RNA Polymerase as a Repressor of Transcription in the *hut(P)* Region of Mutant *Klebsiella aerogenes* Histidine Utilization Operons

ANTHONY J. NIEUWKOOP† AND ROBERT A. BENDER*

Department of Biology, The University of Michigan, Ann Arbor, Michigan 48109-1048

Received 29 February 1988/Accepted 11 July 1988

Mutants of *Klebsiella aerogenes* able to express the *hutUH* operon in the absence of positive effectors were isolated and characterized. These mutations improve the *hutUH* promoter (P_{UH}) by changing the -10 region to match the consensus sequence more closely. These mutations also affect another, oppositely oriented promoter in this region, P_C . Although the mutations lie far outside P_C , they cause P_C to be inactive, apparently because binding of RNA polymerase to the P_{UH} promoter blocks the overlapping P_C site. Thus, in the mutants, RNA polymerase bound at the strong (mutant) P_{UH} site effectively represses the P_C promoter.

The *hutUH* operon of the *Klebsiella aerogenes* histidine utilization genes is subject to many layers of regulation. Even when the *hut*-specific repressor is inactivated (6) and an adequate electron transport acceptor is available (4), *hutUH* is poorly expressed unless either the catabolite gene activator protein-cyclic AMP (CAP-cAMP) complex or the Ntr regulatory factors act to increase transcription of *hutUH* in response to carbon or nitrogen limitation of growth (12). Analyses of the transcripts produced in vitro from the *hut(P)* region (the region controlling *hutUH* expression) suggested that the stimulation of *hutUH* transcription by CAP-cAMP might involve a novel "double-negative" mechanism (11). In this model (Fig. 1), RNA polymerase bound at a site, P_C , within *hut(P)* prevents RNA polymerase from binding to the *hutUH* promoter (P_{UH}) by steric hindrance. If CAP-cAMP were bound to a site near to and interfering with P_C , then CAP-cAMP would prevent RNA polymerase from binding at P_C and would thus lessen the barrier to binding of RNA polymerase at P_{UH} . This model postulates two kinds of competition: between CAP-cAMP and RNA polymerase at P_C , and between RNA polymerases at P_C and P_{UH} . The CAP-cAMP competition with P_C was demonstrated by the observation that CAP-cAMP could prevent transcription from P_C if the CAP-cAMP was bound to DNA before RNA polymerase was added; CAP-cAMP could not repress transcription from P_C if RNA polymerase had already formed an open complex at P_C when CAP-cAMP was added (11). The second kind of competition, between P_C and P_{UH} , was suggested by the observation that the sites occupied by RNA polymerase bound at P_C or P_{UH} overlapped by several base pairs (10). To confirm the competition between P_C and P_{UH} , we studied a class of mutants in which *hutUH* could be efficiently expressed even in the absence of CAP-cAMP (or the Ntr regulatory factors) both in vivo (see below) and in vitro (11).

Isolation of *hut(P)* mutants. Efficient expression of the *hut* operons of *K. aerogenes* requires activation either by the CAP-cAMP complex in response to carbon starvation or by the Ntr regulatory system in response to nitrogen starvation (6). The Ntr regulatory system is not yet well understood; however, it is clear that the product of the *glnG* (*ntrC*) gene is absolutely required for Ntr activation of *hut* (15). Strain

KB770 (Table 1) carries a Tn5 insertion in the *glnLG* operon (14), creating a *glnG* null phenotype and abolishing Ntr activation of *hut*. Growth with glucose as the carbon source eliminates CAP-cAMP-mediated activation, so strain KB770 is unable to use histidine as the sole nitrogen source in the presence of glucose (glucose-histidine medium). Mutants of strain KB770 able to grow on glucose-histidine medium arose spontaneously at a frequency of about 10^{-9} . After single colony purification, these mutants were tested for growth on a variety of nitrogen sources and fell into two classes with about equal numbers in each class. Class I mutants were able to use all the poor nitrogen sources tested and were not studied further. Class II mutants were able to use only histidine and were assumed to affect *hut* operon expression only.

Regulation of histidase formation in mutants. Five independent class II mutants were chosen for analysis. The *hut* region from each of the five was transferred to strain KG2006 (*hutI*) by P1-mediated transduction, and the regulation of histidase formation was assayed in the transductants. Strain KB803 carried the *hut* region from the starting strain, KB770, and histidase formation was regulated as expected of a wild-type *hut* operon. Specifically, histidase formation required inactivation of the *hut* repressor (Table 2, compare lines 1 and 5 or lines 2 and 6), and histidase formation was activated either by carbon or nitrogen limitation (compare lines 2 through 4 with line 1). All five of the transductants showed abnormal regulation of histidase formation. The five were essentially identical, and two are shown in Table 2 for illustration. In the mutants, histidase expression was very active even in the presence of glucose and ammonium (compare GNH cultures). Three observations follow from the data above. (i) All five of the mutants carried *hut*-linked mutations. (ii) In each case, the mutation allowed high-level expression of histidase, even in the absence of carbon or nitrogen activation. (iii) The *hut* repressor was significantly less effective in blocking histidase expression in the mutants than it was in the wild type (compare lines 1 and 5 or lines 2 and 6).

The fact that the initial mutants were still unable to use poor nitrogen sources other than histidine suggested that *hut* expression did not result from some kind of alternative (*glnG*-independent) Ntr activation. Nor did the *hut*-linked mutation cause a constitutive carbon activator, since β -galactosidase expression (which requires CAP-cAMP) was regulated normally (Table 2).

* Corresponding author.

† Present address: Department of Biology, Hope College, Holland, MI 49423.

TABLE 1. Bacterial strains employed

Strain ^a	Genotype or description	Origin
KB770	<i>gltB200 glnL45 gln-228::Tn5</i>	This paper
KB783	<i>gltB200 glnL45 gln-228::Tn5</i> <i>hutP101</i>	This paper
KB784	<i>gltB200 glnL45 gln-228::Tn5</i> <i>hutP102</i>	This paper
KB785	<i>gltB200 glnL45 gln-228::Tn5</i> <i>hutP103</i>	This paper
KB786	<i>gltB200 glnL45 gln-228::Tn5</i> <i>hutP104</i>	This paper
KB787	<i>gltB200 glnL45 gln-228::Tn5</i> <i>hutP105</i>	This paper
KG2004	<i>hutC516 hutH518 gal-1</i>	5
KG2006	<i>hutC516 hutI519 gal-2</i>	5
KB794	<i>hutP101</i>	P1 · KB783 × KG2006 ^b
KB796	<i>hutP102</i>	P1 · KB784 × KG2006
KB798	<i>hutP103</i>	P1 · KB785 × KG2006
KB800	<i>hutP104</i>	P1 · KB786 × KG2006
KB802	<i>hutP105</i>	P1 · KB787 × KG2006
KB803	Prototroph	P1 · KB770 × KG2006
RH202	<i>hss-1 thi-1 lacY1 tonA21</i> <i>supE44</i>	R. Helling

^a All strains, except RH202, are derived from *K. aerogenes* W70. RH202 is derived from *E. coli* K-12.

^b A lysate of phage P1clr100KM (3) from strain KB783 was used to transduce strain KG2006 to growth with histidine as the sole carbon source.

Mapping the *hut(P)* mutations. Since all five of the mutations were linked to the *hut* region, a three-factor cross was used to map the location of *hutP104* and *hutP105* with respect to *hutC* and *hutH*. The map order of the *hut* genes, (M)IGC(P)UH, dictates that *hut(P)* mutations should lie between *hutC* and *hutH* (5). The data in Table 3 show that *hutP104* and *hutP105* are tightly linked to *hutH* (92 and 90%, respectively, versus 88% for *hutC*). If we demand recombination between *hutH* and *hutC* (the $H^+ C$ class), we find that both donor and recipient alleles are found at about equal frequency; if we demand recombination between *hutH* and *hutP* (the $H^+ P^+$ class), we find that the majority carry mutant *hutC*, the recipient allele. These data exclude the order *P-C-H* and support the order *C-P-H*. The only remaining order, *C-H-P*, is also excluded because the $C H^+ P^+$ class, which would require recombination in both the *C-H* and the *H-P* intervals is nearly as frequent (7 to 9%) as the classes requiring recombination in either of the intervals singly (2 to 13% and 8 to 11%, respectively). Similarly, the rarest class of recombinants ($C^+ P^+ H^+$) should represent the class requiring a quadruple crossover event, again giving the order *C-P-H*. Therefore the *hutP104* and *hutP105* mutations appear to lie between *hutC* and *hutH*, consistent with

FIG. 1. Double-negative control model for CAP-cAMP regulation of *hutUH* transcription. This model was first presented by Nieuwkoop et al. (11).

the hypothesis that they are mutations in the regulatory region of the *hutUH* operon.

The ability of the *hutP104* mutation to allow *hutUH* transcription in the absence of positive effectors was also demonstrated in vitro (11). These in vitro transcription experiments demonstrated that the *hutP104* mutation lay within the 247-base-pair (bp) region between a *SalI* restriction site at map position 3.35 and a *PvuII* restriction site at map position 3.59, since material outside this region could be removed without changing the effect of *hutP104* on transcription (11). Thus, *hutP104*, probably *hutP105*, and perhaps all five of the mutations lie within *hut(P)*, the control region of the *hutUH* operon.

Molecular analysis of *hutP104* and *hutP105*. The *hutP104* and *hutP105* mutations were transferred from the chromosome onto plasmid pCB103 by P1-mediated transduction as described previously (10). In both cases, heterodimeric products were observed and these were resolved into monomeric forms by digestion and religation with *EcoRI* (see reference 10 for a discussion of this technical detail). The nucleotide sequence of the 247-bp fragment from the *SalI* to *PvuII* sites at map positions 3.35 and 3.59 was determined for each mutation. The sequence was identical to that previously obtained for the wild type, except that *hutP104* lacked the T · A base pair at position 183 in the wild-type sequence, and *hutP105* duplicated the T · A base pair at position 181 in the wild-type sequence (Fig. 2). The effect of each of these changes is to alter the -10 region of the site previously identified as the promoter of *hutUH*. In the wild

TABLE 2. Regulation of histidase and β -galactosidase formation in *hutP* mutants^a

Growth medium ^b	Histidase (sp act)			β -Galactosidase (sp act)		
	KB803 (<i>hutP⁺</i>)	KB800 (<i>hutP104</i>)	KB802 (<i>hutP105</i>)	KB803 (<i>hutP⁺</i>)	KB800 (<i>hutP104</i>)	KB802 (<i>hutP105</i>)
1. GNH	80	408	505	<5	<5	<5
2. CNH	200	420	496	340	350	260
3. GH	230	460	550	<5	<5	<5
4. H	365	581	881	835	697	590
5. GN	2	41	43	<5	<5	<5
6. CN	5	93	103	575	640	650

^a Histidase and β -galactosidase were assayed in whole cell preparations as described previously (12), except that the β -galactosidase reaction was at 30°C.

^b Abbreviations: G, 0.4% glucose; C, 0.4% sodium citrate; N, 0.2% ammonium sulfate; H, 0.4% L-histidine. All cultures contained 1 mM isopropyl- β -D-thiogalactopyranoside to induce the *lac* genes.

TABLE 3. Map location of *hutP* mutations

Donor strain (genotype) ^a	Recipient strain (genotype)	Selected character (no. examined)	Unselected characters ^b (%)
KB800 (<i>C</i> ⁺ <i>P104</i> <i>H</i> ⁺)	KG2004 (<i>C</i> <i>P</i> ⁺ <i>H</i>)	<i>H</i> ⁺ (335)	<i>C</i> ⁺ <i>P104</i> (87) <i>C</i> ⁺ <i>P</i> ⁺ (1) <i>C</i> <i>P104</i> (5) <i>C</i> <i>P</i> ⁺ (7)
KB802 (<i>C</i> ⁺ <i>P105</i> <i>H</i> ⁺)	KG2004 (<i>C</i> <i>P</i> ⁺ <i>H</i>)	<i>H</i> ⁺ (329)	<i>C</i> ⁺ <i>P105</i> (86) <i>C</i> ⁺ <i>P</i> ⁺ (2) <i>C</i> <i>P105</i> (4) <i>C</i> <i>P</i> ⁺ (9)

^a Three-factors crosses using P1-mediated transduction were carried out as described by Streicher et al. (16). Genotypes are abbreviated here by omitting *hut*, thus, *C*⁺ is *hutC*⁺, *C* is *hutC*, etc.

^b The unselected characters were scored by comparing the amount of histidase found in cells grown in two different media: glucose-ammonia-histidine (GNH) or citrate-ammonia (CN). The microtiter well assay (5) was used, and the pattern of the four recombinant classes is as follows. The patterns of the four recombinant classes, *C*⁺ *P*, *C*⁺ *P*⁺, *C* *P*, and *C* *P*⁺, respectively, were +, +/–, +, and +/– on GNH medium and +/–, –, ++, and + on CN medium.

type, the sequence TATATGT is a poor match to the –10 consensus and, as a result, this is a weak promoter. The *hutP104* deletion improves the –10 sequence to TATAGT as does the *hutP105* duplication (TATTAT). Both of these are excellent matches to the consensus sequence and should be strong promoters (9), especially since the –35 region of this promoter (TTGCGC) is already quite similar to the consensus sequence (10).

A DNA protection experiment (10) was used to determine the effect of *hutP104* and *hutP105* on the binding of RNA polymerase to the *hut(P)* region. RNA polymerase was incubated with a fragment of either *hutP104* DNA or *hutP105* DNA, radioactively labeled at one end. Exonuclease III was then allowed to digest from the opposite end up to the point where the bound RNA polymerase blocked the progress of the exonuclease III. The length of the remaining radioactive fragment reflects the extent of RNA polymerase protection on one side. When the opposite end of the fragment was radioactively labeled, the other boundary of RNA polymerase protection was determined. RNA polymerase binds to and protects a 57-bp region from position 151 to 207 or 209 on the DNA sequence (Fig. 3). This site corresponds exactly to the *hutUH* promoter. It is significant that no binding of RNA polymerase to the *P_C* site (positions 107 to 154 [10]) was detected, in contrast to wild-type *hut(P)*, where the majority of the DNA fragments had RNA polymerase bound at *P_C* and only a minority showed binding at the *hutUH* promoter.

A similar result was obtained by in vitro transcription with *hutP104* DNA. The *hutP104* mutation strengthened transcription from *P_{UH}* and abolished transcription from *P_C* (11).

<i>hutP104</i>	T A T A G T
<i>hutP</i> ⁺	T A T A T G T
<i>hutP105</i>	T A T T A T G T
Consensus	T A T A A T

FIG. 2. The sequence surrounding the –10 consensus region of *P_{UH}* from the wild type and from the mutants. In all other regions, the sequence of both mutants was identical to the wild-type sequence. Note that both the deletion generated by *hutP104* and the insertion generated by *hutP105* improve the match to consensus by having a T at the 3' end of the hexanucleotide, as is found in all but 4 of 112 sequenced promoters (9). The wild-type sequence is from Nieuwkoop et al. (10).

The relative positions of the two promoters, *P_{UH}* and *P_C*, within the wild-type *hut(P)* region results in a 4-bp region of overlap (10), strongly suggesting that RNA polymerase bound to one site prevents RNA polymerase from binding to the other site. The observations that the *hutP104* and *hutP105* mutations abolish RNA polymerase binding at *P_C* (Fig. 3) and that *hutP104* abolishes transcription from *P_C* (11) support the argument of mutually competitive binding sites in *hut(P)*. The mutations (at nucleotides 181 or 183) lie more than 20 bp outside the RNA polymerase-binding region at *P_C* (nucleotides 107 to 154) and thus can have no direct effect on RNA polymerase binding at *P_C* (Fig. 4). However, the mutations greatly strengthen the *P_{UH}* binding site (9). Since this improvement in *P_{UH}* prevents binding at *P_C*, it appears that RNA polymerase bound at *P_{UH}* acts as a classical repressor of *P_C* by blocking the *P_C* binding site and thus excluding RNA polymerase from this site. In the wild type, *P_C* is stronger than *P_{UH}*, and so RNA polymerase bound at *P_C* may act as a repressor of *P_{UH}*, the *hutUH* promoter.

A similar argument may explain why the *hutP104* and *hutP105* mutations render *hutUH* expression more resistant to repressor compared with the wild type. If we assume that *hut* repressor and RNA polymerase compete for binding at the *hutUH* promoter, *P_{UH}*, then mutations that increase the affinity of *P_{UH}* for RNA polymerase without a comparable increase in affinity for repressor would make RNA polymerase a better competitor and hence more resistant to repression. Of course, it remains possible that the *hutUH* operator overlaps the –10 region of *P_{UH}* and the two mutations directly affect repressor-operator interactions.

The observation that a tightly bound RNA polymerase at the mutant *P_{UH}* site can repress that *P_C* promoter raises the question of the relative roles of the (stronger) *P_C* and (weaker) *P_{UH}* promoters in the wild type. It is tempting to speculate (11) that, in the wild type, RNA polymerase bound at *P_C* might exert repression on the *P_{UH}* promoter, with CAP-cAMP acting as a kind of double-negative regulator repressing *P_C* and thus passively allowing expression from *P_{UH}* (10). However, the match of the wild-type *P_{UH}* promoter to the consensus sequence is so poor that it is extremely unlikely that the *P_{UH}* promoter can function as an active promoter in the absence of some directly activating factor (CAP-cAMP), whether the putative repression by RNA polymerase bound at *P_C* is lifted or not. Thus, although repression by RNA polymerase can function in the backward direction on the *P_C* promoter in the mutants, and, by symmetry arguments, ought to function in the forward direction, the physiological significance of such an interac-

FIG. 3. Protection of *hutP104* and *hutP105* DNA from exonuclease III digestion. Binding and digestion conditions were described previously (10). Lanes 1 through 5, DNA was 5' end labeled at the *Sal*I site (left end in the drawings in Fig. 1 and 4) to identify the rightmost extent of a binding site; lanes 6 through 10, DNA was 5' end labeled at the *Pvu*II site (right end in the drawings) to identify the leftmost extent of sites. Lanes 4 and 9 contain *hutP104* DNA; lanes 5 and 10 contain *hutP105* DNA. Lanes 1, 2, 3, 6, 7, and 8 are controls with wild-type DNA showing RNA polymerase protection against exonuclease III (lanes 3 and 8), exonuclease III digestion in the absence of RNA polymerase (lanes 2 and 7), and a Maxam-Gilbert A+G reaction (8) as the size standard (lanes 1 and 6). Data for *hutP*⁺ (wild type) have been reported and discussed previously (10) and are shown here only for comparison. The weak signal of *hutP105* at 150 nucleotides (lane 10) is an artefact of this particular experiment. In other gels, both the signal at 150 and the undigested material near the top are equal for *hutP104* and *hutP105*.

tion (if any) remains obscure. In vitro transcription experiments (currently in progress) using DNA templates with *P_C* deleted should help answer this question.

The phenomenon of multiple, overlapping, mutually competitive promoters is by no means rare in the enteric bacte-

FIG. 4. Illustration of the effects of *hutP* mutations on RNA polymerase binding to the *hut(P)* region. The upper drawing represents the strong (frequent) binding of RNA polymerase to the wild-type *P_C* site and the rarer binding of RNA polymerase to *P_{UH}* (10). The small rectangles represent the -10 and -35 sequences within each promoter. The *hutP104* and *hutP105* mutations (at 181 and 183) lie far outside the *P_C* site, yet they result in *P_C* being inactive both in transcription (11) and in RNA polymerase binding. The binding of RNA polymerase to the mutant *P_{UH}* sites is so strong that it almost totally represses RNA polymerase binding at *P_C*.

ria. For example, the *Escherichia coli gal* operon is transcribed for either of two overlapping promoters, one of which, *P₁*, is activated by CAP-cAMP and the other, *P₂*, is repressed by CAP-cAMP (1). The net result of this curious arrangement is to reduce the degree of regulation by CAP-cAMP, making the promoter more or less constitutive. The *E. coli lac* operon also has a CAP-cAMP repressible promoter (*P₂*) overlapping and competing with the CAP-cAMP activated promoter, *P₁* (7). However, *P₂* is inactive in vivo and mutational destruction of *P₂* has no effect on *lac* expression (2), suggesting that *P₂* is at best an evolutionary relic. In bacteriophage lambda, the *Prm-Pr* region contains two overlapping divergent promoters, one of which is activated by the *cI* product at physiological concentrations and the other of which is repressed (13). In this case, the regulatory significance of both promoters is clear; one is required for stable lysogeny, and the other is required for lytic growth.

In short, the phenomenon of multiple, often overlapping, mutually competing promoters is a surprisingly common motif in the enteric bacteria, the regulatory significance of which (either in the present or in evolutionary history) is still not well understood.

This work was supported by Public Health Service grant AI15822 from the National Institutes of Health.

LITERATURE CITED

1. deCrombrughe, B., and I. Pastan. 1978. Cyclic AMP, the cyclic AMP receptor protein, and their dual control of the galactose operon. In J. H. Miller and W. S. Reznikoff (ed.), *The operon*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. Donnelly, C. E., and W. R. Reznikoff. 1987. Mutations in the *lac* P2 promoter. *J. Bacteriol.* **169**:1812–1817.
3. Goldberg, R. B., R. A. Bender, and S. L. Streicher. 1974. Direct selection for P1 sensitive mutants of enteric bacteria. *J. Bacteriol.* **118**:810–814.
4. Goldberg, R. B., and R. Hanau. 1980. Regulation of *Klebsiella pneumoniae* *hut* operons by oxygen. *J. Bacteriol.* **141**:745–750.
5. Goldberg, R. B., and B. Magasanik. 1975. Gene order of the histidine utilization (*hut*) operons in *Klebsiella aerogenes*. *J. Bacteriol.* **122**:1025–1031.
6. Magasanik, B. 1978. Regulation in the *hut* system, p. 373–387. In J. H. Miller and W. S. Reznikoff (ed.), *The operon*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. Malan, T. P., and W. R. McClure. 1984. Dual promoter control of the *Escherichia coli* lactose operon. *Cell* **39**:173–180.
8. Maxam, A. M., and W. Gilbert. 1977. A new method for sequencing DNA. *Proc. Natl. Acad. Sci. USA* **74**:560–564.
9. Mulligan, M. E., D. K. Hawley, R. Entriken, and W. R. McClure. 1984. *Escherichia coli* promoter sequences predict in vitro RNA polymerase selectivity. *Nucleic Acids Res.* **12**:789–800.
10. Nieuwkoop, A. J., S. A. Baldauf, M. E. S. Hudspeth, and R. A. Bender. 1988. Bidirectional promoter in the *hut(P)* region of the histidine utilization (*hut*) operons of *Klebsiella aerogenes*. *J. Bacteriol.* **170**:2240–2246.
11. Nieuwkoop, A. J., S. A. Boylan, and R. A. Bender. 1984. Regulation of *hutUH* operon expression by the catabolite gene activator protein-cyclic AMP complex in *Klebsiella aerogenes*. *J. Bacteriol.* **159**:934–939.
12. Prival, M. J., and B. Magasanik. 1971. Resistance to catabolite repression of histidase and proline oxidase during nitrogen-limited growth of *Klebsiella aerogenes*. *J. Biol. Chem.* **246**:6288–6296.
13. Ptashne, M., A. Jeffrey, A. D. Johnson, R. Maurer, B. J. Meyer, C. O. Pabo, T. M. Roberts, and R. T. Sauer. 1980. How the λ repressor and *cro* work. *Cell* **19**:1–12.
14. Quinto, M., and R. A. Bender. 1984. Use of bacteriophage P1 as a vector for Tn5 insertion mutagenesis. *Appl. Environ. Microbiol.* **47**:436–438.
15. Rothman, N., D. Rothstein, F. Foor, and B. Magasanik. 1982. Role of *glnA*-linked genes in regulation of glutamine synthetase and histidase formation in *Klebsiella aerogenes*. *J. Bacteriol.* **150**:221–230.
16. Streicher, S. L., R. A. Bender, and B. Magasanik. 1975. Genetic control of glutamine synthetase in *Klebsiella aerogenes*. *J. Bacteriol.* **121**:320–331.