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Roles of Catabolite Activator Protein Sites Centered at −81.5 and −41.5 in the Activation of the *Klebsiella aerogenes* Histidine Utilization Operon *hutUH*

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The *Klebsiella aerogenes hutUH* operon is preceded by a promoter region, *hut(P)*, that contains two divergent promoters (*hutUp* and Pc) which overlap and are alternately expressed. In the absence of the catabolite gene activator protein-cyclic AMP (CAP-cAMP) complex, Pc is predominantly expressed while *hutUp* is largely repressed. CAP-cAMP has the dual effect of repressing transcription from Pc while simultaneously activating transcription from *hutUp*. DNA deletion mutations in this region were used to identify DNA sequences required for transcription of these two promoters. We showed that inactivation of Pc by DNA deletion did not result in activation of *hutUp* in vitro or in vivo. In addition, *Escherichia coli* CAP mutants that are known to bind and bend DNA normally but are unable to activate various CAP-dependent promoters were also unable to activate *hutUp* in vivo. These results invalidate an indirect activation model by which CAP-mediated repression of Pc in itself would lead to activation of *hutUp*. Gel retardation assays with various deletion mutations of *hut(P)* and DNase I protection analyses revealed a high-affinity CAP binding site (CAP site 1) centered at −81.5 relative to the *hutUp* start of transcription and a second low-affinity CAP site (CAP site 2) centered at about −41.5. CAP site 1 is essential for activation of *hutUp*. Although CAP site 2 by itself is unable to activate *hutUp* in vivo under catabolite-activating conditions, it appears to be required for maximal transcription activation. Our observation that the double CAP mutant *crp*H159L,K52N, which is known to activate transcription from a site centered at −41.5, does not activate *hutUp* suggests that the role of CAP-cAMP at the weaker CAP site may be different from that of other promoters containing a similarly positioned site. We propose that CAP directly stimulates the activity of RNA polymerase at *hutUp* and that this reaction is completely dependent on a naturally occurring CAP site centered at −81.5 and also involves a second CAP site centered at about −41.5 for maximal activation.

In enteric bacteria, high intracellular levels of adenosine 3′,5′-cyclic monophosphate (cAMP) are part of a complex signal indicating a state of carbon source starvation. The catabolite activator protein (CAP) acts as a sensor of cAMP levels and relays this information to a number of "catabolite-sensitive" genes or operons (54). The CAP-cAMP complex specifically recognizes and binds a DNA sequence resembling the consensus AANTGTGAN$_6$TCACANTT and can thereby activate or repress the transcription of a number of genes or operons (for reviews, see references 6, 8, 18, and 44). Considerable insight into the role of CAP-cAMP in regulating transcription has been gained from studies of promoters such as those in *lac*, *gal*, *mal*, and *ara* (7, 8, 15, 22, 23, 27, 44, 47), from the crystal structure of CAP and its complex with DNA (48, 56), and from mutagenic analysis of the protein (2, 4, 10, 29, 58). The generation of CAP mutants that are able to bind and bend DNA efficiently but are defective in transcription activation function provides evidence for a direct role of CAP-cAMP in activating transcription, which is thought to involve contacts between CAP and RNA polymerase (4, 10, 58). Additional evidence is provided by mutations in the α subunit of RNA polymerase, which prevent CAP-activated transcription in *lac*P1 and *uxuAB* while allowing transcription of other CAP-independent promoters, such as the *lacUV5*, *lac*P2, *trp*, and *rplJ* promoters (16, 17).

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The primary structure of CAP in *Klebsiella aerogenes*, deduced from DNA sequence analysis of the gene (*crp*), was shown to be almost identical to that of *Escherichia coli*, varying in only one amino acid at residue 118 (serine versus alanine, respectively). Both CAP proteins were shown to be completely interchangeable with respect to activation of the *E. coli lacZ* and the *K. aerogenes hutU* promoters (38). The CAP-dependent *hutU* promoter (*hutUp*) drives the expression of the histidine utilization operon *hutUH* in *K. aerogenes* (35). In the absence of CAP-cAMP, RNA polymerase interacts poorly with *hutUp* but readily transcribes a divergent, overlapping promoter (Pc) whose function is unclear in vitro. The CAP-cAMP complex has the dual effect of repressing transcription from Pc and activating *hutUp*. Thus, within this control region [the *hut(P)* region], CAP-cAMP appears to act as both an activator and repressor of transcription.

The observation that a single mutation within *hutUp* which dramatically increased its activity also resulted in the complete repression of Pc led to the conclusion that the two promoters were mutually exclusive (34). A reasonable match to the CAP-binding consensus sequence, extending from −71 to −92 relative to the *hutUp* start of transcription, appeared to reside too far away from *hutUp* to allow direct contacts between CAP bound at this site and RNA polymerase bound at *hutUp*. However, the observation that this CAP-binding consensus overlapped the RNA polymerase binding site at Pc led to the suggestion that the positive activation of *hutUp* by CAP-cAMP might be achieved by an indirect activation mechanism (double-negative control [34, 36]). By this model, RNA polymerase

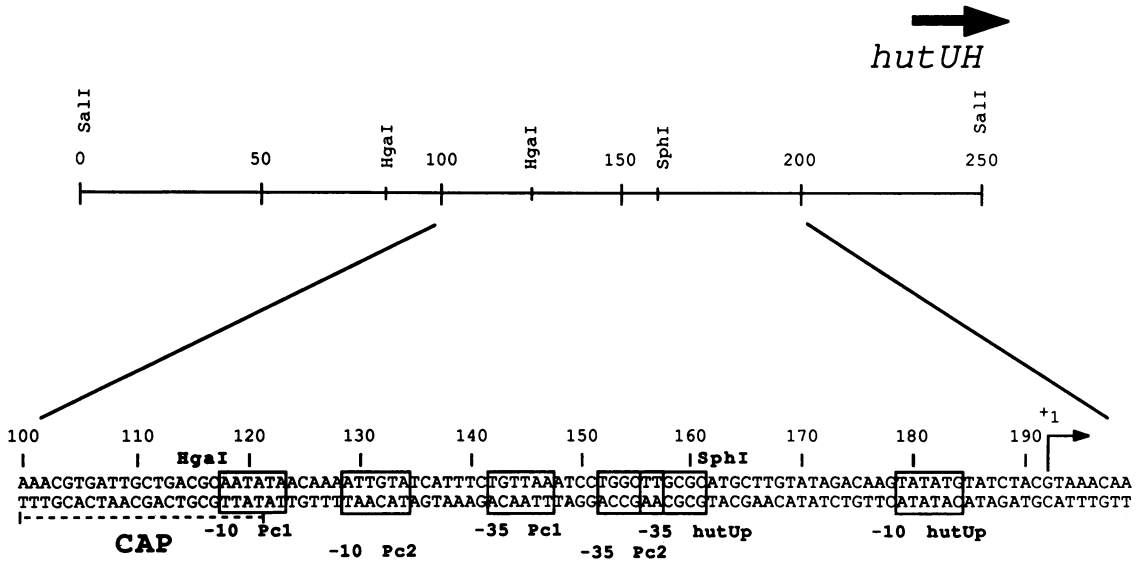

FIG. 1. *hutUH* promoter region [*hut(P)*]. The upper line represents the 250-bp *Sal*I DNA fragment, obtained from pOS2, which contains *hut(P)*. Relative positions of relevant restriction sites are indicated. Numbers above the line denote number of nucleotides from the left *Sal*I end. The lower section shows the *hut(P)* DNA sequence from nucleotides 100 to 199 as reported by Nieuwkoop et al. (33). An arrow indicates the *hutUp* transcriptional start site (+1). The −10 and −35 promoter sequences predicted for *hutUp*, Pc1, and Pc2 are indicated by open boxes. The dotted line highlights a match to the CAP consensus sequence.

bound at Pc would prevent binding of RNA polymerase at *hutUp*. The CAP-cAMP complex bound at the predicted site would prevent binding of RNA polymerase at Pc, allowing free access of RNA polymerase to *hutUp* and resulting in indirect activation of the latter.

The first part of this double-negative control model (that the binding of RNA polymerase to both promoters was mutually exclusive) received support from several lines of evidence showing that any treatment that increased RNA polymerase binding at *hutUp* concomitantly decreased binding at Pc (33, 35, 39). However, we had no direct support for the converse assumption, that RNA polymerase bound at Pc interferes with RNA polymerase binding at *hutUp*. In fact, the slow kinetics of open complex formation at Pc relative to the activated *hutUp* (39) argued that Pc may not be occupied fast enough to enable it to serve as an efficient competitor for RNA polymerase binding. Thus, it was necessary to test the second part of the double-negative control model, that is, that inactivation of Pc results in activation of *hutUp*.

In this report, we present a DNA deletion analysis of regulatory sites within *hut(P)* that are potentially involved in the CAP-cAMP activation of *hutUp*. DNA sequences required for transcription of *hutUp* and Pc and for binding of CAP-cAMP are identified. Our results demonstrate that inactivation of Pc is not sufficient for activation of *hutUp* and suggest a direct role for CAP-cAMP in activating transcription, involving a naturally occurring CAP binding site centered at −81.5 and a weaker CAP binding site centered at about −41.5 relative to *hutUp*.

MATERIALS AND METHODS

Enzymes and chemicals. *K. aerogenes* CAP was prepared as previously described (39). *E. coli* CAP, used in the DNase I footprints, was a kind gift from A. Revzin (Michigan State University). *E. coli* RNA polymerase holoenzyme (EC 2.7.7.11) was purchased from Du Pont NEN Products, Inc. DNase I was from Boehringer Mannheim Biochemicals. All other enzymes were purchased from Boehringer Mannheim Biochemicals or from Bethesda Research Laboratories and were used according to the specifications of the supplier. All other chemicals were purchased as described elsewhere (39).

Strains and plasmids. β-Galactosidase assays of *hutUp-lacZ* operon fusion plasmids were performed in *E. coli* RZ211 [*ara* Δ(*lac-pro*) *recA56 srl str thi*; from R. C. Johnson] or in *K. aerogenes* KC2668 (*hutC* Δ*bla-2*). Histidase assays were performed in *K. aerogenes* KC1669 (*crp-4021*) (38).

Plasmids containing the various right-entering (pOSRΔ) or left-entering (pOSLΔ) deletions within the *hutUH* promoter region [*hut(P)*] were constructed from plasmids pOS1 and pOS2, respectively, and are described in the accompanying paper (41). Plasmids pOS1 and pOS2 consisted of plasmid pUC18 with a 250-bp DNA fragment [containing the *hut(P)* region] inserted within the single *Sal*I restriction site in opposite orientations. The plasmid series pOSRΔ and pOSLΔ are numbered according to the last nucleotide of the *hut(P)* region remaining at the deletion boundary. For example, the deletion that removes the DNA to the right of nucleotide 150 from the *hut(P)* region, shown in Fig. 1, is named pOSRΔ150; likewise, a construct removing the DNA to the left of nucleotide 105 (Fig. 1) is named pOSLΔ105; and so forth. The *Eco*RI and *Hin*dIII sites of the pUC18 polylinker region are retained in each construct.

The *hutUp-lacZ* operon fusion plasmids were constructed by using plasmid pRJ800 (3). pRJ800 is identical to pRZ5202 (32, 45) except that it contains the polylinker sequence from pUC18. In this vector, DNA fragments were cloned upstream of the (*trp-lac*)*W200* fusion. The *Eco*RI-*Hin*dIII fragments of pOS2, pOSLΔ105, pOSLΔ125, and pOSLΔ150 were blunt ended at their *Hin*dIII sites and cloned into the *Eco*RI and *Sma*I restriction sites of pRJ800 to produce the *hutUp-lacZ* operon fusion plasmids pRO80, pRO81, pRO82, and pRO83, respectively. Plasmid pCB540 is essentially the same as pRO80 except that it was constructed by inserting the *Sal*I-*Sal*I frag-

ment from pOS1 into the *Sal*I site of pRJ800 to produce a *hutUp-lacZ* operon fusion.

Plasmids pCB610 and pCB611 both carry an 8-bp DNA sequence with an *Eco*RI restriction site (CGAATTCG) partially replacing a region of CAP site 2 from nucleotides 142 to 149 (underlined nucleotides denote changes from the wild-type sequence). To construct this plasmid, we first cloned the *Hin*dIII-*Eco*RI fragment of pORΔ141 [which contains the *hut(P)* region to the left of nucleotide 142] into the *Sma*I and *Eco*RI sites of pBluescript SK− to make pCB609. The *Eco*RI-*Hin*dIII fragment of pOLΔ150 [which contains the *hut(P)* region to the right of nucleotide 149] was cloned into the *Eco*RI and *Hin*dIII sites of pCB609 to make pCB610. The *Bam*HI-*Hin*dIII fragment of pCB610 was then cloned into the *Bam*HI and *Hin*dIII sites of pRJ800 to construct pCB611. pCB611 carries a *lacZ* operon fusion to the *hut(P)* region mutated at CAP site 2.

The plasmids carrying wild-type (pLG339/CRP) or mutant (pLG339/CRPH159L and pLG339/CRPH159L,K52N) *crp* genes from *E. coli* have been described (4) and were a kind gift from S. Busby (University of Birmingham, Birmingham, United Kingdom). pLG339 is a low-copy-number vector carrying the pSC101 origin of replication and conferring resistance to kanamycin (53); it was used to clone the wild-type and mutant *crp* genes from *E. coli* (4) and served as a control for the experiment shown in Fig. 6.

Enzyme assays. For β-galactosidase activity assays, *E. coli* cells (RZ211) carrying plasmid pRO80, pRO81, pRO82, pRO83, or pCB611 were grown overnight in M9 salts (0.1% NH_4Cl, 0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.05% NaCl) supplemented with 1 μg of thiamine per ml and either 0.4% glucose (catabolite-repressed conditions) or 0.4% glycerol (catabolite-activated conditions) and in the presence of 100 μg of ampicillin per ml. Cells were subcultured in the same medium, grown to an optical density at 600 nm of between 0.1 and 0.4, and assayed for β-galactosidase activity as described before (28). β-Galactosidase activity assays in *K. aerogenes* KC2668 containing pCB540 or pCB611 were performed in cells grown to the mid-logarithmic phase (50 Klett units) in W4 salts (1.05% K_2HPO_4, 0.45% KH_2PO_4, 0.01% $MgSO_4$ [pH 7.4]) supplemented with 0.4% glucose, 0.4% succinate, or 0.2% ammonium sulfate (as indicated in Table 2), 0.2% glutamine, and 100 μg of ampicillin per ml. Cells were resuspended in 1% KCl and assayed for β-galactosidase activity as described before (28) except that cells were made permeable with 0.2% hexadecyl trimethylammonium bromide and 0.02% sodium deoxycholate and assayed at 30°C.

For histidase activity assays, *K. aerogenes* KC1669 *crp* cells (38), transformed with the various plasmids containing wild-type or mutant *crp*, were grown to the mid-logarithmic phase in W4 salts supplemented with 0.2% $(NH_4)_2SO_4$, 0.4% galactose (catabolite-active conditions), and 0.2% histidine to inactivate the *hut* repressor (*hutC*). In addition, kanamycin (25 μg/ml) was added to the medium to ensure plasmid retention. Histidase activity, generated from chromosomal *hutUH*, was assayed essentially as described by Smith et al. (51) except that cells were washed and permeabilized as described elsewhere (21). Specific activity of histidase is given as nanomoles of urocanate produced per minute per milligram of protein. Total protein concentration was measured by the method of Lowry et al. (20).

In vitro transcription. In vitro transcription was performed with linear DNA templates by a procedure described by Maquat and Reznikoff (25). The precise reaction conditions were as previously described (39). Deleted DNA fragments used in the synthesis of run-off transcripts were obtained from various pOSRΔ or pOSLΔ plasmids. These plasmids were cleaved at two *Pvu*II restriction sites, and the DNA fragments containing deletions within *hut(P)* were purified as described before (39) and used for the experiment shown in Fig. 2. The portion of *hut(P)* deleted in these *Pvu*II-*Pvu*II DNA fragments was replaced, in each case, by 120 bp of pUC18-derived DNA containing the *lacZ* promoter (*lacZp*), which is inactive in the absence of CAP-cAMP. However, in the presence of CAP-cAMP, the undeleted 580-bp *Pvu*II-*Pvu*II fragment (used for the experiment shown in Fig. 5) transcribed both the *hutU* and *lacZ* promoters in the same direction; *lacZp*, which initiated transcription at about 280 bp upstream from the *hutUp* initiation site, generated the larger of the two run-off transcripts.

The run-off transcription products were separated by electrophoresis in 6 or 8% polyacrylamide gels containing 7 M urea and TBE (0.1 M Tris-borate [pH 8.3], 1 mM EDTA). Autoradiography was performed at −70°C with intensifying screens on Kodak XAR-5 X-ray film.

Gel retardation assays. The procedures for gel retardation assays have been described (11, 12). Precise conditions for CAP-DNA binding reactions and polyacrylamide gel electrophoresis were as previously described (38).

Deleted DNA fragments used in these assays were also obtained from the pOSRΔ and pOSLΔ plasmid series, which, for this purpose, were prepared by the small-scale alkaline lysis procedure (24). Following cleavage with *Eco*RI and *Hin*dIII restriction enzymes, the resulting two DNA fragments were labeled at their 3′ ends with [α-^{32}P]dATP and Klenow enzyme (24). The larger DNA fragment contained *lacZp*, and the smaller fragment contained *hut(P)* deleted to various extents. Since the *Eco*RI site was 1 bp away from each *hutUp* deletion boundary (41), cleavage with *Eco*RI followed by end-filling with Klenow enzyme resulted in a 6-bp stretch of DNA added to each deletion boundary. In some cases (i.e., Fig. 3A, lanes WT, LΔ97, and LΔ105), the large fragment containing *lacZp* was further digested with *Pvu*I to generate an additional 120-bp ^{32}P-labeled DNA fragment, which served as a negative control for CAP-cAMP binding.

DNase I footprint. DNase I protection analysis was performed essentially as described before (3) with some modifications. The indicated amounts of *E. coli* CAP were combined with 50,000 cpm of ^{32}P-end-labeled DNA in 45 μl of solution containing standard transcription buffer (30 mM Tris-HCl [pH 7.9], 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol) and 1 mM cAMP. The DNA template was a 300-bp *Eco*RI-*Hin*dIII fragment containing the *hut(P)* region (obtained from pOS2) which was labeled with ^{32}P at the 3′ end of the *Hin*dIII site (top strand). Following the binding reaction, which was at room temperature for 10 min, samples were treated with 5 μl of 0.3-μg/ml DNase I for 30 s at room temperature. DNase I cleavage was stopped by addition of 6 μl of DNase I quenching mixture (0.66 M Tris-HCl [pH 9.5], 66 mM *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*,-tetraacetic acid, 3.3% sodium dodecyl sulfate), and the sample was quick frozen in a dry ice-ethanol bath. DNA samples were extracted once with phenol-chloroform (1:1, vol/vol) and once with ether. Following precipitation by addition of 5 μl of 3 M sodium acetate, 5 μg of sonicated salmon sperm DNA, and 220 μl of 95% ethanol and incubation at −80°C for 10 min, the DNA pellet was collected by centrifugation and resuspended in appropriate volumes of formamide solution (99% formamide, 1 mM NaOH, 1 mg of bromophenol blue per ml, 1 mg of xylene cyanol per ml) so that a 3-μl gel loading volume contained 20,000 cpm of ^{32}P. Samples were heated to 90°C for 2 min before being loaded on an 8% polyacrylamide gel made

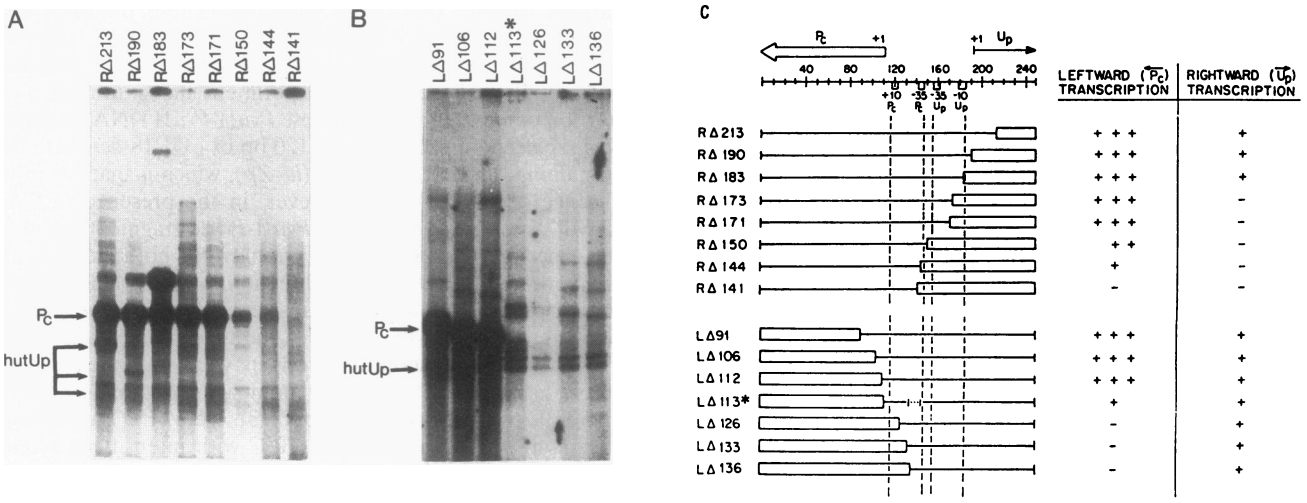

FIG. 2. In vitro transcription of DNA deleted within *hut(P)*. Run-off transcription reactions from linear DNA were performed with 1 pmol of RNA polymerase holoenzyme and 0.08 pmol of either (A) right-entering deletion DNA templates (RΔ) or (B) left-entering deletion DNA templates (LΔ), as indicated above each lane. The RNA signals representing transcripts generated from *hutUp* or from Pc were identified from their expected sizes and are indicated by arrows. (C) Schematic representation of DNA deletions used in the transcription reactions. The top line represents the *hut(P)* region shown in Fig. 1. Numbers above the line indicate nucleotide positions from left to right. The −10 and −35 regions of *hutUp* and Pc1 are indicated with open boxes, and their outermost boundaries are represented by vertical dashed lines. The arrows above the line represent the relative strength and orientation of these promoters in the absence of CAP-cAMP. The extent of each deletion (indicated on the left) is represented by an open rectangle; the deleted sequence in each case was replaced by DNA from pUC18. The solid lines represent undeleted DNA from *hut(P)*. The small dotted line in LΔ113* represents an internally deleted 10-bp DNA region from nucleotides 136 to 145. The relative intensities of transcription signals from Pc and *hutUp* obtained from each construct are indicated in the columns on the right and are qualitatively described as follows: +++, strong signal; ++, moderately strong signal; +, weak signal, −, little or no signal detected.

with 8 M urea and TBE. DNA sequencing reactions specific for nucleotide G (26) were electrophoresed in parallel.

RESULTS

Deletion analysis of *hutUp* and Pc. Our identification of the promoter sequences *hutUp* and Pc, the two promoters residing within the *hut(P)* region (Fig. 1), was based on (i) the sizes of the run-off transcripts obtained from each promoter (35), (ii) exonuclease III protection studies of RNA polymerase bound to *hutUp* and Pc (33, 34), and (iii) resemblance of DNA sequences to the canonical promoter sequence $TTGACAN_{17}TATAAT$ (33). However, no mutations that abolish the activity of either *hutUp* or Pc promoters directly have been obtained. To identify sequences that were required for the function of these promoters, we examined a set of DNA templates deleted within the *hut(P)* region from either direction (41) for their ability to direct transcription in vitro.

When DNA to the right of nucleotide 190 in *hut(P)* was deleted and replaced by pUC18 vector DNA sequence (i.e., RΔ213 and RΔ190), *hutUp* activity remained virtually unaffected (Fig. 2A and C). The deletion RΔ183, in which the DNA to the right of nucleotide 183 was replaced with vector sequence, allowed the synthesis of a very weak signal from *hutUp*. All other right-entering deletions affecting the DNA region to the left of position 183 (RΔ173, RΔ171, RΔ150, RΔ144, and RΔ141) abolished transcription from *hutUp*. Thus, the region between positions 184 and 173 contained the rightmost boundary of the sequence necessary for *hutUp* activity. This is in agreement with our identification of the sequence TATATG at positions 179 to 184 as the *hutUp* −10 promoter sequence (33).

In deletion LΔ113*, both the DNA to the left of nucleotide 113 and a 10-bp DNA segment from nucleotides 136 to 145 were deleted. Neither this deletion nor deletions LΔ126, LΔ133, and LΔ136 abolished transcription from *hutUp* (Fig. 2B and C), demonstrating that the *hutUp* promoter sequences are located to the right of nucleotide 145. This is consistent with our identification of the sequence TTGCGC from nucleotides 156 to 161 as the *hutUp* −35 region (33).

Initial identification of the divergent promoter (Pc) was complicated because two putative promoter-like sequences, Pc1 and Pc2, could potentially have accounted for the observed Pc transcripts (Fig. 1). Because the expected transcripts from these two putative promoters would differ only by about 10 bp, and because mRNA sizing by comparison with DNA size standards is reliable within about 5 to 8 bp, it was uncertain whether one or both promoters were giving rise to this highly intense transcript. However, analysis of DNA deletions in this region allowed us to resolve this difficulty. DNA to the left of nucleotide 112 was not required for Pc activity, since DNA templates LΔ91, LΔ106, and LΔ112 had no effect on Pc (Fig. 2B and C). Deletion LΔ126, which completely removed the Pc1 −10 region but left the Pc2 −10 region intact, completely abolished Pc transcription. Likewise, deletions LΔ133 and LΔ136, which extended still further into the Pc region, also abolished transcription from Pc (Fig. 2B and C). These results strongly argued that Pc1 but not Pc2 promoter sequences are essential for Pc transcription and thus indicate that Pc1 is the primary (if not the only) divergent promoter in *hut(P)*.

Deletions removing DNA to the right of nucleotide 171 (i.e., RΔ213, RΔ190, RΔ183, RΔ173, and RΔ171) had no effect on the activity of Pc (Fig. 2A and C). The additional high-intensity transcript observed for pORΔ183 is attributed to a new artifactual "backward" promoter created by the fusion of *hut(P)* and plasmid DNA sequences in this construct. The deletion RΔ150, which completely removed the putative Pc2 −35 region but left the Pc1 −35 region intact, still generated

TABLE 1. Effect of *hut(P)* deletions on the activity of *hutUp* in vivo[a]

Plasmid	Deletion	β-Galactosidase activity[b]		Fold activation
		M9 glucose	M9 glycerol	
pRO80	Undeleted *hut(P)*	2,277	15,450	6.8
pRO81	LΔ105	2,018	7,212	3.6
pRO82	LΔ125	2,065	2,070	1.0
pCB611	Mut 142–149	887	2,267	2.5
pRJ800	No insert	21	24	1.1

[a] Plasmids were constructed from pRJ800 and contain *lacZ* fusions to the indicated deleted DNA fragments from the *hut(P)* region. The extent of left-entering deletions within *hut(P)* used to construct the *hutUp-lacZ* fusions is indicated. Undeleted *hut(P)* is the complete 250-bp DNA represented in Fig. 1. In Mut 142–149, nucleotides 142 to 149 were replaced with the 8-bp sequence CGAATTCG, which affects part of CAP site 2.

[b] Miller units (28). Results are averages for at least three independent assays.

the same size of transcript from Pc, although less efficiently than the above-mentioned deletions. The larger deletion RΔ144, which removed the Pc2 −35 region and changed the Pc1 −35 sequence from TTAACA to TCGACA (matches to consensus are underlined), allowed transcription of Pc at levels comparable to (but slightly less than) those by RΔ150. However, deletion RΔ141, which completely removed the Pc1 −35 sequence, completely abolished Pc activity in vitro, suggesting that the Pc1 −35 sequence is absolutely required for Pc activity. The DNA deletion LΔ113* resembled LΔ112 in that the −10 regions of both Pc1 and Pc2 remained intact, but differed from LΔ112 in that the −35 region of Pc1 was completely replaced by the −35 region of Pc2. The hybrid Pc in LΔ113* resulted in very poor transcription activity (Fig. 2B and C), suggesting that the putative Pc2 −35 sequence is inefficient for promoting transcription in vitro.

A key observation from these experiments was that none of the left-entering deletions that decreased or abolished the activity of Pc (LΔ113*, LΔ126, LΔ133, and LΔ136) caused an enhancement of the activity of *hutUp* (Fig. 2B and C), as is the case when CAP-cAMP is added to undeleted *hut(P)* (see Fig. 5). Thus, elimination of Pc does not cause a "derepression" of *hutUp*, as predicted from the double-negative control model in its simplest form (35). This observation was confirmed in vivo by using *lacZ* fusions to *hutUp* on multicopy plasmids (Table 1). In cells grown under non-catabolite-activating conditions (M9 salts supplemented with glucose), a plasmid carrying a *lacZ* fusion to LΔ125 (which inactivates Pc) yields β-galactosidase at a level similar to that of *lacZ* fusions to LΔ105 and to undeleted *hut(P)*, in which Pc sequences are intact and active in vitro. Deletion LΔ150, which removes the Pc promoter but leaves the *hutUp* −35 and −10 sequences intact, results in a fourfold-lower *hutUp* activity. Thus, sequences between nucleotides 125 and 150 are somehow also required for normal *hutUp* activity in vivo. Nevertheless, these results suggest that the activity of *hutUp* in vivo is not subject to repression by Pc.

Identification of high- and low-affinity CAP binding sites within *hutUp*. To identify the site(s) of CAP-cAMP binding, we used the set of DNA deletion mutations across *hut(P)* in a gel retardation assay with CAP, in the presence and absence of cAMP, and buffer conditions normally used for in vitro transcription. A previously reported CAP-cAMP titration experiment showed that CAP bound to both the *hut* and *lac* promoter regions at concentrations ranging from 25 to 50 nM (38). As shown in Fig. 3A, at 50 nM CAP, both the *hutUp*- and *lacZp*-containing DNA fragments from pOS1 showed a cAMP-dependent gel mobility retardation (Fig. 3A). As a control, we show that the mobility of a smaller DNA fragment derived from pOS1, which contained no known CAP sites, was not retarded in the presence of CAP-cAMP (lanes WT). Deletions removing DNA to the left of nucleotide 97 (LΔ97) showed cAMP-dependent CAP binding, but when DNA to the left of nucleotide 105 (LΔ105) was deleted, no binding was detected (Fig. 3A and D). Likewise, left-entering deletions extending beyond nucleotide 105 (LΔ112 [Fig. 3A], LΔ125, LΔ133, LΔ136, LΔ148, and LΔ180 [not shown]) showed no binding at 50 nM CAP. This demonstrated that the upstream boundary for the sequence required for CAP-cAMP binding was located between nucleotides 97 and 105. A similar analysis with right-entering deletions showed that the downstream boundary of the CAP site resides between nucleotides 120 and 127. The largest right-entering deletion that showed CAP binding was RΔ127; RΔ120 failed to bind CAP (Fig. 3A and D).

A previously reported gel retardation assay with *Hga*I restriction fragments from the *hut(P)* region (38) suggested that a second CAP site (with less sequence similarity to the CAP consensus) might also exist downstream of nucleotide 125. Therefore, we tested for the presence of a weaker CAP binding site in this region with a higher CAP concentration. A careful titration (not shown) demonstrated that at a 300 nM concentration or greater, CAP bound to DNA fragments deleted for the high-affinity CAP site (CAP site 1, located between nucleotides 97 and 127, as defined above). Deletions LΔ105, LΔ112, LΔ125, and LΔ133, which failed to bind CAP at 50 nM, bound CAP at 300 nM in the presence (but not in the absence) of cAMP; deletions LΔ136, LΔ148, and LΔ180 failed to bind CAP at any concentration (Fig. 3B, and data not shown). Thus, the upstream boundary of this lower-affinity CAP binding site (CAP site 2) resides between nucleotides 133 and 136.

The downstream boundary of CAP site 2 could not be estimated with the right-entering deletions because CAP site 1 is present in these deletions. However, we observed that the *Hga*I-*Sph*I DNA fragment, which extends from nucleotides 125 to 160 and lacks CAP site 1, could not bind CAP at 300 nM (Fig. 3C, fragment a) or even at 575 nM (not shown). In contrast, the *Hga*I-*Sal*I fragment, which extends from nucleotides 125 to 250 (fragment e) and is comparable in *hut(P)* sequence to LΔ125, bound CAP-cAMP at a 300 nM concentration. Thus, the downstream boundary of CAP site 2 appears to extend at least into the *Sph*I site at nucleotides 160 to 165. A CAP site within this DNA region (from 133 to 165) might be centered at about −41.5 relative to the *hutUp* start of transcription. A weak match to a CAP half-site is noted within this region (Fig. 3D), but its contribution to the binding of CAP at site 2 has not been studied in detail.

DNase I protection analysis confirms the presence of the strong and weak CAP binding sites. CAP site 1 (from −67 to −96, centered at −81.5) can be fully protected from DNase I cleavage at CAP concentrations at which only weak protection at CAP site 2 is observed (Fig. 4, lane 3). As the CAP concentration is increased 10-fold, protection at CAP site 2 tends to increase (Fig. 4, lanes 4 and 5). However, DNase I protection at this second site is unusual in that it does not yield hypersensitive sites characteristic of CAP binding sites (1, 7, 13, 30, 42, 43, 50, 52) and its downstream boundary at about −32 is ill defined, possibly extending farther, to about −20 or −16 relative to *hutUp*. The fact that a significant difference in extent of CAP binding is noted at the lower CAP concentration used, even when both CAP sites remain intact, suggests that binding to these sites may not be cooperative.

CAP requirement for transcription of *hutUp*. Previous gel retardation experiments suggested that the affinities of CAP-

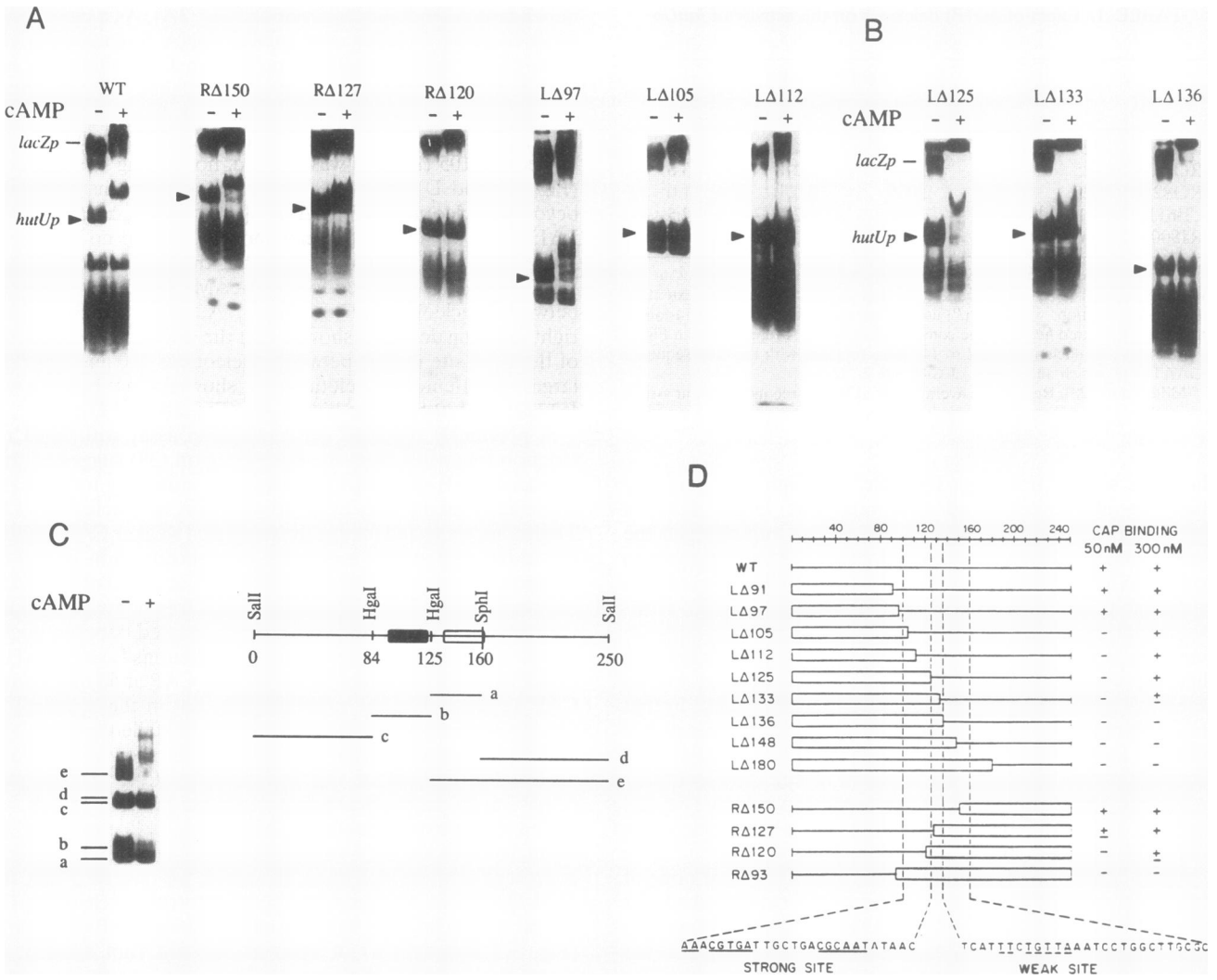

FIG. 3. Identification of CAP binding sites within *hut(P)*. Gel retardation assays were done in the presence of (A) 50 nM or (B) 300 nM purified CAP from *K. aerogenes*. The DNA constructs used in each pair of binding reactions (about 0.15 pmol) are indicated above the gels. Binding reactions were performed in the absence (−) or presence (+) of 1 mM cAMP. Unbound DNA fragments containing *lacZp* or *hutUp* (present in equimolar amounts) are indicated on the side; arrowheads indicate the positions of unbound *hutUp*-containing DNA fragments in each pair of lanes. In some lanes (WT, LΔ97, and LΔ105), a smaller DNA fragment lacking a CAP binding site was also present as a negative control. WT, wild type. (C) Binding reactions were performed with 300 nM CAP and 20 ng (ca. 0.1 pmol) of DNA fragments obtained from an *Hga*I-*Sph*I triple digest of the 250-bp *Sal*I fragment that contains *hut(P)*. The products of this digest are schematically represented on the right; letters used to label each DNA fragment are also used to identify the corresponding bands on the gel. Relative positions of the relevant restriction sites and of the strong (solid box) and weak (open box) CAP binding sites are also indicated. (D) Schematic representation of DNA deletions used in gel retardation assays. Details are as in Fig. 2C except that no DNA was used to replace the deleted portions. Instead, only a 6-bp stretch of DNA is connected to each deletion boundary. The results for CAP binding activity with each deletion tested (including some not shown in panels A and B) at 50 and 300 nM CAP are summarized in the columns on the right as follows: +, complete binding; ±, weak or partial binding; −, no binding detected. Dashed vertical lines indicate the approximate boundaries of the strong and weak CAP binding sites as determined by gel retardation assays, and the DNA sequences of these two sites are shown at the bottom. The solid and dotted underlined sequences show a good and a poor match, respectively, to the CAP consensus sequence.

cAMP for the *hutUp* region and *lacZp* region were similar (38). We compared the requirement for CAP-cAMP in both *hutUp* and *lacZp* more directly by using run-off transcription from linear DNA fragments. In this assay, both the *hut(P)* region (containing *hutUp* and Pc) and the *lac* promoter (*lacZp*) were located on the same DNA fragment to ensure that all three promoters were present in equimolar amounts (Fig. 5). The *lacZp* transcriptional activity was saturated at about 40 ng (35 nM) of CAP and showed half-maximal activity at about 15 ng (13 nM) of CAP (Fig. 5A). For *hutUp* activity, the system was saturated with about 120 ng (106 nM) of CAP and required about 55 ng (49 nM) of CAP for half-maximal activation of transcription. As noted previously (39), the transcriptional activity of Pc was a mirror image of that of *hutUp*, with a similar amount of CAP required to repress Pc as to activate *hutUp*. The fact that the CAP concentration required for half-maximal *hutUp* transcription was about 3.7 times higher than that required for *lac* P, even when their CAP binding affinities were comparable (38), suggests that occupancy of CAP site 1 alone may not be sufficient for efficient activation of

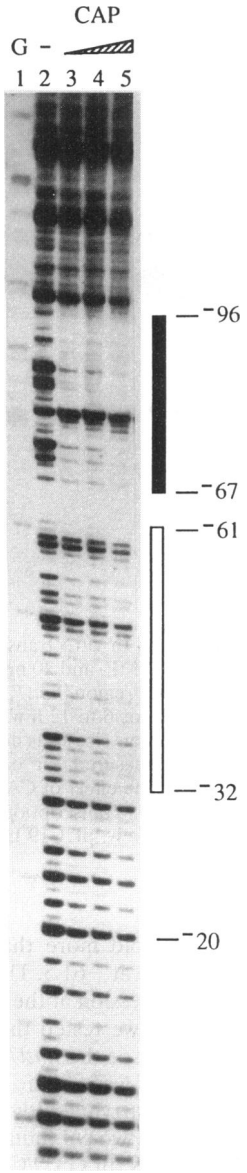

FIG. 4. DNase I protection of *hut(P)* by CAP. A 300-bp *Eco*RI-*Hin*dIII fragment containing *hut(P)* was reacted with 0 (lane 2), 325 nM (lane 3), 1.62 μM (lane 4), or 3.25 μM (lane 5) purified CAP from *E. coli* and 1 mM cAMP for 10 min at room temperature prior to cleavage with DNase I. Products of a Maxam and Gilbert DNA cleavage reaction specific for G (26) were electrophoresed in parallel and used as size standards (lane 1). The region strongly protected by CAP is indicated on the right with a solid bar; the open bar indicates a region of weak protection by CAP. The ends of protected regions are denoted as nucleotide positions relative to the *hutUp* start of transcription.

hutUp. Maximum *hutUp* transcription was observed at 106 nM CAP (Fig. 5A), even when as little as 30 nM CAP can completely bind CAP site 1 (38). These observations can be more easily explained if both CAP sites are playing a role in efficient transcription activation in vitro. It is conceivable that, in the presence of RNA polymerase, CAP-cAMP binding at site 2 is stabilized at 106 nM CAP or that the kinetics of the transcription reaction are such that they do not require prolonged occupancy at CAP site 2, as is required in the DNA-binding assays.

Previous determinations of the *hutP* mRNA start site in vivo (by S1 nuclease mapping) demonstrated that CAP-activated transcription has the same start site as that identified from in vitro experiments (33, 39). Thus, CAP-cAMP activates the same promoter in vivo and in vitro. β-Galactosidase assays of *lacZ* fusions to *hutUp* suggest that the presence of CAP site 2 alone (without CAP site 1) has no effect on the activity of *hutUp* under catabolite-activating conditions (Table 1). The *lacZ* fusion to *hutUp* in LΔ125 (pRO82), which contains an intact CAP site 2 and *hutUp* but completely lacks CAP site 1, shows no increase in β-galactosidase activity when cells are grown in M9-glycerol medium over that when grown in M9-glucose medium. No increase in *hutUp* promoter activity was observed even when cells were grown in M9 glycerol medium in the presence of 2 mM cAMP (not shown). In contrast, when both CAP sites 1 and 2 are present (pRO80), a 6.8-fold activation of *hutUp* by CAP-cAMP is observed (Table 1). The deletion LΔ105 in pRO81 still allowed a 3.6-fold activation of *hutUp* by CAP-cAMP, even though DNA upstream of nucleotide 105 was shown to be required for CAP binding at site 1 (Fig. 3A). However, because substitution of the deleted DNA in LΔ105 with plasmid DNA sequence simply changed the CAP site 1 sequence from AAACGTGAN$_6$ACGCAATA to AATTCTGAN$_6$ACGCAATA (matches to consensus are underlined), we attribute this residual CAP-cAMP activation to the presence of a partially active CAP site 1. In contrast, the portion of DNA deleted in the LΔ105 DNA fragment used in the gel retardation assay was not replaced and thus contained a disrupted CAP site 1. These results showed that CAP site 1 is essential for *hutUp* activation. On the other hand, CAP site 2, by itself, cannot effect the activation of *hutUp* in vivo, even if additional cAMP is added to the growth medium.

To test whether CAP site 2 was involved in activation of *hutUp*, we replaced a portion of the region containing CAP site 2 (nucleotides 142 to 149) with an 8-bp DNA sequence containing an *Eco*RI site (CGAATTCG) in pCB610. Gel retardation analysis with this mutated *hut(P)* showed a fourfold reduction in CAP-cAMP binding at site 2 (not shown). *hut(P)* containing this mutated CAP site 2 in pCB610 was fused to *lacZ* in pCB611. Transcription activation of this promoter under catabolite-activating conditions was only 2.5-fold, compared with the 6.8-fold observed for wild-type *hutUp* (Table 1), suggesting that CAP site 2 plays a role in activating *hutUp* transcription in vivo.

β-Galactosidase activity from pCB611 under nonactivating conditions resembled that from LΔ150 in that they were noticeably lower than the activity from wild-type *hut(P)* or LΔ105 and LΔ125. Both pCB611 and LΔ150 lack the wild-type sequence from nucleotides 142 to 149, which suggests that this sequence may play an important role in achieving maximum transcription of *hutUp* in a manner that is independent of CAP-cAMP. In *K. aerogenes*, activation of *hutUp* can also be achieved by nitrogen starvation (33). When cells are grown with limited nitrogen source, a nitrogen-controlled transcription activator, NAC, binds to a single site in *hut(P)* centered at −64 to activate *hutUp* (5, 14, 49). The results in Table 2 show that, despite the lower transcription activity of *hutUp* in pCB611, nitrogen activation of this promoter (3.82-fold) was the same as that for wild-type *hut(P)* in pCB540 (3.88-fold). Thus, inefficient activation of *hutUp* in pCB611 is observed only when CAP-cAMP serves as the activator. These results are consistent with CAP site 2 playing a role in achieving maximum transcription activation of *hutUp*.

Effect of CAP mutations on *hutUp* activity. Several CAP

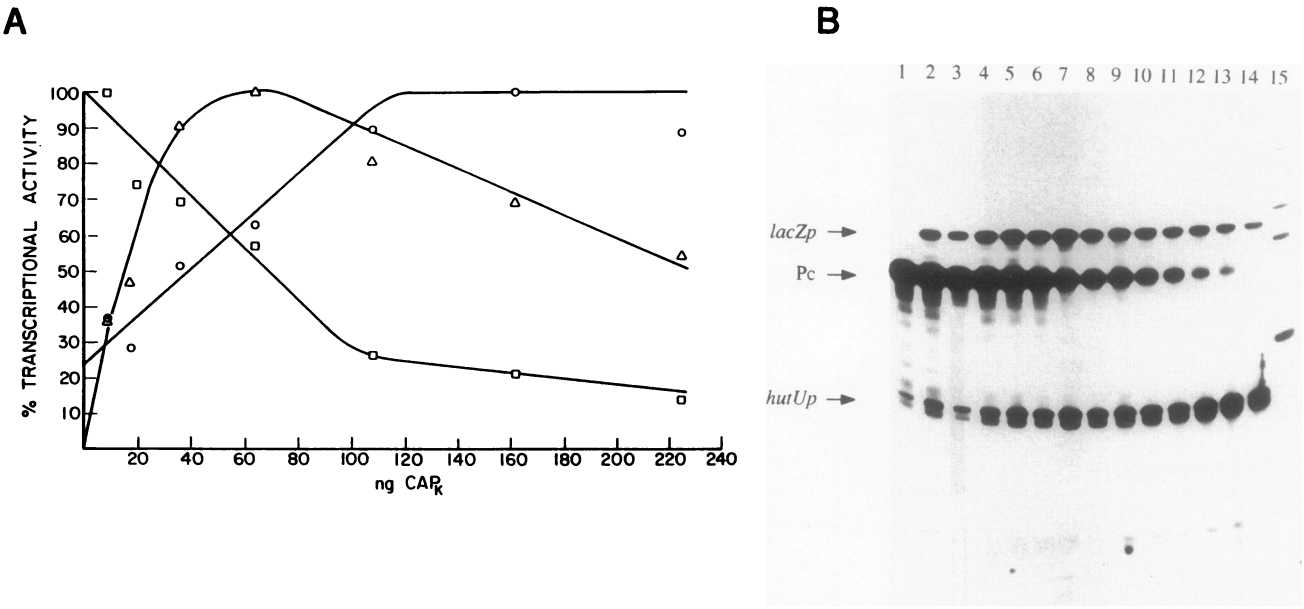

FIG. 5. Effect of CAP concentration on relative transcription activities from *hutUp*, Pc, and *lacZp*. (A) In vitro transcription reactions were performed with the indicated amounts of *K. aerogenes* CAP (CAP_K), 0.48 μg of RNA polymerase, 1 mM cAMP, and 20 ng of a *Pvu*II-*Pvu*II DNA template (580 bp) which contained both the *E. coli lacZ* promoter region and the *K. aerogenes hutU* promoter region [*hut*(*P*)]. Samples were loaded on a 6% polyacrylamide–7 M urea gel for electrophoresis, and autoradiography was performed at −70°C for 6 to 12 h with intensifying screens. The relative intensity of the bands corresponding to the transcripts from each promoter (distinguished by their size) was determined with an LKB laser beam densitometer. A 100% value was arbitrarily assigned to the maximum band intensity measured for each promoter. Percent transcriptional activity is indicated for *lacZp* (△), *hutUp* (○), and Pc (□). (B) Representative gel showing the effect of CAP concentration on the activity of *lacZp*, Pc, and *hutUp*. The transcripts corresponding to these promoters are indicated on the side. Transcription reactions in lanes 1 to 14 were done with 0, 12.5, 25, 37.5, 50, 75, 100, 125, 150, 175, 200, 225, 250, and 275 ng of CAP_K, respectively. pUC19 DNA cleaved with *Hin*fI was used as size standards (lane 15).

mutations that result in loss of transcription activation from *lac*P1 and *gal*P1 while retaining its normal DNA-binding and bending functions, as well as its ability to repress transcription (4, 10, 58), have been obtained. These observations have provided evidence for direct activation of RNA polymerase function by CAP-cAMP, involving contacts between CAP and RNA polymerase. The mutant *crp*H159L was shown to be incapable of activating a synthetic promoter containing a CAP binding site centered at either −41.5 or −61.5, nor did it activate *gal*P1, whereas wild-type *crp* activated all these promoters (4). On the other hand, the double mutant *crp*H159L,K52N activated transcription from a CAP site at −41.5 approximately threefold more than wild-type *crp* but had no effect from a CAP site at −61.5. This same CAP mutant was reported to activate expression at the *gal* promoter but not the *lac* promoter (4). Thus, we tested the effect of these two CAP mutants on the activity of the *hutUH* operon in vivo. *K. aerogenes* KC1669 (*crp-4021*) was transformed with a pSC105-based plasmid (pLG339) carrying either the wild-type *crp* (pLG339/CRP) or one of the two *crp* mutants H159L (pLG339/CRPH159L) and H159L,K52N (pLG339/CRPH159L,K52N) and assayed for histidase activity (encoded by the *hutH* gene) under catabolite-activating conditions. The results shown in Fig. 6 indicate that only wild-type *crp* significantly stimulated *hutUH* expression above levels obtained in cells carrying no plasmid or plasmid pLG339; neither *crp*H159L nor *crp*H159L,K52N caused a significant increase in *hutUH* expression. This suggests that the normal DNA-binding and bending activities that characterize these mutant CAP proteins (4) are not sufficient for *hutUH* activation, which is consistent with the notion that activation of *hutUp* by CAP-cAMP requires a direct activation mechanism. The observation that the double *crp* mutant H159L,K52N, reported to be more active at position −41.5 than the wild-type *crp* (4), did not increase but rather decreased *hutUH* expression suggests that the role played by this *crp* mutant at site 2 is different from that of the reported synthetic CAP site at −41.5. The decrease in *hutUp* activity caused by this *crp* mutant was not observed in pCB611 (not shown), suggesting that the negative effect of *crp*H159L,K52N is largely due to binding at CAP site 2. Potential interactions between this mutant CAP protein and RNA polymerase may tend to hinder, rather than stimulate, transcription initiation.

TABLE 2. Activation of *hutUp* by carbon and nitrogen starvation[a]

Plasmid	Growth medium[b]	β-Galactosidase activity[c]	Fold activation
pCB540	GNgln	3,166	1.0
	Ggln	12,308	3.89
	SNgln	17,433	5.51
pCB611	GNgln	1,523	1.0
	Ggln	5,827	3.83
	SNgln	3,583	2.35

[a] Plasmids were constructed in pRJ800 and contain *lacZ* fusions to wild-type *hut*(*P*)(pCB540) and to *hut*(*P*) containing a mutation in CAP site 2. Plasmids were assayed in *K. aerogenes* KC2668.

[b] Growth medium was W4 salts supplemented with 0.2% glutamine (gln), 0.4% glucose (G), 0.2% NH_4SO_4 (N), or 0.4% succinate (S), as indicated.

[c] Shown in nanomoles of *o*-nitrophenyl-β-D-galactopyranoside per minute per milligram of protein. Results are averages for at least three independent assays.

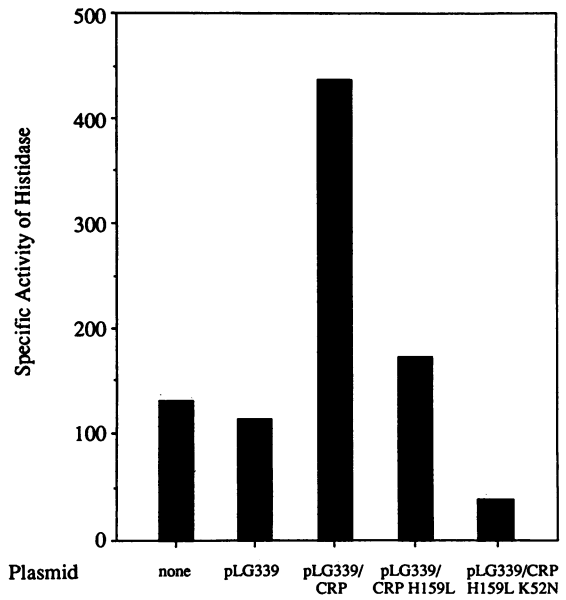

FIG. 6. Effect of *crp* mutants on the in vivo activity of *hutUp*. *K. aerogenes* KC1669 containing the indicated *crp* plasmids constructed by Bell et al. (4) was grown in catabolite-activating medium (W4 salts supplemented with 0.4% galactose and 0.2% histidine) to the mid-logarithmic growth phase and assayed for histidase activity (the product of *hutH*), which is a reflection of transcription from chromosomal *hutUp*. Specific activity of histidase is given as nanomoles of urocanate produced per minute per milligram of protein, and the results are averages of three independent assays. pLG339 is the pSC105-based cloning vector, which confers resistance to kanamycin (53), and was used as a control. Moderately high background levels of histidase activity are attributed to the presence of histidine in the growth medium, which causes inactivation of the *hut* repressor (HutC).

DISCUSSION

The data presented in this work led us to propose a direct role for CAP-cAMP in activation of *K. aerogenes hutUp*. Repression of the divergent promoter Pc, which normally accompanies *hutUp* activation in the presence of CAP-cAMP, cannot in itself result in activation of the latter, since none of the deletions that inactivated Pc caused a detectable increase in transcription from *hutUp* in vitro (Fig. 2) or in vivo (Table 1). Thus, the role of CAP-cAMP cannot be limited to an exclusion of the RNA polymerase binding at Pc (as was hypothesized by the double-negative control model [36]), but must involve a direct activation of *hutUp*.

Data from exonuclease III protection studies (33) and from in vitro transcription (39) suggested that the RNA polymerase binding sites at *hutUp* and Pc overlapped within a 4- to 10-bp region and implied that these two promoters were mutually exclusive. Each of the methods used to achieve activation of *hutUp*, whether by addition of CAP-cAMP or glycerol (a helix destabilizing agent), or by an up-promoter mutation at the *hutUp* −10 region (34, 35, 39), resulted in a repression of Pc, suggesting that RNA polymerase bound at *hutUp* could act as a direct repressor of Pc. This led to the assumption that, under nonactivating conditions, the reverse relationship between these two promoters might exist: that RNA polymerase bound at Pc might cause a repression of *hutUp*. However, the data presented here suggest that, even in the absence of Pc, *hutUp* is too weak a promoter and requires direct activation by CAP-cAMP. In fact, according to the rules of Mulligan et al. (31), the *hutUp* DNA sequence is predicted to be a very weak promoter. Thus, the potential *hutUp* repression that might, in theory, be exerted by RNA polymerase bound at Pc may not be relevant, because the *hutUp* activity cannot, by itself, exceed its basal transcription levels.

A similar case was found for the overlapping P1 and P2 *lac* promoters in *E. coli*. In the absence of CAP-cAMP, *lac*P2 is strongly expressed in vitro while *lac*P1 is completely repressed. However, in the presence of CAP-cAMP, P1 is completely active while P2 is strongly repressed (23). Since the CAP-binding site positioned at −61.5 relative to P1 overlapped P2, it was originally hypothesized that repression of P2 by CAP-cAMP would suffice for a substantial activation of P1. However, inactivation of P2 by a single point mutation or DNA deletion did not result in detectable activation of P1 in vitro, suggesting that a direct activation mechanism by CAP was required for P1 (9, 57). Although a role for CAP in reducing the occupancy of RNA polymerase at P2 cannot be excluded, a role for P2 in regulating P1 in vivo is not certain.

We cannot exclude the possibility that Pc may act as a repressor of *hutUp* at a level not detectable by our methods. For example, a deletion of Pc may allow an increased occupancy of RNA polymerase at *hutUp* to form closed or open complexes which may not result in the synthesis of run-off transcripts without further intervention of CAP-cAMP. In the case of *malT*, it was shown that RNA polymerase readily forms open complexes with the *malT* promoter but cannot make the transition to transcription elongation unless CAP-cAMP is present (27).

In vitro transcription from deleted *hut(P)* DNA templates (Fig. 2) confirmed the *hutUp* sequence TTGCGCN$_{17}$TAT ATG and distinguished the Pc1 promoter sequence TTAA CAN$_{18}$TATATT from that of Pc2 as the active divergent promoter. The fact that deletion RΔ150 partially decreased Pc transcription suggested that some sequence to the right of nucleotide 150 in Fig. 1 is involved in (but not absolutely required for) Pc transcription. This is consistent with data from exonuclease III protection studies (33) or from in vitro transcription using *Sph*I-cleaved *hut(P)* DNA (37), both of which suggested that DNA sequences to the right of nucleotide 150 could be involved in RNA polymerase activity at Pc. Similarly, in vivo transcription data suggested that DNA upstream of the *hutUp* −35 sequence, between nucleotides 150 and 125, is somehow required for *hutUp* transcription (Table 1). When the sequence from nucleotide 142 to 149 (−50 to −43 relative to *hutUp*) was substituted with an 8-bp sequence containing an *Eco*RI site, *hutUp* activity was similarly decreased. This sequence may be similar to the UP promoter element recently reported to be essential for maximal transcription of the *rrnB* P1 and P2 promoters (46). This UP sequence was shown to be located outside the "core" promoter elements and includes the region from −41 to −60 relative to the start of transcription. It appears that, when present, this highly A-T-rich UP sequence interacts with the α subunit of RNA polymerase to increase transcription (46).

The *hutUp* activation process requires a CAP binding site (CAP site 1), centered at −81.5 relative to *hutUp*, that is comparable in binding affinity to the *lac* promoter CAP site centered at −61.5. Deletion of this CAP site results in complete loss of CAP-cAMP activation of *hutUp* in vivo (Table 1). A second lower-affinity yet cAMP-dependent CAP binding site centered at about −41.5 relative to *hutUp* (CAP site 2) was also detected by gel retardation and DNase I protection analyses. CAP site 2, by itself, was unable to activate transcription in vivo, as evidenced by β-galactosidase levels of pRO82 in cells grown under catabolite-activated conditions (Table 1).

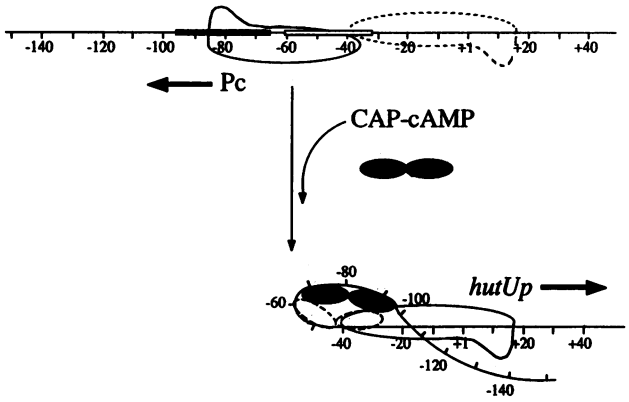

FIG. 7. Model for direct activation of *hutUp* by CAP. The upper section represents a situation in the absence of CAP-cAMP. RNA polymerase transcribes predominantly from the divergent promoter Pc (solid-line delineation) and very weakly from the forward promoter *hutUp* (dotted-line delineation). The *hut(P)* region from −150 to +40 is indicated by the horizontal solid line. CAP sites 1 and 2 are indicated by solid and open rectangles, respectively. The lower section illustrates what might occur in the presence of CAP-cAMP. CAP bound at site 1 (solid ovals) interacts with RNA polymerase at *hutUp* via DNA looping to stimulate *hutUp* transcription. CAP bound at site 2 (dotted-line open ovals) may play a more passive role in helping to stabilize the DNA loop or may not play a role at all.

When 8 bp within the region containing CAP site 2 (from nucleotides 142 to 149) are replaced with 8 bp (CGAATTCG) carrying an *Eco*RI site (in pCB611), which leaves CAP site 1 intact, only a 2.5-fold activation of *hutUp* transcription is observed in vivo. Compared with the 6.8-fold activation observed for wild-type *hutUp*, this value represents a significant decrease in efficiency of activation. It is possible that CAP site 1 alone can still activate transcription to some extent or that a less efficient occupancy of CAP site 2 is responsible for the decrease in transcription activation. These results are consistent with the notion that CAP site 1 is essential for activation of *hutUp* and that CAP site 2 is also required for maximal transcriptional activation.

The fact that *hutUp* activation by nitrogen starvation was the same in pCB611 as in pCB540 suggests that the decrease in *hutUp* activation by carbon starvation in pCB611 is related to the mutated CAP site 2 and not to a less active promoter. It is also possible, however, that a mutation in the UP element could lead to inappropriate binding of the α subunit of RNA polymerase, which could, in turn, hinder potential interactions between CAP-cAMP and RNA polymerase (i.e., with the α subunit). NAC, on the other hand, may not interact with the same region of RNA polymerase as CAP; hence, it may not depend on the presence of the UP element.

The observation that neither of the *E. coli* CAP mutants *crp*H159L or *crp*H159L,K52N caused the increase in *hutUH* expression observed with wild-type CAP (Fig. 6) may indicate that activation of *hutUp* might involve direct contacts between CAP and RNA polymerase. Ushida and Aiba (55) demonstrated that a single CAP site positioned at −82.5 was sufficient to generate substantial transcription activation from a synthetic promoter in vivo. Thus, it is possible that CAP bound at −81.5 might contact RNA polymerase at *hutUp* by DNA looping of the intervening DNA sequence (Fig. 7). DNA looping may be facilitated by the highly A-T-rich composition of the region between CAP site 1 and *hutUp* and by the binding of a second CAP protein bound at site 2. In this case, CAP bound at site 2 would be envisioned to play a purely architectural role (Fig. 7). The affinity of CAP at site 2 may be further stabilized by the presence of RNA polymerase and by the formation of a DNA loop structure. Alternatively, CAP bound at site 2 may directly contact RNA polymerase at *hutUp* to activate transcription. However, our observation that the double *crp* mutant H159L,K52N has a negative rather than a positive effect on *hutUp* suggests either that the role of CAP at site 2 is not mediated through contacts with RNA polymerase or that its putative interaction with RNA polymerase is different from that observed at synthetically engineered sites at −41.5 (4).

It is also possible that under conditions of DNA supercoiling in vivo, other proteins might assist CAP in this reaction. We have noted, for example, the existence of two adjacent binding sites for the small DNA-binding and bending protein Fis in the region between CAP site 1 and *hutUp*, based on DNase I protection studies (36). Perhaps Fis-mediated DNA bending in this region may further help facilitate DNA looping in vivo, as has been suggested for its role in stimulating λ phage DNA excision (19, 40). Although the model for CAP activation presented in Fig. 7 is purely hypothetical at this point, it serves to focus future investigations. It would be of interest to generate *crp* mutants specifically defective in *hutUp* activation function, as it is conceivable that novel sites of contact between CAP and RNA polymerase may be uncovered that are unique for a naturally occurring CAP site at −81.5. The generation of point mutations in the region between CAP site 1 and *hutUp* will also be important to more carefully assess the role of CAP site 2 or any other important sequence in this region.

Recently, a novel DNA-binding protein, NAC, has been shown to be an activator of *hutUH*, independently of CAP-cAMP in vivo and in vitro, in response to nitrogen starvation (14, 21, 33, 49). The NAC protein binds a single site centered at −64 to stimulate σ^{70} RNA polymerase-dependent transcription from *hutUp*, most likely also by a direct activation mechanism (5, 14). It will be of interest to investigate the extent to which NAC- and CAP-mediated activation of *hutUp* has similar mechanistic features.

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