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 (*hutP* 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 *hutP* is largely repressed. CAP-cAMP has the dual effect of repressing transcription from *Pc* while simultaneously activating transcription from *hutP*. 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 *hutP* 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 *hutP* in vivo. These results invalidate an indirect activation model by which CAP-mediated repression of *Pc* in itself would lead to activation of *hutP*. 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 *hutP* 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 *hutP*. Although CAP site 2 by itself is unable to activate *hutP* in vivo under catabolite-activating conditions, it appears to be required for maximal transcription activation. Our observation that the double CAP mutant *crph159L,K52N*, which is known to activate transcription from a site centered at −41.5, does not activate *hutP* 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 *hutP* 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 AANTGTGAN6TCACANTT 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 *lacP1* and *uacAB* while allowing transcription of other CAP-independent promoters, such as the *lacUV5, lacP2, trp*, and *rplJ* promoters (16, 17). 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* lac*Z* and the *K. aerogenes hutU* promoters (38). The CAP-dependent *hutU* promoter (*hutP*) drives the expression of the histidine utilization operon *hutUH* in *K. aerogenes* (35). In the absence of CAP-cAMP, RNA polymerase interacts poorly with *hutP* 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 *hutP*. 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 *hutP* 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 *hutP* start of transcription, appeared to reside too far away from *hutP* to allow direct contacts between CAP bound at this site and RNA polymerase bound at *hutP*. 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 *hutP* by CAP-cAMP might be achieved by an indirect activation mechanism (double-negative control [34, 36]). By this model, RNA polymerase

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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 hutUp 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 Δbla-2). Histidase assays were performed in *K. aerogenes* KC1669 (cer-4021) (38).

Plasmids containing the various right-entering (pOSRA) or left-entering (pOSLD) 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 *SalI* restriction site in opposite orientations. The plasmid series pOSRA and pOSLD 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 pOSRA150; likewise, a construct removing the DNA to the left of nucleotide 105 (Fig. 1) is named pOSLA105; and so forth. The EcoRI and HindIII 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 (rp-lac)W200 fusion. The EcoRI-HindIII fragments of pOS2, pOSLA105, pOSLA125, and pOSLA150 were blunt ended at their HindIII sites and cloned into the EcoRI and *SmaI* restriction sites of pRJ800 to produce the hutUp-lacZ operon fusion plasmids pR080, pR081, pR082, and pR083, respectively. Plasmid pCBS40 is essentially the same as pR080 except that it was constructed by inserting the *SalI*-SalII frag-
ment from pOS1 into the SalI site of pRJ800 to produce a hutUp-lacZ operon fusion.

Plasmids pCB610 and pCB611 both carry an 8-bp DNA sequence with an EcoRI 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 HindIII-EcoRI fragment of pORA141 [which contains the hut(P) region to the left of nucleotide 142] into the SmalI and EcoRI sites of PBulgescript SK+ to make pCB609. The EcoRI-HindIII fragment of pOLΔ150 [which contains the hut(P) region to the right of nucleotide 149] was cloned into the EcoRI and HindIII sites of pCB609 to make pCB610. The BamHI-HindIII fragment of pCB610 was then cloned into the BamHI and HindIII 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) cpr 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 cpr 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 pR080, pR081, pR082, pO50, or pCB611 were grown overnight in M9 salts (0.1% NH₄Cl, 0.3% KH₂PO₄, 0.6% Na₂HPO₄, 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 pCBS40 or pCB611 were performed in cells grown to the mid-logarithmic phase (50 Klett units) in W4 salts (1.05% K₂HPO₄, 0.45% KH₂PO₄, 0.01% MgSO₄ [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% K₂HPO₄ for β-galactosidase activity assays described before (28) except that cells were made permeable with 0.2% hexadeyl trimethylammonium bromide and 0.02% sodium deoxycholate and assayed at 30°C.

For histidine activity assays, K. aerogenes KC1669 ccr cells (38), transformed with the various plasmids containing wild-type or mutant ccr, were grown to the mid-logarithmic phase in W4 salts supplemented with 0.2% (NH₄)₂SO₄, 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. Histidine 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 histidine is given as n mole 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 pOSΔ or pOSLΔ plasmids. These plasmids were cleaved at two PvuII 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 PvuII-PvuII 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 PvuII-PvuII 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 pOSΔ and pOSLΔ plasmid series, which, for this purpose, were prepared by the small-scale alkaline lysis procedure (24). Following cleavage with EcoRI and HindIII restriction enzymes, the resulting two DNA fragments were labeled at their 3' ends with [α-32P]dATP and Klenow enzyme (24). The larger DNA fragment contained lacZp, and the smaller fragment contained hut(P) deleted to various extents. Since the EcoRI site was 1 bp away from each hutUp deletion boundary (41), cleavage with EcoRI 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, ΔA97, and ΔA105), the large fragment containing lacZp was further digested with PvuI to generate an additional 120-bp 32P-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 32P-end-labeled DNA in 45 μl of solution containing standard transcription buffer (30 mM Tris-Cl [pH 7.9], 40 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.1 mM dithiothreitol) and 1 mM CAP. The DNA template was a 300-bp EcoRI-HindIII fragment containing the hut(P) region (obtained from pOS2) which was labeled with 32P at the 3' end of the HindIII 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-Cl [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 bromphenol blue per ml, 1 mg of xylene cyanol per ml) so that a 3-μl loading volume contained 20,000 cpm of 32P. Samples were heated to 90°C for 2 min before being loaded on an 8% polyacrylamide gel made
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_7 TATAAT (33). However, no mutations that abolish the activity of either hutUp or Pc promoters directly have been described. 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., RA213 and RA190), hutUp activity remained virtually unaffected (Fig. 2A and C). The deletion RA183, 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 (RA173, RA171, RA150, RA144, and RA141) 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 LA113*, 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 LA126, LA133, and LA136 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 LA9i and LA112 had no effect on Pc (Fig. 2B and C). Deletion LA126, which completely removed the Pc -10 region but left the Pc -10 region intact, completely abolished Pc transcription. Likewise, deletions LA133 and LA136, 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., RA213, RA190, RA183, RA173, and RA171) had no effect on the activity of Pc (Fig. 2A and C). The additional high-intensity transcript observed for pORA183 is attributed to a new artificial "backward" promoter created by the fusion of hut(P) and plasmid DNA sequences in this construct. The deletion RA150, 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 vitro a

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<thead>
<tr>
<th>Plasmid</th>
<th>Deletion</th>
<th>β-Galactosidase activity b</th>
<th>Fold activation</th>
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<tr>
<td></td>
<td></td>
<td>M9 glucose</td>
<td>M9 glycerol</td>
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<tr>
<td>pRO80</td>
<td>Undeleted hut(P)</td>
<td>2,277</td>
<td>15,450</td>
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<tr>
<td>pRO81</td>
<td>LA105</td>
<td>2,018</td>
<td>7,212</td>
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<td>LA125</td>
<td>2,065</td>
<td>2,070</td>
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<tr>
<td>pCB611</td>
<td>Mut 142–149</td>
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<td>2,267</td>
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<tr>
<td>pJR800</td>
<td>No insert</td>
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a Plasmids were constructed from pJR800 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 CGAATTCC, 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 Pc -35 region and changed the Pc -35 region 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 Pc -35 sequence, completely abolished Pc activity in vitro, suggesting that the Pc -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 CAP-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, LΔ132, 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 Hgal 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 Hgal-SphI 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 Hgal-SullI 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 SphI 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 the 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 further, 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-
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 (15 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. 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, LA97, and LA105), 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 HpaI-SphI triple digest of the 250-bp Sall 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.
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 LA125 (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 LA105 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 LA105 with plasmid DNA sequence simply changed the CAP site 1 sequence from AAAGTGANACGGCAATA to AA TJCCTGANACGGCAATA (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 LA105 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 EcoRI site (CGAATTGC) in pCB610. Gel retardation analysis with this mutated hutP showed a fourfold reduction in CAP-cAMP binding at site 2 (not shown). hutP 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 LA150 in that they were noticeably lower than the activity from wild-type hutP) or LA105 and LA125. Both pCB611 and LA150 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 hutP 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 hutP in pCB540 (3.88-fold). Thus, inefficient activation of hutUp in pCB611 is observed only when CAP-cAMP is used 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<sub>r</sub>), 0.48 μg of RNA polymerase, 1 mM cAMP, and 20 ng of a PvuII-PvuII 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<sub>r</sub>, respectively. pUC19 DNA cleaved with HindIII was used as size standards (lane 15).

mutations that result in loss of transcription activation from lacP1 and galP1 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 crpH159L 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 galP1, whereas wild-type crp activated all these promoters (4). On the other hand, the double mutant crpH159L,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-402J) 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 histidine 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 crpH159L nor crpH159L,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 hutUH 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 hutUH activity caused by this crp mutant was not observed in pCB611 (not shown), suggesting that the negative effect of crpH159L,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 hutUH by carbon and nitrogen starvation

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Growth medium</th>
<th>β-Galactosidase activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fold activation</th>
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<tr>
<td>pCB540</td>
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<tr>
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<td>SNgln</td>
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<td>GNgln</td>
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<td></td>
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<tr>
<td></td>
<td>SNgln</td>
<td>3,583</td>
<td>2.35</td>
</tr>
</tbody>
</table>

<sup>a</sup> Plasmids were constructed in pRJ800 and contain lacZ fusions to wild-type hutUH(pCB540) and to hutUH containing a mutation in CAP site 2. Plasmids were assayed in K. aerogenes KC2668.

<sup>b</sup> Growth medium was W4 salts supplemented with 0.2% glutamine (gln), 0.4% glucose (G), 0.2% NH<sub>4</sub>SO<sub>4</sub> (N), or 0.4% succinate (S), as indicated.

<sup>c</sup> Shown in nanomoles of d-nitrophenyl-β-D-galactopyranoside per minute per milligram of protein. Results are averages for at least three independent assays.
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The data presented in this work led us to propose a direct role for CAP-cAMP in repression of the K. aerogenes hutUH. The role of the divergent promoter Pc, which normally accompanies hutUH 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 hutUH 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 hutUH.

Data from exonuclease III protection studies (33) and from in vitro transcription (39) suggested that the RNA polymerase binding sites at hutUH 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 hutUH, whether by addition of CAP-cAMP or glycerol (a helix destabilizing agent), or by an up-promoter mutation at the hutUH −10 region (34, 35, 39), resulted in a repression of Pc, suggesting that RNA polymerase bound at hutUH 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 hutUH. However, the data presented here suggest that, even in the absence of Pc, hutUH is too weak a promoter and requires direct activation by CAP-cAMP. In fact, according to the rules of Mulligan et al. (31), the hutUH DNA sequence is predicted to be a very weak promoter. Thus, the potential hutUH repression that might, in theory, be exerted by RNA polymerase bound at Pc may not be relevant, because the hutUH 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, lacP2 is strongly expressed in vitro while lacP1 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 hutUH at a level not detectable by our methods. For example, a deletion of Pc may allow an increased occupancy of RNA polymerase at hutUH 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 hutUH sequence TTTGCCG(N7)ATG and distinguished the Pc1 promoter sequence ITAA(CAN15)TATT from that of P2 as the active divergent promoter. The fact that deletion R1A50 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 Sphl-cleaved hutUH 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 hutUH −35 sequence, between nucleotides 150 and 125, is somehow required for hutUH transcription (Table 1). When the sequence from nucleotide 142 to 149 (−50 to −43 relative to hutUH) was substituted with an 8-bp sequence containing an EcoRI site, hutUH activity was similarly decreased. This sequence may be similar to the UP promoter element recently reported to be essential for maximal transcription of the mmB 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 hutUH activation process requires a CAP binding site (CAP site 1), centered at −81.5 relative to hutUH, 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 hutUH in vivo (Table 1). A second lower-affinity yet CAP-dependent CAP binding site centered at about −41.5 relative to hutUH (CAP site 2) was also detected by gel retardation and DNase I protection analysis. CAP site 2, however, was used only in transcription in vivo, as evidenced by β-galactosidase levels of pR082 in cells grown under catabolite-activated conditions (Table 1).
When 8 bp within the region containing CAP site 2 (from nucleotides 142 to 149) are replaced with 8 bp (CGAATTCG) carrying an EcoRI 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 crpH159L or crpH159L,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 binding 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 the crp mutants more clearly distinctive in hutUp transcription 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 σ70 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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