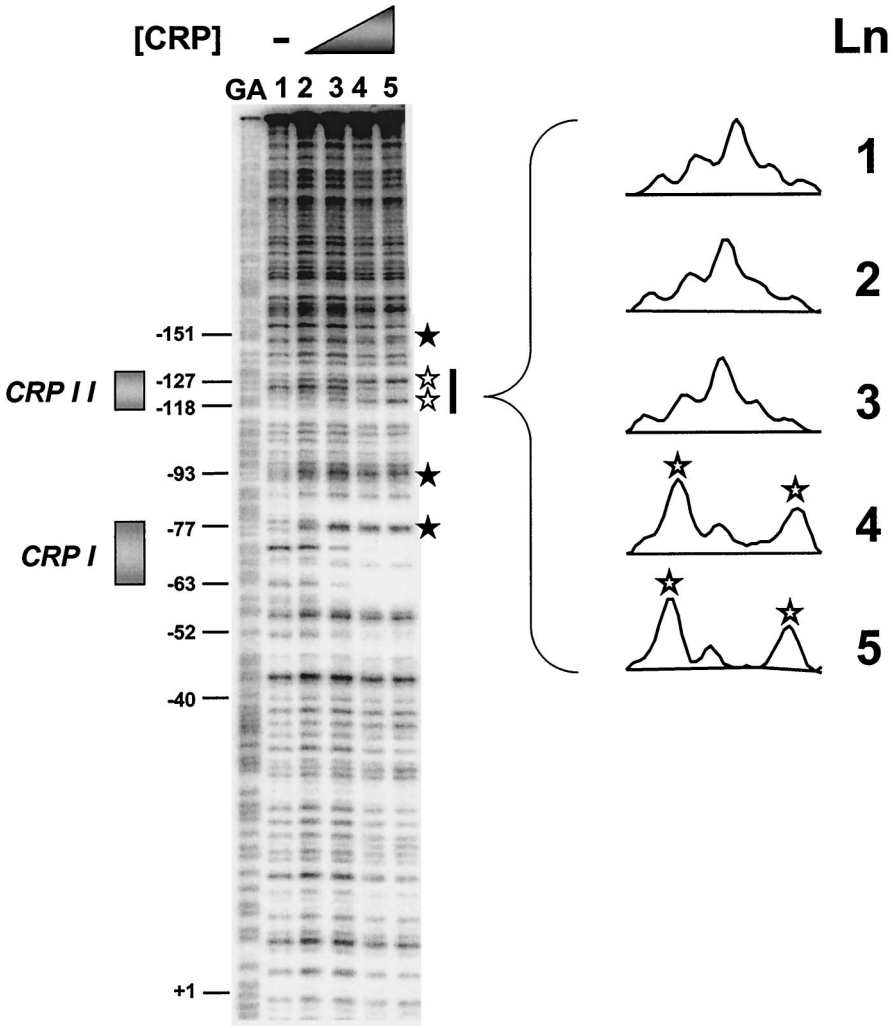

FIG. 3. CRP binds specifically to two sites within the *acs* regulatory region. (A) End-labeled *pacs444 Aat*II-*Hin*dIII fragment (carrying *acs* promoter sequences from positions −379 to +65) was incubated with increasing concentrations of CRP protein and subjected to DNase I footprint analysis. The concentration of CRP in each reaction was as follows: lane 1, no protein; lane 2, 30 nM; lane 3, 125 nM; lane 4, 250 nM; lane 5, 500 nM. The gel was calibrated with Maxam-Gilbert G+A sequencing reactions of the labeled fragment (GA). The locations of CRP I and CRP II are shown by boxes, and hypersensitive sites are indicated by stars. The region encompassing the CRP II signature is designated by the thick bar to the right of the figure. (B) A graphic representation of the CRP II region as quantified with ImageQuant (version 5.2) software (Molecular Dynamics). The hypersensitive sites are indicated by stars.

of 4 nM template DNA. The buffer composition was 20 mM HEPES (pH 8.0), 5 mM $MgCl_2$, 50 mM potassium glutamate, 1 mM dithiothreitol, 500 μg of bovine serum albumin per ml, and 25 μg of herring sperm DNA per ml. When CRP was present in the reactions, 0.2 mM cAMP also was included. For potassium permanganate footprint experiments, herring sperm DNA was omitted and *E. coli* RNA polymerase (Epicentre Technologies) was used at a final concentration of 50 nM. All samples were analyzed by denaturing gel electrophoresis. Gels were calibrated with Maxam-Gilbert G+A sequencing reactions of the labeled fragment and quantified with a Typhoon 8600 variable-mode imager and ImageQuant software (Molecular Dynamics).

In vitro transcription assays. In vitro transcription reactions (20 μl) were performed in a buffer containing 100 mM KCl, 40 mM Tris-acetate (pH 7.9), 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg of bovine serum albumin per ml, 200 μM ATP, 200 μM CTP, 200 μM GTP, 10 μM UTP, and 2 μCi of [α-^{32}P]UTP (Perkin Elmer). When CRP was present in the reactions, 0.2 mM cAMP was also included. CsCl-ethidium bromide gradient-purified supercoiled template DNA (pSR*acs444*) was added at a final concentration of 10 nM. Reactions were initiated by the addition of RNA polymerase (Epicentre Technologies) to a final concentration 50 nM and incubated at 30°C for 10 min. Samples were analyzed by denaturing gel electrophoresis and quantified with a Typhoon 8600 variable-mode imager and ImageQuant software (Molecular Dynamics).

RESULTS

P2 can function as the major *acs* promoter. On the basis of reverse transcription-PCR (14), in vivo reporter assays (5), in vitro transcription, and potassium permanganate footprint analyses (data not shown), we previously proposed the existence of two *acs* promoters, the distal P1 and a proximal P2. To determine the relative contribution of P1 and P2 to *acs* transcription, we generated point mutations in the predicted −10 element of each promoter. For P1, we generated A214G/A215G; for P2, we generated C16G/A11C (Fig. 2A) and C16G/T10C (data not shown). We incorporated each mutant allele into an *acs*::*lacZ* transcriptional fusion (λCB12, Table 1)

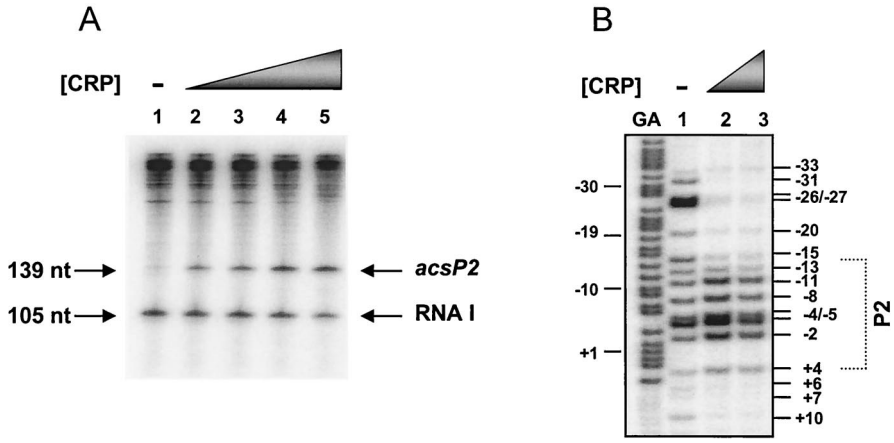

FIG. 4. CRP activates transcription in vitro by focusing RNAP to P2. (A) CRP is required for transcription of P2 in vitro. In vitro multiround transcription assays of the P2 promoter carried by plasmid pSR*acs444*. Reactions contained purified RNA polymerase and increasing concentrations of purified CRP. The concentration of CRP was as follows: lane 1, no protein; lane 2, 25 nM; lane 3, 50 nM; lane 4, 100 nM; lane 5, 300 nM. Transcription initiates from P2 and terminates at a strong transcriptional terminator within the vector backbone (pSR), producing a single 139-nucleotide (nt) transcript. The 105-nucleotide RNA I transcript, encoded by pSR, served as an internal transcriptional control. Arrows indicate the P2 and RNA I transcripts. (B) Potassium permanganate footprint analysis of complexes formed at the *acs* promoter region. The figure shows the cleavage products produced when the end-labeled *pacs444 Aat*II-*Hin*dIII fragment was incubated with purified RNA polymerase and purified CRP and then subjected to potassium permanganate footprint analysis. All reactions contained 50 nM RNAP polymerase and the following CRP concentration: lane 2, no protein; lane 3, 25 nM; lane 4, 100 nM. The gel was calibrated with Maxam-Gilbert G+A sequencing reactions of the labeled fragment (GA). The location of the P2 promoter is shown.

that encompasses sequences from position −379 to position +65 of *acs* (Fig. 1B). We then constructed a set of lysogens in a wild-type strain (AJW678, Table 1), with each lysogen carrying a single copy of either λCB12 (AJW1941) or one of the mutant derivatives (Table 1). We grew the resultant lysogens in tryptone broth (TB), harvested cells at several intervals throughout growth, and determined the β-galactosidase activity.

The C16G/A11C allele resulted in a complete loss of activity, while the A214G/A215G allele exhibited no loss of activity (Fig. 2B). To generate a restriction site, the C16G/A11C allele combines a mutation (A11C) at a highly conserved position within the −10 element with a mutation (C16G) outside the −10 element. To determine whether the C16G mutation exerts an effect on transcription, we constructed a second mutant allele (C16G/T10C) that included a mutation (T10C) at a less conserved position within the −10 element and was thus predicted to cause less severe consequences to promoter activity. The C16G/T10C allele behaved like its wild-type parent (data not shown), supporting the conclusion that the T11C mutation was responsible for the decrease in transcription. A 3′ deletion from the middle of the −10 element resulted in activity similar to that exhibited by the C16G/A11C allele, whereas a 5′ deletion that removed the P1 −10 region exhibited transcription comparable to that of the wild type (data not shown). Taken together, these results support the hypothesis that P2 functions as the major *acs* promoter, at least under the conditions tested.

CRP activates P2 in vitro. Previously, we used electrophoretic mobility shift analysis to demonstrate the existence of one CRP binding site in the *acs* regulatory region (13). To pinpoint this binding site, we performed DNase I footprint analysis. We incubated the *acs* promoter fragment *pacs444*, an *Aat*II-*Hin*dIII fragment that carries *acs* sequences from position −379 to position +65 (Table 1), with increasing concentrations of purified CRP in the presence of cAMP (Fig. 3). Surprisingly, we observed two regions of protection, each with associated hypersensitive sites. At the lowest CRP concentration (Fig. 3, compare lane 1 with lane 2), two hypersensitive sites appeared (−77 and −93). With increasing CRP concentration (Fig. 3, lanes 3 and 4), protection appeared between positions −60 and −83. These results support the existence of a site (CRP I) centered at −69.5, as predicted by sequence analysis (Fig. 1). At higher CRP concentrations (Fig. 3, lanes 3 to 5), hypersensitive sites appeared at positions −120, −130, and −151, followed by a second region of protection in the region between −118 and −127 (inset). These results suggest the existence of a second, lower-affinity upstream DNA site for CRP (CRP II). Sequence inspection revealed a site centered at position −122.5 that resembled the consensus CRP sequence (Fig. 1).

Using an in vitro transcription assay, we determined that CRP activates transcription of *acs*. Alone, RNAP did not transcribe *acs* efficiently (Fig. 4A, lane 1), while producing a robust 105-nucleotide band that corresponds to the RNA 1 control. In the presence of CRP, however, RNAP transcribed from P2 quite efficiently (Fig. 4A, lanes 2 to 5), producing a 139-nucleotide band with no change in the intensity of the RNA 1 control.

Although RNAP alone cannot transcribe from P2, it can bind and melt DNA in the region that includes P2. Note that permanganate footprint analysis revealed a long stretch of modified and cleaved residues from −33 to +10 (Fig. 4B, lane 1). In contrast, we observed no cleaved residues in the absence of RNAP (data not shown). Permanganate footprint analysis modifies and cleaves T's within single-stranded DNA, such as those present in a transcription open complex. Open complexes tend to be 10 to 14 bp in length; thus, these results suggest the potential for multiple open complexes. Permanga-

nate footprint analysis also showed that CRP favored open-complex formation between nucleotides +4 and −15 (Fig. 4B, lanes 2 and 3), a region that includes the P2 −10 element (TATTAT, Fig. 1). Taken together, these results suggest that CRP facilitates transcription by "focusing" RNAP to P2.

Both CRP I and CRP II contribute to *acs* transcription in vivo. To assess the regulatory role of each DNA site for CRP (CRP I and CRP II), we generated mutations predicted, on the basis of analogous mutations in the site for CRP within the *lac* promoter that reduce affinity to as much as 1/70th that of the wild-type site (6), to reduce the ability of CRP to bind. We incorporated each mutation into an *acs*::*lacZ* fusion (λCB12, Table 1) that encompasses the entire *acs* regulatory region, as described above (Fig. 1B). We then constructed a set of lysogens in a strain wild type for *crp* (AJW678, Table 1), with each lysogen carrying a single copy of either λCB12 (AJW1941) or one of the mutant derivatives (Table 1).

For the proximal, higher-affinity site CRP I, we generated substitutions in the upstream half-site (G75C, T76A, and G77C) or inserted 1 bp into the spacer (+1A) (Fig. 5A). Electrophoretic mobility shift assays showed that each mutation significantly diminished the ability of CRP to bind (data not shown). Compared to the wild-type fusion (λCB12), all four mutant fusions yielded significantly lower activity (Fig. 5B). Substitution at the critical G75 position or insertion into the spacer yielded ≈6-fold-lower activity, only slightly higher than the activity exhibited by a λCB12 lysogen of a strain deleted for *crp* (13). This result suggests the existence of low-level basal CRP-independent activity and clearly supports the hypothesis that optimal CRP-dependent activation requires CRP I.

For the upstream, lower-affinity site CRP II, we generated the substitution G126C in the upstream half-site (Fig. 5A). Electrophoretic mobility shift assays did not detect CRP binding to CRP II (data not shown). However, this substitution is analogous to G75C, the most severe mutation generated in CRP I. Therefore, we predict that it severely impacted the ability of CRP to bind. Compared to the wild-type fusion (λCB12), this mutant fusion yielded 1.7-fold lower activity (Fig. 5B). This moderate decrease in transcription, together with the fact that −10 element and CRP I substitutions resulted in significant decreases in transcription, suggests that CRP bound to CRP II functions as a coactivator of the CRP bound at CRP I.

To confirm that the G126C mutation in CRP II does not exert a CRP-independent effect upon transcription, we generated Δ*crp* cells carrying fusions to the wild-type, G75C mutant, and G126C mutant promoters. In the absence of CRP, the G126C mutant promoter yielded activity almost identical to that of its wild-type parent and the G75C mutant (Fig. 5C). This lack of a difference in promoter activities supports the notion that the loss of activity due to G126C is CRP dependent. Complementation with wild-type CRP yielded activities similar to that observed in the wild-type background. Strains carrying the wild-type fusion regained wild-type levels of transcription, those carrying the G75C mutant fusion gained no additional activity, and the G126C mutant fusion regained 50 to 60% of wild-type activity. These results further support the importance of CRP binding to CRP I and the contribution of CRP binding to CRP II.

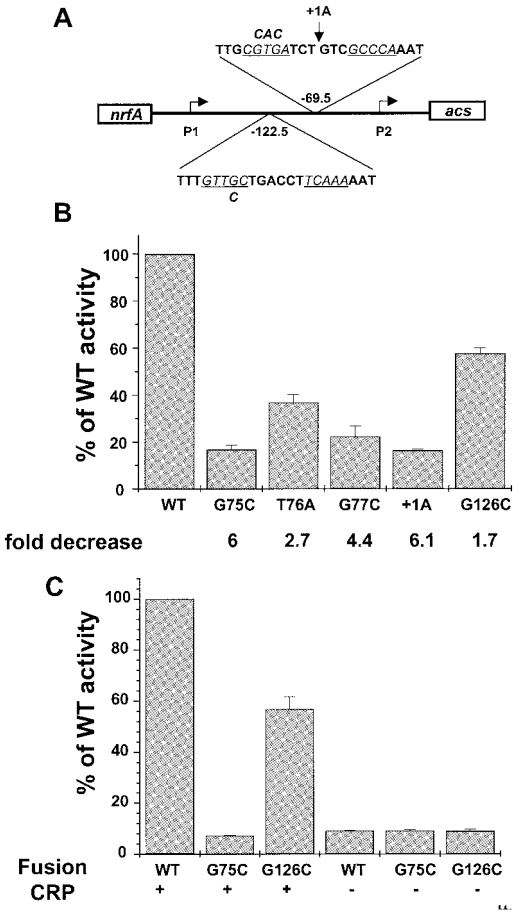

FIG. 5. *acs* transcription requires CRP I, while optimal transcription also involves CRP II. (A) Schematic of the *acs* promoter region showing the sequences of CRP I and CRP II. The italic letters and numbers above the sequences indicate site-directed mutations. (B) β-Galactosidase activity of *acs*::*lacZ* transcriptional fusions, either wild type or mutant for CRP I (G75C, T76A, G77C, +1A) or CRP II (G126C). Wild-type strain AJW678 was lysogenized with a hybrid λ that carried either the wild-type *acs*::*lacZ* fusion (λCB12) or one of its mutant derivatives (λCB15, λCB20, λCB21, λCB21a, or λCB36). The resultant strains were grown in TB, samples were harvested during the transition from exponential growth to stationary phase, the β-galactosidase activity was determined, and the activity was expressed as a percentage of the wild-type value. Each value represents the mean ± SEM of at least three independent measurements. Fold changes in relation to the wild type are noted below the histogram. (C) β-Galactosidase activity of *acs*::*lacZ* transcriptional fusions, either wild type (λCB12), mutant (G75C) for CRP I (λCB20), or mutant (G126C) for CRP II (λCB26) carried by cells with *crp* deleted (strains AJU2163, AJW2181, or AJW2182, respectively) and transformed. The resultant transformants were grown in TB, samples were harvested during the transition from exponential growth to stationary phase, the β-galactosidase activity was determined, and the activity was expressed as a percentage of the wild-type value. Each value represents the mean ± SEM of at least three independent measurements.

CRP-dependent activation requires activating region I. Given the location of CRP I (−69.5) and CRP II (−122.5), we predicted that CRP activates *acs* transcription by class I interactions. To test this prediction, we constructed a strain deleted for *crp* and lysogenized with λCB12 (AJW2163). We transformed this strain with plasmids that express wild-type CRP or

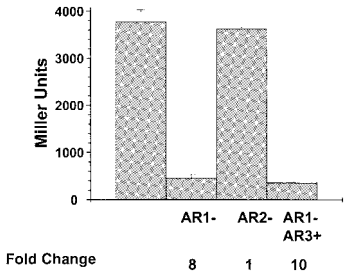

FIG. 6. CRP-dependent activation requires activating region I. β-Galactosidase activity of a λCB12 lysogen deleted for *crp* (strain AJW2163) and transformed with plasmids expressing either wild-type CRP, CRP H159L, CRP K101E, or CRP H159L/K52N was determined. The resultant transformants were grown in TB, samples were harvested at the transition from exponential to stationary phase, and the β-galactosidase activity was determined and expressed in Miller units. Each value represents the mean ± SEM of at least three independent measurements. Changes in relation to the wild type are noted below the histogram.

its mutant variants. The variant CRP H159L possesses a defective AR1 and therefore activates class I and class II promoters poorly. The variant CRP K101E possesses a defective AR2 and thus activates class II promoters poorly. The double mutant CRP H159L/K52N is defective in AR1 and possesses AR3, a nonnative activation region that compensates for the lack of AR1 in class II interactions but not in class I interactions (reviewed in reference 5). Figure 6 shows that the AR1 substitution (regardless of AR3 status) resulted in an 8- to 10-fold reduction in β-galactosidase activity, while the AR2 substitution exerted no significant effect. These results support the hypothesis that CRP activates *acs* transcription by means of class I interactions and not via class II interactions.

Multiple α-CTD residues influence transcription. Class I interactions also require specific, surface-exposed α-CTD determinants (reviewed in reference 5). To identify α-CTD residues involved in *acs* transcription, we used a collection of α-subunit mutants containing single alanine substitutions at each nonalanine position in the α-CTD (residues 255 to 329). With this collection of mutants, we transformed the λCB12 lysogen AJW1941. We expressed the β-galactosidase activity of each transformant as a percentage of wild-type activity and considered those altered by at least 25% significant. By these criteria, we identified 22 residues whose substitution resulted in decreased activity (D259, E261, N268, C269, I275, Y277, I278, D280, L281, Q283, T285, V287, E288, P293, N294, L295, G296, K297, K298, S299, V306, and R317) and five residues

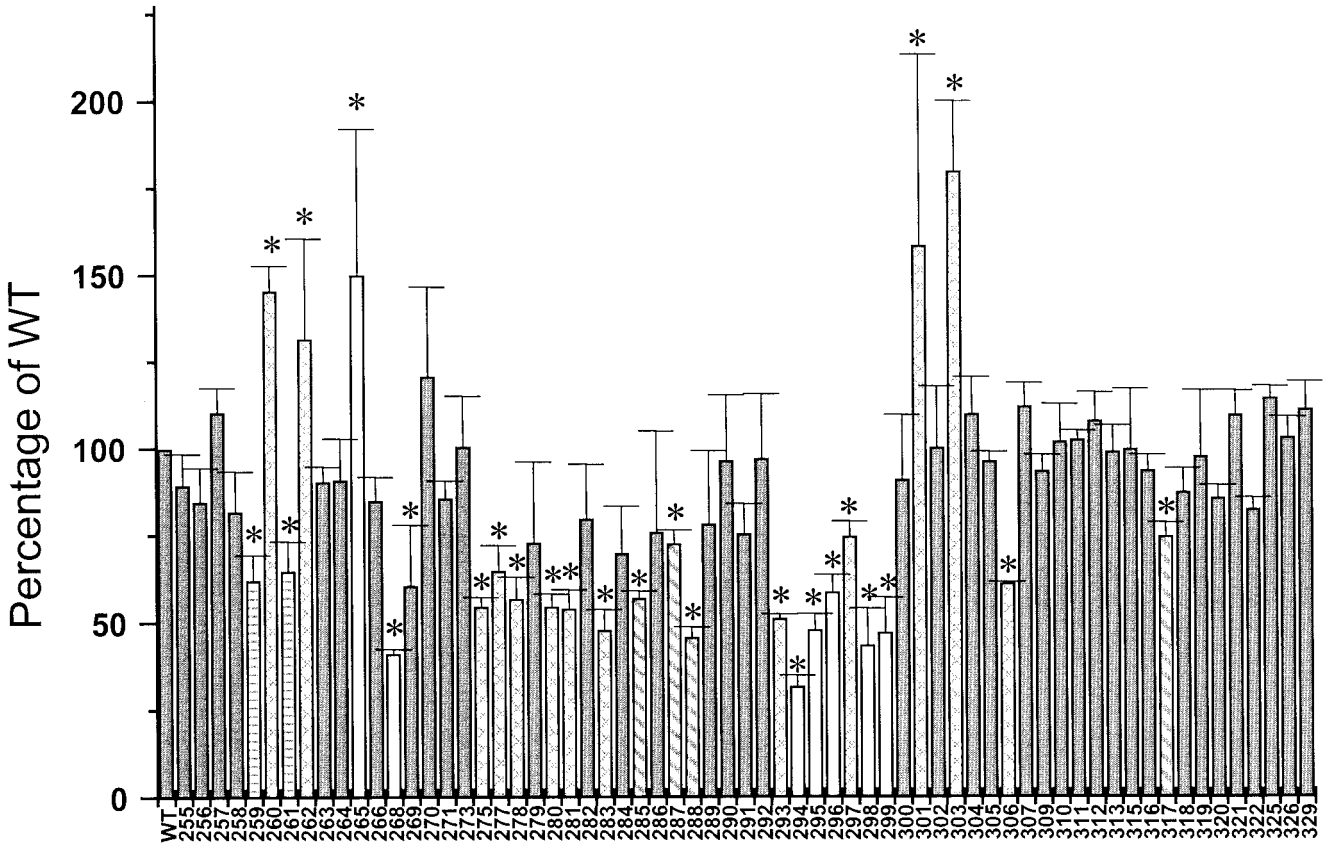

FIG. 7. Effect of single alanine substitutions within α-CTD. The relative β-galactosidase activity of a λCB12 lysogen wild type for *crp* (AJW1941) and transformed with a set of plasmids expressing a library of alanine-substituted variants of the α-CTD of RNAP (residues 255 to 329) was determined. Residues 267, 272, 274, 308, 324, and 327 are naturally alanines. The resultant transformants were grown in TB, samples were harvested during the transition from exponential growth to stationary phase, and the β-galactosidase activity was determined. Activities are expressed as a percentage of that of the transformant containing a plasmid expressing wild-type α. Each value represents the mean ± SEM of at least two independent measurements. All alanine substitutions that significantly altered expression of the *acs*::*lacZ* fusion are marked with an asterisk above the bar. Horizontally striped bars, the 261 determinant; open bars, the 265 determinant; diagonally striped bars, the 287 determinant; cross-hatched bars, residues outside a known determinant.

TABLE 2. α-CTD substitutions that affect *acs* transcription

α-CTD determinant	Substitutions that decreased *acs* transcription	Substitutions that increased *acs* transcription	Substitutions adjacent to known determinants
287	T285A, V287A, E288A		
261	D259A, E261A		L260A, L262A (both increased transcription)
265	N268A, N294A, G296A, K298A, S299A	R265A	P293A, K297A (decreased transcription); T301 (increased transcription)
Other residues	C269A, I275A, Y277A, I278A, D280A, L281A, Q283A, V306A	I303A	

whose substitution resulted in increased activity (L260, L262, R265, T301, and I303) (Fig. 7). These results suggest that multiple, distinct regions of the α-CTD contribute to *acs* transcription (Table 2).

DISCUSSION

Previously, we demonstrated that CRP plays a critical role in the activation of *acs* transcription (13). In this study, we determined that P2 can act as the major *acs* promoter and examined the mechanism by which CRP controls its transcription. In vitro, RNAP alone melted an extensive region in the vicinity of P2 yet failed to transcribe. In contrast, CRP "focused" RNAP to form a single, productive, open complex at P2. In vitro, we defined two DNA sites for CRP, the higher-affinity CRP I site (centered at −69.5) and the lower-affinity CRP II site (centered at −122.5). In vivo, we showed that *acs* activation absolutely requires a functional CRP I, while optimal transcription also requires CRP II. Gaston et al. (9) demonstrated that a DNA site for CRP located at −69.5 resulted in weak activation compared to the preferred −61.5 site. The addition of a UP element, however, renders the −69.5 position optimal for CRP activation (17). P2 lacks a good consensus UP element proximal to the CRP I site (7). CRP bound to the lower-affinity upstream CRP II site, however, may substitute for the UP element to enhance activation of P2 from the suboptimal −69.5 site.

CRP I and CRP II both reside in classic class I positions (5). Therefore, we hypothesized that class III CRP-dependent activation of *acs* might consist of simple class I interactions between RNAP and each of two CRP dimers (11, 16, 23), and the evidence supported this conclusion. In vivo, *acs* transcription required AR1, a surface patch of CRP residues that contacts its α-CTD counterpart, the 287 determinant. Notably, activation did not involve AR2 or AR3, surfaces important for activation of class II promoters (5). While our experiments clearly demonstrated a requirement for AR1, they did not show unequivocally that both dimers require a functional activating region. Using an alanine scanning library of the α-CTD, we showed that *acs* transcription involves residues within the 287 and 265 determinants. Importantly, activation also involved residues within or adjacent to the 261 determinant, a surface patch known to be important for activation of class I but not class II promoters (Table 2) (20).

Four of the alanine substitutions that significantly increased *acs* promoter activity (L260A, L262A, R265A, and T301A) reside in or around previously defined determinants (Table 2). R265, the surface-exposed eponymous residue of the 265 determinant, interacts directly with UP elements (1). T301, a surface-exposed residue, resides immediately adjacent to this determinant. In contrast, residues L260 and L262, located adjacent to the 261 determinant, are generally buried, suggesting that they alter the structure of the α-CTD.

Relative to the extensively characterized CRP-dependent class I promoters *lac*P2 and *CC*(61.5), we observed some differences in the identity and involvement of residues within determinants. For example, the R265A substitution diminishes transcription from *lac*P2 and *CC*(61.5) but enhanced transcription from *acs* (20). This and other differences probably result from architectural differences between promoters. At *acs*, however, they may also reflect the involvement of proteins other than CRP and RNAP.

Intriguingly, several substitutions that either significantly decreased (C269A, I275A, Y277A, I278A, D280A, L281A, Q283A, and V306A) or significantly increased (I303A) *acs* promoter activity did not correspond to α-CTD surfaces known to be involved in class I activation (Table 2). This observation suggests that the α-CTD plays a more complex role at *acs* than it does at simple class I promoters, possibly making additional interactions with proteins other than CRP. Candidate proteins include the nucleoid proteins FIS (factor for inversion stimulation) and IHF (integration host factor), both of which participate directly in the regulation of *acs* transcription (D. F. Browning, C. M. Beatty, S. J. W. Busby, and A. J. Wolfe, unpublished data).

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