JOURNAL OF BACTERIOLOGY, Oct. 2010, p. 4801–4811 Vol. 192, No. 19
0021-9193/10/$12.00 doi:10.1128/JB.00266-10

MINIREVIEW

A NAC for Regulating Metabolism: the Nitrogen Assimilation Control Protein (NAC) from *Klebsiella pneumoniae*[▽]

Robert A. Bender*

Department of Molecular, Cellular, and Developmental Biology, the University of Michigan, Ann Arbor, Michigan 48109

The nitrogen assimilation control protein (NAC) is a LysR-type transcriptional regulator (LTTR) that is made under conditions of nitrogen-limited growth. NAC's synthesis is entirely dependent on phosphorylated NtrC from the two-component Ntr system and requires the unusual sigma factor σ54 for transcription of the *nac* gene. NAC activates the transcription of σ70-dependent genes whose products provide the cell with ammonia or glutamate. NAC represses genes whose products use ammonia and also represses its own transcription. In addition, NAC also subtly adjusts other cellular functions to keep pace with the supply of biosynthetically available nitrogen.

NITROGEN REGULATION

In 1952, Boris Magasanik noticed that a histidine auxotroph of *Klebsiella pneumoniae* (then known as *Aerobacter aerogenes*) required 30 times more histidine for growth in minimal medium (rich in ammonium salts) when inositol was supplied as the carbon source than it did when glucose was supplied (76). This led to the double assumption that there must be an enzymatic pathway to catabolize histidine and that the expression of this pathway must be repressed by glucose. A series of publications in the 1950s proved both of these conclusions correct and demonstrated both the histidine utilization enzymes encoded by the *hut* operons (36, 43, 64) and their repression by glucose (40, 52, 53). In pursuing the repression, Magasanik coined the term catabolite repression to describe the absence of the enzymes responsible for catabolizing a poorer carbon source when a more effective carbon source is being catabolized (41). Histidine can be used as the sole source of carbon, but growth is slower than that with glucose as the carbon source. Inositol is also a poor carbon source, so less repression of the histidine degrading enzymes is seen when inositol replaces glucose. Histidine can also serve as a nitrogen source for *K. pneumoniae* when a poor carbon source (like inositol) is present, with two of its nitrogen atoms available for incorporation into cellular material (40, 43). Thus, it was predicted that *K. pneumoniae* would be able to use histidine as a sole nitrogen source only when glucose was absent. However, surprisingly, *K. pneumoniae* grows well in glucose minimal medium with histidine as the nitrogen source, and the enzymes of histidine degradation are derepressed under these conditions (53).

In 1956, Neidhardt and Magasanik (53) showed that the *hut* enzymes could be derepressed in glucose minimal medium, but only if ammonium was absent. They showed that either nitrogen limitation (N limitation) or carbon limitation would allow expression of the *hut* enzymes. Moreover, the derepression in response to N limitation did not result from elimination of catabolite repression. In fact, in glucose minimal medium, N limitation leads to an increased catabolite repression of traditional indicators like β-galactosidase and tryptophanase (63). This phenomenon was termed "relief of catabolite repression" and appeared to be specific for pathways that yielded ammonium or glutamate and to be independent of the effects of CRP-cAMP.

But the exploration reached a dead end at that point. There was no system of genetic exchange characterized for *K. pneumoniae* at that time. And the two enterobacteria with good genetic systems, *Escherichia coli* and *Salmonella enterica* (then called *S. typhimurium*), were unsuitable. *E. coli* lacks the *hut* operons and cannot catabolize histidine under any growth condition. *S. enterica* has a *hut* system, but in *S. enterica* the catabolite repression of *hut* is not relieved by nitrogen limitation (8, 45), and thus, *S. enterica* cannot use histidine as the sole source of nitrogen when glucose is the carbon source. So, in the absence of good genetics, the question of how *hut* could be derepressed by nitrogen limitation lay unanswered for a dozen or more years.

THE Ntr SYSTEM

The discovery of a transducing phage for *K. pneumoniae* (38) and the subsequent adaptation of the coliphage P1 tools for use with *K. pneumoniae* (17) allowed a reopening of the study of this nitrogen regulatory effect (63). A study of *K. pneumoniae* glutamine auxotrophs and their revertants (7, 62) led ultimately to the discovery of the Ntr system. The Ntr system has been reviewed extensively elsewhere (42, 61), and a detailed discussion of it is beyond the scope of this review. A few basic features of the Ntr system (Fig. 1) are pertinent to this discussion. (i) The Ntr system is a two-component regulatory system in which the transcriptional regulator, NtrC, is phosphorylated to its active form by NtrB under conditions of N-limited growth (30, 56). (ii) The signal for nitrogen limitation is a low intracellular pool of glutamine (3, 25). (iii) NtrC~P activates transcription by RNA poly-

* Mailing address: Department of Molecular, Cellular, and Developmental Biology, the University of Michigan, Ann Arbor, MI 48109-1048. Phone: (734) 975-1776. Fax: (734) 647-0884. E-mail: rbender@umich.edu.

▽ Published ahead of print on 30 July 2010.

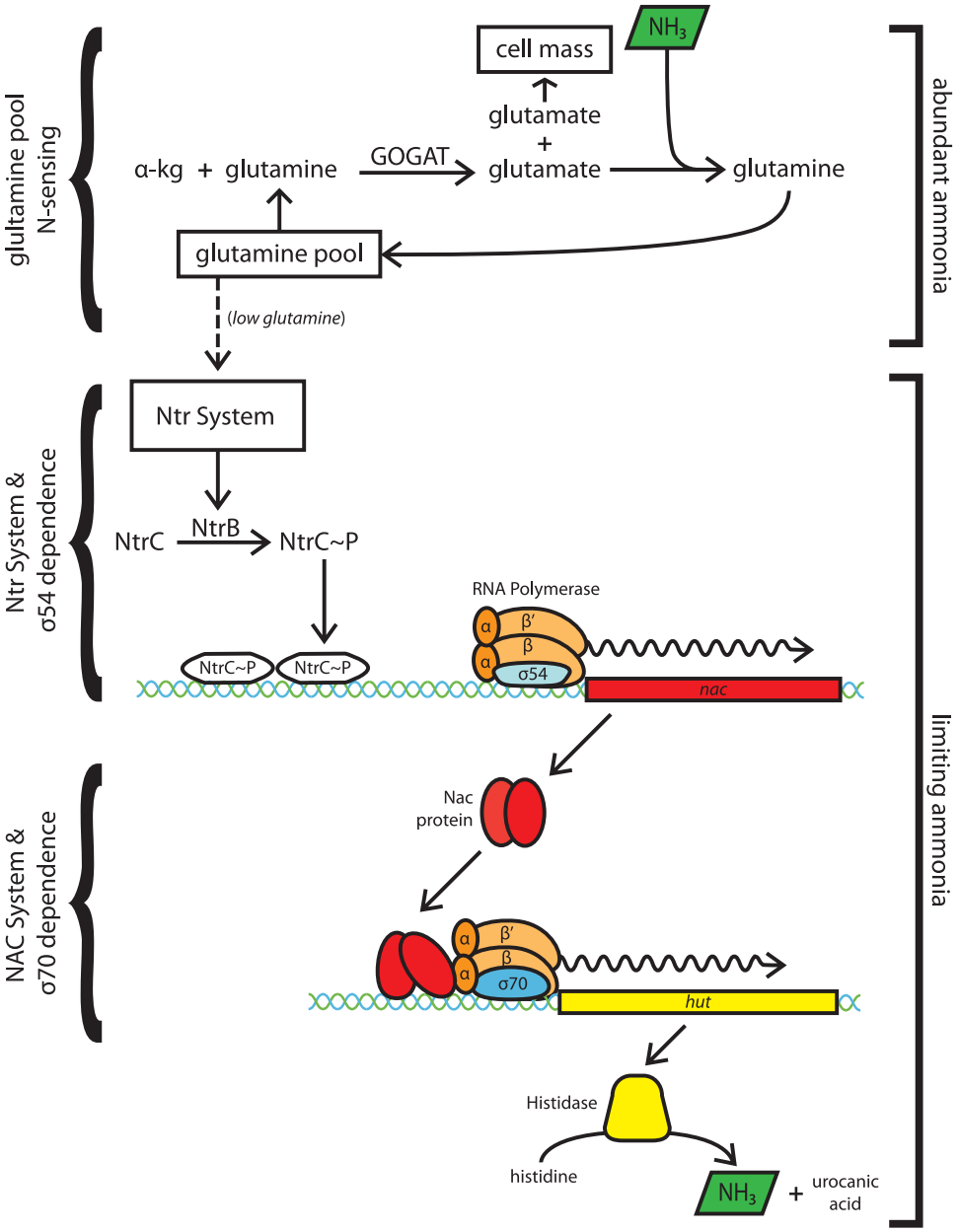

FIG. 1. A cartoon illustrating the nitrogen regulation of the histidine utilization (*hut*) genes of *K. pneumoniae*. Step one: under conditions of ammonia limitation, the intracellular pool of glutamine is filled by the action of glutamine synthetase and depleted by the action of glutamate synthase (GOGAT). The glutamine pool is also depleted by the biosynthetic needs of the cell, both directly and by incorporation of glutamate into cellular material. Step two: when the intracellular pool of glutamine is low, the *glnD* and *glnB* products (not shown) signal NtrB to phosphorylate (activate) NtrC to NtrC~P. NtrC~P activates transcription of the *nac* gene by RNA polymerase bearing the unusual sigma factor σ54. Step three: the NAC protein activates transcription of the *hutUH* operon by RNA polymerase bearing the "housekeeping" sigma factor σ70. The products of the *hut* operons cleave histidine to generate ammonia, which is then assimilated into glutamine, restoring the cycle. (Urocanic acid, the other product of histidine cleavage, is further degraded to glutamate by the remaining *hut*-encoded enzymes.)

merase bearing the unusual sigma factor σ54 (23, 24), which recognizes an unusual, GC-rich promoter sequence (22) that is not recognized by σ70.

K. pneumoniae is a "nitrogen generalist," capable of using a wide range of organic and inorganic compounds as its sole source of nitrogen (75). The metabolism of nearly all of these alternative N sources is controlled in response to N limitation, and this N regulation requires the Ntr system in almost every case. This regulation by the σ54-dependent Ntr system can be direct, as it is for the *glnA* gene, or indirect, as it is for the *hut* (histidine utilization) operon.

DISCOVERY OF NAC

As part of a study of nitrogen fixation in a related *Klebsiella* strain, Valentine and his coworkers isolated the first true ni-

trogen regulatory mutation, which they called *asm-1* (51). This mutation was in the *gltBD* operon, which encodes GOGAT (glutamate synthase), one of the two enzymes that allow *de novo* synthesis of glutamate from α-ketoglutarate (44, 74). These *gltBD* mutants were unable to activate expression of the *nif* (nitrogen fixation) or *hut* operon in response to nitrogen limitation (51). Similar *gltB* mutants that could not activate *hut* or *put* expression were then identified in *K. pneumoniae* (7). These mutants were still able to activate *hut* in response to carbon limitation, and thus, the defect seemed to be specific to N regulation. The reason for the inability to activate *hut* expression was not known at that time (21), but revertants that could activate *hut* expression were easily obtained, and these were constitutively active for *hut* expression, even in the presence of excess ammonia (7). The mutations responsible for the constitutive expression of *hut* were mapped to the *ntrB* gene (69), tightly linked to *glnA*, the structural gene for glutamine synthetase (73). Unexpectedly, these revertants were unable to grow in glucose minimal medium with ammonia as the sole source of nitrogen (7). This inability was caused by a repression of the *gdhA* gene, encoding the enzyme glutamate dehydrogenase, the only enzyme other than GOGAT that can effect a net assimilation of ammonia into glutamate. Once again, revertants that could grow with ammonia as the sole nitrogen source were easily isolated. These revertants were constitutive for *gdhA* expression and constitutively repressed for *hut* expression (4). The mutations responsible for some of the revertants were located in *ntrC*, again tightly linked to the *glnA* locus, but the majority defined a new locus called *nac.*

Under conditions of N limitation, *K. pneumoniae nac* mutants are still able to activate expression of *glnA* and a variety of other N-regulated genes (e.g., *nif*, *nas*, a catabolic asparaginase gene, and a tryptophan permease gene) but are not able to activate expression of several others, e.g., *hut*, *put*, and *ure* (4, 37). Even when the Ntr system was constitutively active (due to an *ntrB* mutation), *nac* mutants could not activate this subset of nitrogen-regulated genes. The glutamate dehydrogenase (GDH) gene, *gdhA*, also fell into the class of NAC-regulated genes, but in the opposite direction. GDH was expressed at high levels in the absence or presence of ammonia in *nac* mutants (4, 37). Operons whose regulation did not require NAC (*glnA-ntrB-ntrC* and *nifLA*) had σ54-dependent promoters, as expected for Ntr-regulated genes (22, 42). However, operons whose regulation did require NAC (*hutUH* and *gdhA*) had σ70-dependent promoters (28, 54), which should not be recognized by the Ntr system. Studies with *nac-lacZ* fusions showed that *nac* was itself regulated by the Ntr system (37). This was confirmed by *in vitro* transcription using purified components of the Ntr system (11). Finally, experiments with mutants that put the *nac* gene under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter proved that *nac* expression was both necessary and sufficient for activation of *hutUH* and repression of *gdhA* expression *in vivo* (72).

Thus, the basic picture of N regulation in *K. pneumoniae* (Fig. 1) was understood (1): in the absence of the preferred N source (ammonia), the glutamine pool falls and leads to activation of the Ntr system and the consequent phosphorylation of the NtrC protein. NtrC~P activates expression of a number of genes, all of which are dependent on σ54. One of those genes is *nac*. NAC in turn activates or represses expression of a number of operons, whose expression is dependent on the normal sigma factor, σ70. One of those operons is *hutUH*.

SCOPE OF NAC REGULON

A survey of related enterobacteria showed that *E. coli* has a *nac* gene but *S. enterica* does not. The location of *nac* between two direct repeats of an asparaginyl tRNA gene explains the origin of the *nac-1* mutation of *K. pneumoniae* and the lack of a *nac* gene in *S. enterica*. In both cases, there is only one of those Asn-tRNA genes, and the genes between them are either missing or located elsewhere on the chromosome (20). This explains why the catabolite repression of *hut* in *S. enterica* is not relieved by N limitation even though it has a functioning Ntr system.

Genetic and physiological studies of individual catabolic pathways led to an initial list of 8 operons regulated by NAC in *K. pneumoniae* (Table 1). A comparable survey for *E. coli* identified 4 operons. Zimmer et al. (78) used an *E. coli* microarray to examine the scope of the NAC regulon and found 9 operons whose regulation depends on NAC. As expected, each of the operons whose products are known is involved in N metabolism. We used chromatin immunoprecipitation (ChIP) to identify potential NAC-regulated genes in *K. pneumoniae* and found at least 89 unique DNA regions that bound NAC (16). Primer extension assays with 16 of these genes showed that 15 required NAC for their regulation. (The 16th gave no signal under the growth conditions tested.) Of the genes for which a function was known or could be inferred, about half were clearly related to N metabolism. The remaining genes affect the mobilization of carbon sources, the synthesis of macromolecules, and cell division. In other words, the NAC regulon of *K. pneumoniae* is large and broad, that of *E. coli* is much smaller and may be limited to nitrogen metabolism, and *S. enterica* lacks a *nac* gene. However, it should be noted that the *hutUH* operon of *S. enterica* retains the sites needed for regulation by NAC. If its *hutUH* operon is transferred to *K. pneumoniae*, it is regulated by nitrogen (63). Conversely, transfer of a cloned *nac* gene from *K. pneumoniae* to *S. enterica* allows relief of catabolite repression in response to N limitation (5).

The differences in the size of *K. pneumoniae*'s NAC regulon and that of *E. coli* or *S. enterica* probably reflect the different ecology of *K. pneumoniae. K. pneumoniae* is at best a minor component of the human gut and, despite its fearsome species name, is merely an opportunistic pathogen. It contrast to *E. coli* and *S. enterica*, *K. pneumoniae* survives and thrives in soil and aquatic environments, where nutrient limitation, especially N limitation, would favor an expanded repertoire of pathways that could be expressed when needed. It is telling that several of the genes that are regulated by NAC in *K. pneumoniae* are either not regulated (26) or weakly regulated (48) in *E. coli*. In at least one case (26), this loss in *E. coli* resulted from a single base pair change in an otherwise recognizable activation consensus sequence. Thus, it appears that the *E. coli* set of NAC-regulated genes is shrinking while that of *K. pneumoniae* may be expanding. *S. enterica* has lost NAC entirely but still contains remnants of a NAC regulon in that *hutUp* of *S. enterica* carries all the information for regulation by NAC. Of course, it is possible that *S. enterica* acquired *hut* recently from a NAC-containing source. However, the location of *hut*, between the *gal* and *bio* operons, is identical in *K. pneumoniae* and *S. en-*

TABLE 1. Evidence for NAC regulation of gene expression

Category and operon	Function	Reference(s) for evidence							
		In vivo				*In vitro*			
		Enzyme assay[a]	*lacZ* fusion[b]	Primer extension[c]	NBS mutant[d]	EMSA[e]	DNase I footprint[f]	Transcription[g]	ChIP[h]
Activated by NAC									
hut	Histidine → NH_3 + glutamate + formamide	4, 3			60	19	19	19	
put	Proline permease	37			9	9	19		
ure	Urea → NH_3 − CO_2	37	33	16, 33	15, 33	15, 68	19		16
cod	Cytosine → NH_3 + uracil	50	50		50	50, 66	50	50	16
dad	Alanine → NH_3 + pyruvate	26		26		26			16
folA	Dihydrofolate reductase (thymine synthesis)			16					16
gyrA	DNA gyrase			16					16
ldcA	L,D-Carboxypeptidase (murein integrity)			16					16
oppA	Oligopeptide transport			16					16
rpmI	Ribosomal protein L35			16					16
ybjI	(Putative lipoprotein)			16					16
yceO	(Biofilm and acid stress)			16					16
Repressed by NAC									
gdhA	NH_3 + αkg[i] → glutamate	4, 37	20	28	20	20, 29, 68	20		16
gltB	Glutamine + αkg[i] → glutamate	4, 21							
nac	Transcription regulation		37	11		12, 66, 67	12, 67	12	
argP	Transcription regulation			16					16
holE	DNA replication			16					16
secY	Protein secretion			16					16
glgX	Glycogen debranching			16					16
ygdQ	(Putative transport protein)			16					16
yobA	(Unknown)			16					16

[a] An enzyme encoded by the operon was assayed in a *nac*$^+$ and *nac* mutant background.
[b] A promoterless *lacZ* gene was fused to the promoter of the operon, and β-galactosidase expression was measured in a *nac*$^+$ and *nac* mutant background.
[c] Primer extension was performed using RNA extracted from cells grown in excess or limiting nitrogen or from *nac*$^+$ and *nac* mutant cells grown in limiting nitrogen.
[d] A mutation was introduced into the NAC-binding site (NBS) as determined by DNase I footprinting. The enzyme activities of the mutant and wild type were compared under conditions of NAC-regulated expression.
[e] EMSAs were performed with purified NAC and DNA fragments containing the NBS.
[f] DNase I protection of the NBS by purified NAC.
[g] *In vitro* transcription with purified components.
[h] Chromatin immunoprecipitation using purified anti-NAC antibody.
[i] αkg, α-ketoglutarate.

terica, arguing that *hut* is ancestral to both. Although NAC has allowed *K. pneumoniae* to coopt many operons into the N limitation response, NAC is not the only path to a rich metabolic diversity of nitrogen-yielding pathways. The pseudomonads can use a very large number of N sources, and yet no true homolog of NAC has been found (77).

One final observation about the *E. coli nac* gene deserves comment. Although *E. coli nac* appears to complement *K. pneumoniae nac* mutants completely (48), there is a surprising divergence of sequence between these two homologous genes. Many *E. coli* proteins are 90 to 95% identical to their *K. pneumoniae* homologs in their amino acid sequence. The *E. coli* and *K. pneumoniae* NAC proteins are only 79% identical in amino acid sequence (48). However, the *E. coli* and *K. pneumoniae* NAC proteins are more than 90% identical in the N-terminal one-third of the protein, the portion that is sufficient for binding to DNA and activating transcription (49, 68). This divergence in the C-terminal domain of NAC has its explanation in the structure of NAC.

STRUCTURE

NAC is a LysR-type transcriptional regulator with strong similarity to other LysR-type transcriptional regulator (LTTRs). For example, the N-terminal domain of NAC (amino acids 1 to 100) is 39% identical in amino acid sequence to OxyR from *E. coli* and 15% identical in the C-terminal domain (71). So, it is not surprising that NAC shares many of the properties common to most LTTRs (39). NAC is a tetramer in solutions at concentrations above 7 μM (68) and dissociates into dimers at lower concentrations, consistent with the general "dimer of dimers" structure of LTTRs. Like most LTTRs, NAC is quite insoluble, though the *K. pneumoniae* NAC (but not the *E. coli* NAC) is soluble in high salt (19). This differential solubility provided a simple protocol for purification of the *K. pneumoniae* NAC so that both native NAC and C-terminally His-tagged NAC can be isolated with ease. Finally, NAC regulates expression of its own gene (see below), though there is no oppositely directed gene that NAC activates (20, 37), as is the case with many LTTRs whose regulons are more limited (39, 70).

However, NAC differs from the generic LTTR (39, 70) in several key ways. NAC dimers activate transcription at most of the well-characterized promoters (68). Most LTTRs use tetramers to activate transcription. Most LTTRs bind a physiologically relevant coeffector (or undergo a covalent modification) to regulate their function in response to environmental signals. NAC's function is regulated solely at the level of synthesis. NAC is active whether purified from cells grown under

N excess or N limitation (19). NAC's activity *in vitro* is unchanged by likely candidates for coeffectors such as α-ketoglutarate, glutamate, glutamine, and ammonia (19). If NAC is produced *in vivo* from an IPTG-inducible promoter, NAC-dependent gene expression responds to IPTG and not to the nitrogen status of the cell (72). Taken together, these *in vivo* and *in vitro* data argue that the NAC polypeptide (as a dimer or as a tetramer) is necessary and sufficient for regulation of NAC-dependent genes.

The C-terminal domain of most LTTRs contains the sites of coeffector binding, for formation of tetramers from dimers, and for conformational rearrangement of the dimers within the tetramer in response to coeffector binding (39, 70). Thus, it is not surprising that this domain is dispensable for many (though not all) of NAC's functions. Substitution mutants of NAC (NAC^{L111K} and NAC^{L125R}) have been isolated that cannot form tetramers and remain as dimers (68). These mutants activate transcription of *hut* and *ure* about as well as wild-type NAC (NAC^{WT}). In addition, truncation mutants of NAC with only the 125, 100, or even 86 N-terminal amino acids have been isolated. These are dimers in solution and activate as well as NAC^{WT} wherever tested (49, 68). But the C-terminal domain does have a function: tetramer formation, which is essential for the repression of *gdhA* (20, 49, 68) and is also part of the regulation of *codBA* (50) and *nac* (12, 66) as described below.

NAC-ACTIVATED PROMOTERS

The *hutUH* promoter, *hutUp*. At *hutUp*, a dimer of NAC (68) binds to a site centered at −64 relative to the transcription start site (19) and activates transcription of *hutUp* by RNA polymerase bearing σ70 (19). The promoter activated by NAC is the same promoter that is used for basal expression of *hutUp* in the absence of activators. This same promoter is also activated by CRP-cAMP (58), which has binding sites centered at −81.5 and −41.5 relative to the transcription start site (58). In all three cases (basal expression, NAC-mediated activation, and CRP-cAMP-mediated activation), RNA polymerase bearing σ70 is used (19, 55, 58). The common question of whether the two positive activators of *hutUp*, NAC and CRP-cAMP, would act synergistically or antagonistically is moot, since these two activators would never be present in the same cell at the same time.

Several lines of evidence establish that the dimer is the active form of NAC at *hutUp*. The extent of the mobility shift in an electrophoretic mobility shift assay (EMSA) is consistent with a dimer, as is the 28-bp size of the DNase I footprint (19, 68). In addition, NAC mutants that cannot form tetramers (NAC^{L111K}) are fully active at *hutUp* (29, 68). Truncated versions of NAC (NAC^{100ter} and NAC^{86ter}) containing only the 100 or 86 N-terminal amino acids of NAC are fully active at *hutUp in vivo* (49, 68). Thus, the N-terminal domain of NAC is sufficient for NAC activation at *hutUp*. The C-terminal domain plays no known role at *hutUp*.

The nature of the interaction of NAC with RNA polymerase is not known. Genetic analysis of a related LTTR, CysB, identified a positive control (PC) mutant (Y27G, affecting amino acid 27) that still binds normally to DNA but fails to activate transcription of the *cysP* operon (34). Those studies showed that mutations affecting amino acids K271 and E273 of the alpha subunit of RNA polymerase also showed defects in activation by CysB (35). In addition, a two-hybrid assay showed an interaction between a domain of CysB and the alpha subunit of RNA polymerase that is not found with the Y27G substitution of CysB (35). This has suggested a necessary interaction between Y27 of CysB and the "273 determinant" of the alpha subunit as the key to transcriptional activation. A comparable PC mutant of NAC, NAC^{H26D}, has been isolated and shown to have a PC phenotype (67), so there is reason to suspect that many of the features of activation by LTTRs may be generalizable. However, it seems likely that H26 of NAC plays a more complex role in activation (15, 67).

NAC^{H26A} is wild type for activation of *hutUp*, and several other drastic substitution mutants (e.g., NAC^{H26K} and NAC^{H26Y}) also retain a substantial ability to activate *hutUp* (67). NAC activates *ureDp* from a site centered at −47, overlapping the RNA polymerase binding site and on the opposite face of the DNA helix from the site at *hutUp* (33), and yet NAC^{H26D} shows a positive-control phenotype at this promoter as well (67). It seems unlikely that H26 could make the same kinds of contact with RNA polymerase in both cases. So, we favor a model where H26 plays an important role in allowing NAC to undergo the DNA-mediated conformational change that is required for allowing it to activate transcription.

The interaction of NAC with its DNA site is better understood. NAC's 28-bp DNase I footprint is centered on a 15-bp symmetric sequence, ATA-n9-TAT (19). Genetic analysis of the 5 or 6 bp flanking this consensus suggests that they do not provide specific information for binding or activation (60). A more careful analysis of the 15-bp core, including both mutational analysis and comparison with other known NAC-binding sites (NBS), suggests that the critical nucleotides in this more detailed "activation consensus" are ATAA-n5-TnGTAT (19, 60). The asymmetry of this sequence is critical to NAC's ability to activate *hutUp* expression (60). Inverting the 15-bp site from ATA ACA AAA TTG TAT (the wild type) to ATA CAA TTT TGT TAT leaves NAC's binding and DNase I footprint intact but severely reduces activation. A site with symmetric hexamers and based on the promoter-proximal half-site (ATA CAA AAA TTG TAT) bound NAC weakly but still showed activation proportional to the reduced binding. A symmetric site based on the promoter distal half-site (ATA ACA AAA TGT TAT) bound NAC better than the wild-type site but was severely defective for activation.

At *hutUp*, binding of NAC is not sufficient for activation. The sequence of key nucleotides determines whether a bound NAC dimer will activate transcription or not (60). In other words, the DNA binding site is an allosteric effector for NAC. NAC bound to some sites is in an inactive conformation; NAC bound to other sites is active. This agrees with a pattern seen in other LTTRs where the dimers within the tetramers play different roles in mediating activation of transcription (39, 70). One of the dimer-binding sites used by such a tetramer functions in binding but lacks some information that allows a bound protein to activate transcription. The other dimer-binding site used by this tetramer carries information necessary for activation as well as binding.

The *ureDABCEFG* promoter, *ureDp*. The *ureDABCEFG* operon of *K. pneumoniae* encodes an N-regulated urease that provides the cell with ammonia from urea (13). The *ureA*, *ureB*,

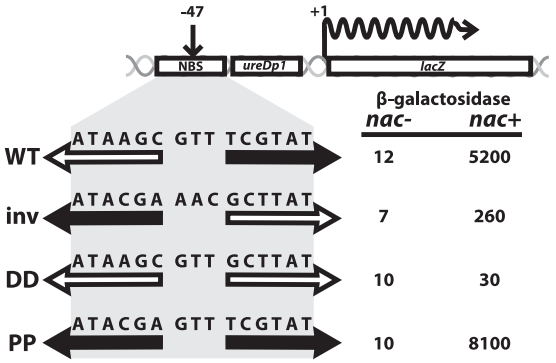

FIG. 2. Sequence specificity and directionality of the NAC-binding site (NBS) within the *ureD* promoter. The upper drawing illustrates the position of the NBS at −47 relative to the start of transcription of a fusion of a promoterless *lacZ* gene to the nitrogen-regulated *ureDp1* promoter. The wild-type (WT) sequence of the NBS yielded 433 times as much β-galactosidase expression in a *nac*$^+$ strain as in a *nac* mutant strain. Inverting the 15-bp core of the NBS (inv) greatly reduced the ability of NAC to activate expression of the fusion. An alteration of the NBS that increased the symmetry of the core by making an inverted repeat based on the promoter-distal half-site (DD) virtually eliminated the ability of NAC to activate expression of the fusion. The converse alteration that increased symmetry based on the promoter-proximal half-site (PP) made the effect of NAC on expression even greater than that in the wild type. All four NBS sites shown here bound NAC well *in vitro*.

and *ureC* genes encode the subunits of the apoenzyme, and the other *ure* genes are involved in the insertion of a required nickel atom to generate an active holoenzyme (32). In contrast to the well-studied urease from *Proteus* spp., whose formation requires the presence of urea, the urease from *K. pneumoniae* is regulated solely by N limitation (46). The *ureDp* region contains a weakly regulated promoter (*ureDp2*), which I shall ignore here, and the NAC-dependent *ureDp1* promoter (33).

Transcription from *ureDp1* is the most tightly regulated of all the characterized NAC-dependent promoters. In fact, in the absence of NAC, β-galactosidase expression from a *ureDp1-lacZ* fusion (Fig. 2) was barely detectable, making *ureDp1* at least 300- to 500-fold inducible by NAC (33). NAC-mediated activation of *ureDp1* shares several features with that of *hutUp*. A dimer of NAC binds to a region near the promoter and protects a 28-bp region of DNA with an "activation consensus" site (ATAA-N5-TnGTAT) at its center, the same activation consensus found at *hutUp* (19). NAC^{L111K}, NAC^{86ter}, and NAC^{100ter}, which cannot form tetramers, activate *ureDp1* about as well as NAC^{WT} (68). The asymmetry of the NBS is important (15). Inverting the site nearly abolishes the ability of NAC to activate *ureDp1* (Fig. 2). A symmetric site based on the promoter-proximal half-site is even more effective than the native site. In contrast, a symmetric site based on the promoter-distal half-site is totally inactive, in spite of the fact that all four types of sites bind NAC well (15).

However, the *ureDp1* promoter differs from *hutUp* in several important features. The NBS at *ureDp1* is centered at −47 relative to the start of transcription (33), and its DNase I footprint extends from −37 to −60 (19), overlapping the RNA polymerase binding site and on a different side of the helix (relative to RNA polymerase) than the NBS at *hutUp*. In addition to being the most NAC-dependent of the NAC-regulated promoters, *ureDp1* is also the most NAC sensitive. When *nac* expression was titrated by varying the IPTG concentrations for an IPTG-inducible *nac* gene construct, we found that urease expression was fully induced at NAC levels that did not induce histidase at all (72). This was also evident from unpublished EMSA experiments with mixtures of *ureDp* and *hutUp* fragments. The *ureDp* fragments were shifted at lower concentrations of NAC than were the *hutUp* fragments. It bears repeating that most of the NAC-regulated promoters we have studied show considerable basal (NAC-independent) expression, and NAC-mediated activation results in a 3- to 10-fold increase in expression (37). For *ureDp1*, there is no detectable expression in the absence of NAC. Unfortunately, early (unsuccessful) attempts to show NAC-mediated activation of *ureDp* were not resumed (19). Thus, *hutUp* from *K. pneumoniae* and *codBp* from *E. coli* remain the only promoters whose NAC regulation has been confirmed *in vitro* with purified components.

THE *codBA* OPERON

K. pneumoniae has two cytosine deaminase genes, one of which is part of a *codBXA* operon that encodes a putative cytosine permease (*codB*), the cytosine deaminase (*codA*), and a gene of unknown function (*codX*). The other cytosine deaminase (*codA2*) is a standalone gene (50). Both of these appear to be regulated by NAC. Experiments using sensitivity to 5-fluorocytosine as an indicator of *codA* expression suggest that cytosine deaminase expression in *K. pneumoniae* is activated in a NAC-dependent manner (50). Cloned *codBXA* from *K. pneumoniae* was transferred to an *E. coli cod* deletion mutant, and the cytosine deaminase expression of the resulting strain was derepressed about 5-fold in response to N limitation (50). Further explorations of NAC-mediated regulation of cytosine deaminase expression were done using the better-characterized *codBA* operon from *E. coli.*

Plasmid-borne *cod-lac* fusions show a NAC-dependent derepression of about 3- to 5-fold. This recapitulates the 3-fold derepression of cytosine deaminase expression by N limitation (50). *In vitro* transcription from a supercoiled plasmid containing the *codB* promoter showed a NAC-dependent activation of the *codBp*-driven transcript (50). Thus, NAC is both necessary and sufficient for activation of *codBp* in response to N limitation.

Both *hutUp* and *ureDp* are activated by NAC dimers. However, both EMSA and DNase I footprinting have shown that NAC binds as a tetramer at *codBp* (50). In contrast to the 26- to 28-bp footprint seen at *hutUp* or *ureDp*, the NAC footprint at *codBp* is about 56 bp, with a single hypersensitive site in the middle (50). The promoter-proximal half of the footprinted region contains a standard NBS that is able to bind NAC dimers in the absence of the rest of the region. The promoter-distal half of the footprinted region cannot bind a NAC dimer on its own, but the presence of an ATA triplet (half of a NBS consensus) is necessary for tetramer binding to the complete site. The length of the DNase I footprint, the mobility in gel shift assays, and the minimal amount of hypersensitivity all suggest that the NAC tetramer bound at *codBp* has a conformation similar to that of a typical LTTR in its active form, the

form with the two dimers binding the DNA with little or no space between them (66).

Mutational analysis showed that the promoter-proximal NBS at *codBp* is essential for activation (50). However, deletion of the promoter-distal NBS fragment within the region, resulting in dimer binding as determined by gel shifts, only slightly reduced the ability of NAC to activate a *cod-lac* fusion on a plasmid (50). Thus, although a NAC tetramer appears to be more effective, it appears that a NAC dimer can also activate *codBp.*

The regulation of *codBA* is complex. Cytosine deaminase provides the cell with ammonia (for general biosynthesis) but also with uracil (on the salvage pathway that leads to UTP and CTP). Expression of *codBA* is regulated by PurR in response to purine levels, and this regulation appears to be independent of NAC (31). Expression of *codBA* is also regulated by the stringent response, with a strong repression mediated by ppGpp and DksA (14). In response to severe N limitation, DksA-mediated repression and NAC-mediated activation counteract each other. In a DksA deletion, *codBA* expression is high and NAC provides little or no further activation. Thus, NAC's role appears to allow the cells to extract ammonia from cytosine, even when the stringent response signals that there is no need for uracil (i.e., pyrimidines) for RNA synthesis.

THE *lac* OPERON

The wild-type *lacZYA* operon of *E. coli* is not activated by N limitation. In fact, it is subject to an especially strong catabolite repression under these conditions (63). When the NBS from the *K. pneumoniae hut* operon (NBS^{hutU}) was inserted into the *lacZp* region, β-galactosidase expression was derepressed about 13-fold by N limitation (59). Primer extension analysis showed that most of the increased expression came from the principal *lacZ* promoter, *lacZp1* (59). However, a second minor *lacZ* promoter, *lacZp2*, was also activated. NBS^{hutU} in this construct was centered at −64 and −42 relative to the start of transcription from *lacZp1* and *lacZp2*, respectively. When the NBS^{hutU} was moved 5 bp farther from *lacZp*, another minor promoter, *lacZp3*, was activated, but *lacZp1* and *lacZp2* were not. When NBS^{hutU} was moved another 5 bp away, only *lacZp2* was activated. A pattern emerged from these data, with NAC activating when NBS^{hutU} was 42, 52, 54, or 64 bp from the start of transcription but not when at 47, 49, 59, or 74 bp from the start of transcription. In other words, NAC activated *lacZp* when NBS^{hutU} was on the correct side of the DNA helix and not too far away (59). It is curious that the NBS at *ureDp1* is located at −47, and yet *ureDp1* is the most responsive of all the NAC-dependent promoters.

This suggested that there may be nothing special to distinguish those promoters that are activated by NAC from those that are not. This also suggested that the position of the NBS is variable but not infinitely so. The NBS from the *gdhA* promoter, where NAC represses transcription, contains the "repression consensus" of ATA-n9-GAT (see below), which differs slightly from the activation consensus discussed thus far. When NBS^{gdhA} was used instead of NBS^{hutU}, no activation was seen (59). Thus, inverting an NBS or using an NBS from a repression site eliminates NAC activation, suggesting that the information needed for activation of transcription includes the sequence, direction, and location of the NBS.

THE *dadAB* OPERON

The *dadAB* operon (*dadAX* in *E. coli*) is responsible for L-alanine catabolism in *K. pneumoniae* (26). The *dadB* gene encodes an alanine racemase, and *dadA* encodes the small subunit of D-amino acid dehydrogenase. The regulation of *dadAB* in *K. pneumoniae* is complex. It is both induced (in the presence of alanine) and repressed (in the absence of alanine) by the leucine-responsive protein, Lrp (27). As a result, cells grown with alanine added to glucose minimal medium have 25 to 30 times as much racemase and dehydrogenase as cells grown in glucose minimal medium without alanine. The *dadAB* operon is also weakly activated (about 2-fold) by CRP-cAMP (26). Neither the *E. coli* nor the *S. enterica dad* operon shows regulation by nitrogen (26). However, the *K. pneumoniae dad* operon is activated by NAC in response to N-limited growth.

Studying the nitrogen regulation of *dadAB* in *K. pneumoniae* was initially complicated by the fact that alanine (both the L and D isomers) strongly inhibits glutamine synthetase activity (2, 26). The resulting glutamine starvation leads to derepression of the entire Ntr system, including NAC expression (Fig. 1). By using *nac* and *ntr* mutants and adding glutamine to the growth medium, it was possible to identify the role of NAC in regulating *dadAB* expression (26). In the absence of alanine (i.e., *dad* repressed by Lrp), artificial induction of *nac* raised *dad* expression about 5-fold. In the presence of alanine, (i.e., *dad* activated by Lrp), constitutive expression of the Ntr system (and NAC) raised *dad* expression about 3-fold. Thus, the addition of alanine to glucose minimal medium has three physiological effects. (i) It lifts the Lrp-mediated repression of *dad*. (ii) It brings about the Lrp-mediated activation of *dad*. (iii) It induces the Ntr system, resulting in NAC-mediated activation of *dad.* Taken together, these effects result in a 50-fold increase in *dad* expression.

NAC-dependent gel shifts of the *dadAp* region and inspection of the DNA sequence of the *dadAp* region suggest that NAC binds to *dadAp* as a dimer (26), though DNase I footprints have not yet confirmed this. The NAC-activated transcript has the same start site as the Lrp-activated one (26), and there is an NBS consensus (ATAA-n5-CnGTAT) that differs by only 1 bp from the activation consensus and is centered at −44 relative to this start site. The *dadAB* regions of *K. pneumoniae* and *E. coli* are nearly identical from −56 to +23 except for the region of the putative NBS. In particular, ATA-n9-TAT of *K. pneumoniae* is replaced by ATA-n9-CAT (26). This explains why NAC fails to bind to the *E. coli dadAp* region *in vitro* and why there is no activation of *E. coli dad* expression *in vivo*.

THE *put* OPERON

The *putP* and *putA* genes of *K. pneumoniae* are a pair of divergently transcribed genes that encode a proline permease and a bifunctional proline oxidase/pyrroline-5-carboxylic acid dehydrogenase, respectively (10). Proline oxidase expression is elevated by N limitation (63), and this elevation is NAC dependent (37). NAC binds to and protects a 25-bp site in the regulatory region between *putP* and *putA* (19). This site con-

tains the NBS of ATA-n9-TAT, and genetic analysis showed that these are important for NAC binding both *in vivo* and *in vitro* (9). Primer extension studies show strong derepression of the *putP* transcript in response to N limitation (10) and suggest that the regulation of proline oxidase expression from *putA* may be indirect. In addition to its two enzymatic functions, the PutA product also represses transcription of the *put* genes in the absence of proline (47). Thus, an increase in proline transport (caused by NAC) might be sufficient to cause derepression of *putA* expression.

OTHER ACTIVATED GENES

ChIP experiments using anti-NAC antibody identified 89 unique DNA regions that are candidates for NAC-mediated regulation (16). Regulation of 15 of these was tested by comparing primer extension products from nac^+ and *nac* mutant strains grown under N limitation. Nine of these 15 had a stronger signal in the nac^+ strain than in the *nac* mutant (i.e., they were activated by NAC). These nine were *folA*, *gyrA*, *ldcA*, *mltE*, *oppA*, *rpmI*, *ureD*, *ybjP*, and *yceO.* Although not characterized in detail, these are likely candidates for NAC-activated genes. The remaining six genes appear to be repressed by NAC (see below).

GENES REPRESSED BY NAC

Like most other LTTRs, NAC represses its own synthesis, but NAC also represses the expression of several other operons and uses a variety of mechanisms to exert that repression. A slightly different NBS consensus seems to be used in these sites. Instead of the ATA-n9-TAT sequence seen within the activation consensus NBS, the NBS consensus for repression seems to be ATA-n9-GAT or its equivalent, ATC-n9-TAT (20, 68).

Glutamate dehydrogenase (*gdhA* gene). The *gdhA* gene of *K. pneumoniae* is activated by ArgP, which binds to the *gdhAp* region and footprints a region from −50 to −100 relative to the transcriptional start site (18). A NAC dimer binds to a NBS and footprints a region from −75 to −100, overlapping the ArgP-binding site (20). Since ArgP and NAC share a binding site, the presence of NAC eliminates ArgP-mediated activation and reduces *gdhA* expression about 3-fold (18). The presence of lysine in the growth medium inhibits ArgP-mediated activation of *gdhA* to the same extent as NAC's binding to the region upstream of the *gdhA* promoter (28). It is clear that a NAC dimer is sufficient for this effect, since NAC^{L111K}, which cannot tetramerize, can exert this effect, sometimes called "weak repression" (68).

NAC also exerts a much stronger repression of *gdhAp* expression, but this "strong repression" requires that NAC be able to form a tetramer (68). The strong repression requires the dimer-binding site centered at −89 (mentioned above), but it also requires a second dimer-binding site centered at +57, about 147 bp away from the other NBS (20). Moreover, it is important that the two dimer-binding sites be on the correct side of the DNA helix. Inserting 5 extra base pairs between them reduces the effect of NAC, but inserting 10 bp between them leaves strong repression largely intact (20). The best model to explain these data is that a tetramer of NAC with one dimer bound at −89 and another at +57 causes a DNA loop to form and this loop interferes with the ability of RNA polymerase to transcribe from *gdhAp.*

Glutamate synthase (*gltBD* operon). The *gltBD* operon of *K. pneumoniae* codes for the two subunits of glutamate synthase (commonly called GOGAT). GOGAT activity is repressed as much as 17-fold under conditions of severe N limitation (21), though somewhat less under less severe limitation (4, 21, 37). This repression is NAC dependent (4, 48). A similar NAC-dependent repression of GOGAT formation (about 8-fold) is seen in *E. coli* (21, 48). The transcription start site for *gltBp* is known in *E. coli* (57), and the strong DNA sequence conservation in this region suggests that it is the same in *K. pneumoniae* (21), though this has not been confirmed. Preliminary experiments from this laboratory suggest that NAC binds to two sites in the *gltBp* region, one of which could overlap the −35 region of *gltBp* and the other of which lies about 270 bp farther upstream. Each of these regions contains a sequence that resembles or matches a known NBS consensus (centered at about −60 and −305 relative to the transcription start site). Little else is known about the nature of this regulation in *K. pneumoniae*, but the presence of two NAC-binding sites might suggest a mechanism similar to that seen at *gdhA.* Alternatively, NAC might directly affect RNA polymerase binding, it might interfere with the essential role of Lrp in activating *gltBp*, or it might act through none of these mechanisms. Clearly this important operon deserves further study.

The *nac* gene. Like most LTTRs, NAC downregulates its own synthesis (5, 12, 37). This autoregulation is important, since NAC is always in its active form and overproduction of NAC severely inhibits growth (6). Most LTTRs limit transcription of their own gene by a simple repression mechanism. However, NAC's autoregulation is more complex. The *nac* gene is transcribed by RNA polymerase bearing the unusual sigma factor σ54 (11, 37). This transcription absolutely requires the presence of an activator (NtrC∼P) bound to two enhancer sites at −155 and −135 relative to the transcription start site (11). NAC is one of the very few negative regulators of σ54-dependent transcription and acts by binding as a tetramer to a site between the enhancer and the promoter (12). DNase I footprints show that NAC protects a region from −58 to −130, occupying almost all the space between bound NtrC∼P and the RNA polymerase (11, 68). NAC does not interfere with the binding of NtrC∼P or RNA polymerase (11), but it does introduce a significant bend (about 113 degrees) into the DNA (12, 68). Because the NtrC∼P-dependent enhancer is so close to the promoter, the flexibility of the "linker DNA" is limited, and anything that interferes with this flexibility will impede the ability of NtrC∼P to activate the polymerase. The severe bend introduced by NAC has precisely this effect (12).

The NAC-binding region at the *nac* promoter is composed of two NBSs (66), one of which contains a match to the repression consensus (ATC-n9-TAT, which is equivalent to ATA-n9-GAT) and the other of which has a one-base mismatch (ATA-n9-GcT). Each of these sites is capable of binding a dimer of NAC (though not a tetramer) in the absence of the other. Furthermore, the nontetramerizing mutant NAC^{L111K} fills both sites but bends the DNA much less than the tetramer (66, 68). Curiously, NAC^{L111K} blocks NtrC∼P-mediated acti-

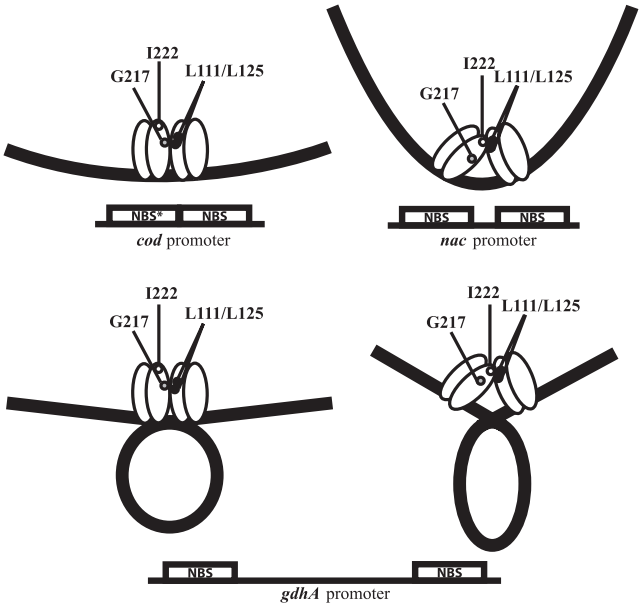

FIG. 3. NAC tetramers bind to three different kinds of sites. NAC tetramers appear to require an interaction between the L111/L125 region of one dimer with either the I222 region or the G217 region of another dimer. The NAC mutants NAC^{L111K} and NAC^{L125R} are dimers under all conditions and cannot form tetramers at any known site. The NAC mutants NAC^{I222R} and NAC^{G217R} bind as tetramers at some sites, but the double mutant $NAC^{I222R,G217}$ is a dimer. When two NBSs are adjacent to each other as they are at the *cod* promoter, NAC assumes a compact conformation that depends on an interaction between the L111/L125 region and a region near I222. NAC^{I222R} can still form tetramers at some sites (*nac*) but binds poorly at *cod.* When two NBSs are separated by one turn of the DNA helix, NAC assumes an extended conformation that depends on an interaction between the L111/L125 region and a region near G217. NAC^{G217R} can still form tetramers at some sites (*cod*) but not at sites separated by one turn of the helix (*nac*). When the NBSs are separated by many turns of the helix (as at *gdh*), both the compact and extended conformations of NAC can bind as a tetramer by forming a DNA loop between the two dimers of the tetramer. Both NAC^{I222R} and NAC^{G217R} can bind and repress *gdh*, but the double mutant $NAC^{I222R,G217R}$ cannot. The asterisk in one of the NBSs at *cod* indicates that this is a weak NBS that cannot independently bind a NAC dimer; all other NBSs here can bind a dimer of NAC in the absence of the other NBS.

vation nearly as well as NAC^{WT} (29). Perhaps the lesser bend induced by a NAC dimer (65) is the reason for this effect. However, it is more likely that filling the space between the bound NtrC~P and RNA polymerase with two dimers of NAC^{L111K} restricts the flexibility of the DNA enough to prevent the interaction of NtrC~P with the RNA polymerase (12). Strictly speaking, the regulation of *nac* by NAC is an antiactivation rather than a repression.

FLEXIBILITY OF NAC TETRAMER CONFORMATIONS

The three sites where NAC has been shown to function as a tetramer (Fig. 3) differ in the position of the two dimer-binding sites that make up the complete tetramer-binding site (66). At *cod*, the two dimer-binding sites are closely apposed (50), at *nac*, the two sites are separated by 10 bp (11, 68), and at *gdh*, the two sites are separated by 148 bp (20). The architectures of the first two types (*cod* and *nac*) resemble sites recognized by the activated and nonactivated forms of most LTTRs (39, 70). Since NAC recognizes both sites without any coeffector signal to change its conformation (66), there was a question of whether NAC did in fact have two different conformations. Using the structures of OxyR and CbnR as a guide, mutants of NAC were generated that should have been unable to assume one or the other predicted conformation (67). One such mutant readily bound *cod* but not *nac*; the other readily bound *nac* but not *cod*. Thus, NAC^{WT} can assume either conformation and can change from one to the other depending only on the spacing of the DNA site presented. In other words, the DNA site is the allosteric effector that determines the conformation of the NAC tetramer (compact and not bending the DNA, as at *cod*, or extended with a significant DNA bend, as at *nac*).

When the distance between the two dimer-binding sites is large enough to allow a DNA loop to form (as it is at *gdhAp* and perhaps also at *gltBp*), either conformation of NAC should be able to function. In fact, the *cod*-specific mutants and the *nac*-specific mutants can each still form tetramers and repress *gdh* expression. However, the double mutant cannot bind as a tetramer at either *cod* or *nac* and does not form the tetramers that repress *gdhAp* (67).

Recall that the DNA sequence of the dimer-binding sites at *hut* and *ure* also serves as an allosteric effector. Binding of a NAC dimer to an NBS is not sufficient for activation of transcription. A specific set of nucleotides in the promoter-proximal half of the NBS is required for the bound NAC to be active at *hut*, *ure*, or even *lacZ.* But this allosteric effect is different from the spacing effects that influence tetramer conformation. However, the question of whether the dimer effects influence the tetramer conformation remains open.

OVERVIEW

NAC is one of the most versatile regulators in bacteria. NAC is promiscuous in that at least four different DNA sequences have been shown to be recognized by the helix-turn-helix of a NAC monomer. As a result, the family of genes regulated by NAC is exceptionally large for an LTTR. It is clear that many of those genes have functions other than nitrogen metabolism, suggesting that NAC provides *K. pneumoniae* with the means to coopt genes or operons into the nitrogen regulon. Moreover, NAC's regulon overlaps the regulons of other global regulators. For example, CAP-cAMP regulates *hut* and *put*, DksA regulates *cod*, Lrp regulates *dad* and *gltB*, and NtrC regulates *nac.*

The degeneracy of the NBS sequence (there are three or four NBSs in pUC19! [19]) suggests that NAC will bind to nearly any AT-rich DNA at some level. This may explain why NAC regulates its own expression and why overproduction of NAC is so detrimental to *K. pneumoniae*. This also makes it easy for evolution to add NAC activation to an operon. A weak binding of NAC to a promoter can become stronger and can acquire the "activation sequence" without too may constraints on the precise location of the NBS or on its sequence. Acquiring repression is even easier.

OPEN QUESTIONS

It has been more than half a century since Magasanik began to ask how N limitation could relieve the catabolite repression of histidase formation in *K. pneumoniae*. The properties of NAC, produced under the control of the Ntr system, have provided the answer. But two pressing questions remain, and both require an analysis of NAC structure for their answers. The first is how the sequence of the NBS can convert NAC from a bound (but inactive) dimer to its activating form. Co-crystals of NAC complexes with activating and nonactivating NBSs will be necessary to answer this question. The second obvious question pertains to all LTTRs, not only NAC. What is the nature of the interaction of NAC with RNA polymerase that results in increased transcription? Findings for the positive-control mutants of CysB, GcvA, and NAC have been interpreted to suggest that amino acid Y27 of CysB (equivalent to H26 of NAC and F31 of GcvA) makes a direct contact with the "273 determinant" of the alpha-subunit C-terminal domain (α-CTD) of RNA polymerase. However, it is unlikely that amino acid H26 of NAC could make the same contact with the α-CTD at both *hutUp*, where NAC lies centered at -64, and *ureDp*, where NAC lies centered at -47. It seems more likely that H26 is important in transmitting a conformation signal from the DNA-binding (helix-turn-helix) region of NAC to the rest of the N-terminal domain of NAC (the N-terminal 86 amino acids). The effect of the H26D substitution remains a mystery. Do NAC^{H26D} dimers bend DNA differently than does NAC^{WT}? Does NAC interact with different subunits of RNA polymerase at *hutUp* and *ureDp*? The fact that very short fragments of NAC (with as few as 86 amino acids) are fully functional makes NAC a good candidate for solving this mystery.

ACKNOWLEDGMENTS

This minireview is dedicated to Boris Magasanik on the occasion of his 90th birthday, in recognition of his more than 5 decades spent unraveling the intricacies of microbial nitrogen metabolism.

This work was supported by Public Health Service grant GM47156 from the National Institutes of Health.

REFERENCES

1. **Bender, R. A.** 1991. The role of the NAC protein in the nitrogen regulation of *Klebsiella aerogenes*. Mol. Microbiol. **5:**2575–2580.
2. **Bender, R. A., K. A. Janssen, A. D. Resnick, M. Blumenberg, F. Foor, and B. Magasanik.** 1977. Biochemical parameters of glutamine synthetase from *Klebsiella aerogenes*. J. Bacteriol. **129:**1001–1009.
3. **Bender, R. A., and B. Magasanik.** 1977. Regulatory mutations in the *Klebsiella aerogenes* structural gene for glutamine synthetase. J. Bacteriol. **132:** 100–105.
4. **Bender, R. A., P. M. Snyder, R. Bueno, M. Quinto, and B. Magasanik.** 1983. Nitrogen regulation system of *Klebsiella aerogenes*: the *nac* gene. J. Bacteriol. **156:**444–446.
5. **Best, E. A., and R. A. Bender.** 1990. Cloning of the *Klebsiella aerogenes nac* gene, which encodes a factor required for nitrogen regulation of the histidine utilization (*hut*) operons in *Salmonella typhimurium*. J. Bacteriol. **172:**7043–7048.
6. **Blauwkamp, T. A., and A. J. Ninfa.** 2002. Nac-mediated repression of the *serA* promoter of *Escherichia coli*. Mol. Microbiol. **45:**351–363.
7. **Brenchley, J. E., M. J. Prival, and B. Magasanik.** 1973. Regulation of the synthesis of enzymes responsible for glutamate formation in *Klebsiella aerogenes*. J. Biol. Chem. **248:**6122–6128.
8. **Brill, W. J., and B. Magasanik.** 1969. Genetic and metabolic control of histidase and urocanase in *Salmonella typhimurium*, strain 15-59. J. Biol. Chem. **244:**5392–5402.
9. **Chen, L. M., T. J. Goss, R. A. Bender, S. Swift, and S. Maloy.** 1998. Genetic analysis, using P22 challenge phage, of the nitrogen activator protein DNA-binding site in the *Klebsiella aerogenes put* operon. J. Bacteriol. **180:**571–577.
10. **Chen, L. M., and S. Maloy.** 1991. Regulation of proline utilization in enteric bacteria: cloning and characterization of the *Klebsiella put* control region. J. Bacteriol. **173:**783–790.
11. **Feng, J., T. J. Goss, R. A. Bender, and A. J. Ninfa.** 1995. Activation of transcription initiation from the *nac* promoter of *Klebsiella aerogenes*. J. Bacteriol. **177:**5523–5534.
12. **Feng, J., T. J. Goss, R. A. Bender, and A. J. Ninfa.** 1995. Repression of the *Klebsiella aerogenes nac* promoter. J. Bacteriol. **177:**5535–5538.
13. **Friedrich, B., and B. Magasanik.** 1977. Urease of *Klebsiella aerogenes:* control of its synthesis by glutamine synthetase. J. Bacteriol. **131:**446–452.
14. **Frisch, R. L.** 2009. The role of the nitrogen assimilation control protein (NAC) in the response of *Klebsiella pneumoniae* to nitrogen limitation, Ph.D. thesis. University of Michigan, Ann Arbor, MI.
15. **Frisch, R. L., and R. A. Bender.** 2010. Properties of the NAC (nitrogen assimilation control protein)-binding site within the *ureD* promoter of *Klebsiella pneumoniae*. J. Bacteriol. **192:**4821–4826.
16. **Frisch, R. L., and R. A. Bender.** 2010. Expanded role for the nitrogen assimilation control protein in the response of *Klebsiella pneumoniae* to nitrogen stress. J. Bacteriol. **192:**4812–4820.
17. **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.
18. **Goss, T. J.** 2008. The ArgP protein stimulates the *Klebsiella pneumoniae gdhA* promoter in a lysine-sensitive manner. J. Bacteriol. **190:**4351–4359.
19. **Goss, T. J., and R. A. Bender.** 1995. The nitrogen assimilation control protein, NAC, is a DNA binding transcription activator in *Klebsiella aerogenes*. J. Bacteriol. **177:**3546–3555.
20. **Goss, T. J., B. K. Janes, and R. A. Bender.** 2002. Repression of glutamate dehydrogenase formation in *Klebsiella aerogenes* requires two binding sites for the nitrogen assimilation control protein, NAC. J. Bacteriol. **184:**6966–6975.
21. **Goss, T. J., A. Perez-Matos, and R. A. Bender.** 2001. Roles of glutamate synthase, *gltBD*, and *gltF* in nitrogen metabolism of *Escherichia coli* and *Klebsiella aerogenes*. J. Bacteriol. **183:**6607–6619.
22. **Gussin, G. N., C. W. Ronson, and F. M. Ausubel.** 1986. Regulation of nitrogen fixation genes. Annu. Rev. Genet. **20:**567–591.
23. **Hirschman, J., P. K. Wong, K. Sei, J. Keener, and S. Kustu.** 1985. Products of nitrogen regulatory genes *ntrA* and *ntrC* of enteric bacteria activate *glnA* transcription in vitro: evidence that the *ntrA* product is a sigma factor. Proc. Natl. Acad. Sci. U. S. A. **82:**7525–7529.
24. **Hunt, T. P., and B. Magasanik.** 1985. Transcription of *glnA* by purified *Escherichia coli* components: core RNA polymerase and the products of *glnF*, *glnG*, and *glnL*. Proc. Natl. Acad. Sci. U. S. A. **82:**8453–8457.
25. **Ikeda, T. P., A. E. Shauger, and S. Kustu.** 1996. *Salmonella typhimurium* apparently perceives external nitrogen limitation as internal glutamine limitation. J. Mol. Biol. **259:**589–607.
26. **Janes, B. K., and R. A. Bender.** 1998. Alanine catabolism in *Klebsiella aerogenes:* molecular characterization of the *dadAB* operon and its regulation by the nitrogen assimilation control protein. J. Bacteriol. **180:**563–570.
27. **Janes, B. K., and R. A. Bender.** 1999. Two roles for the leucine-responsive regulatory protein in expression of the alanine catabolic operon (*dadAB*) in *Klebsiella aerogenes*. J. Bacteriol. **181:**1054–1058.
28. **Janes, B. K., P. J. Pomposiello, A. Perez-Matos, D. J. Najarian, T. J. Goss, and R. A. Bender.** 2001. Growth inhibition caused by overexpression of the structural gene for glutamate dehydrogenase (*gdhA*) from *Klebsiella aerogenes*. J. Bacteriol. **183:**2709–2714.
29. **Janes, B. K., C. J. Rosario, and R. A. Bender.** 2003. Isolation of a negative control mutant of the nitrogen assimilation control protein, NAC, in *Klebsiella aerogenes*. J. Bacteriol. **185:**688–692.
30. **Keener, J., and S. Kustu.** 1988. Protein kinase and phosphoprotein phosphatase activities of nitrogen regulatory proteins NTRB and NTRC of enteric bacteria: roles of the conserved amino-terminal domain of NTRC. Proc. Natl. Acad. Sci. U. S. A. **85:**4976–4980.
31. **Kilstrup, M., L. M. Meng, J. Neuhard, and P. Nygaard.** 1989. Genetic evidence for a repressor of synthesis of cytosine deaminase and purine biosynthesis enzymes in *Escherichia coli*. J. Bacteriol. **171:**2124–2127.
32. **Lee, M. H., S. B. Mulrooney, M. J. Renner, Y. Markowicz, and R. P. Hausinger.** 1992. *Klebsiella aerogenes* urease gene cluster: sequence of *ureD* and demonstration that four accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) are involved in nickel metallocenter biosynthesis. J. Bacteriol. **174:**4324–4330.
33. **Liu, Q., and R. A. Bender.** 2007. Complex regulation of urease formation from the two promoters of the ure operon of *Klebsiella pneumoniae*. J. Bacteriol. **189:**7593–7599.
34. **Lochowska, A., R. Iwanicka-Nowicka, D. Plochocka, and M. M. Hryniewicz.** 2001. Functional dissection of the LysR-type CysB transcriptional regulator. Regions important for DNA binding, inducer response, oligomerization, and positive control. J. Biol. Chem. **276:**2098–2107.
35. **Lochowska, A., R. Iwanicka-Nowicka, J. Zaim, M. Witkowska-Zimny, K. Bolewska, and M. M. Hryniewicz.** 2004. Identification of activating region (AR) of *Escherichia coli* LysR-type transcription factor CysB and CysB contact site on RNA polymerase alpha subunit at the *cysP* promoter. Mol. Microbiol. **53:**791–806.
36. **Lund, P., and B. Magasanik.** 1965. N-formimino-L-glutamate formiminohydrolase of *Aerobacter aerogenes*. J. Biol. Chem. **240:**4316–4319.

37. **Macaluso, A., E. A. Best, and R. A. Bender.** 1990. Role of the *nac* gene product in the nitrogen regulation of some NTR-regulated operons of *Klebsiella aerogenes*. J. Bacteriol. **172:**7249–7255.
38. **MacPhee, D. G., I. W. Sutherland, and J. F. Wilkinson.** 1969. Transduction in *Klebsiella*. Nature **221:**475–476.
39. **Maddocks, S. E., and P. C. Oyston.** 2008. Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. Microbiology **154:**3609–3623.
40. **Magasanik, B.** 1955. The metabolic control of histidine assimilation and dissimilation in *Aerobacter aerogenes*. J. Biol. Chem. **213:**557–569.
41. **Magasanik, B.** 1961. Catabolite repression. Cold Spring Harb. Symp. Quant Biol. **26:**249–256.
42. **Magasanik, B.** 1996. Regulation of nitrogen utilization, p. 1344–1356. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, DC.
43. **Magasanik, B., and H. R. Bowser.** 1955. The degradation of histidine by *Aerobacter aerogenes*. J. Biol. Chem. **213:**571–580.
44. **Meers, J. L., D. W. Tempest, and C. M. Brown.** 1970. Glutamine(amide):2-oxoglutarate amino transferase oxido-reductase (NADP); an enzyme involved in the synthesis of glutamate by some bacteria. J. Gen. Microbiol. **64:**187–194.
45. **Meiss, H. K., W. J. Brill, and B. Magasanik.** 1969. Genetic control of histidine degradation in *Salmonella typhimurium*, strain LT-2. J. Biol. Chem. **244:**5382–5391.
46. **Mobley, H. L., M. D. Island, and R. P. Hausinger.** 1995. Molecular biology of microbial ureases. Microbiol. Rev. **59:**451–480.
47. **Muro-Pastor, A. M., P. Ostrovsky, and S. Maloy.** 1997. Regulation of gene expression by repressor localization: biochemical evidence that membrane and DNA binding by the PutA protein are mutually exclusive. J. Bacteriol. **179:**2788–2791.
48. **Muse, W. B., and R. A. Bender.** 1998. The *nac* (nitrogen assimilation control) gene from *Escherichia coli*. J. Bacteriol. **180:**1166–1173.
49. **Muse, W. B., and R. A. Bender.** 1999. The amino-terminal 100 residues of the nitrogen assimilation control protein (NAC) encode all known properties of NAC from *Klebsiella aerogenes* and *Escherichia coli*. J. Bacteriol. **181:**934–940.
50. **Muse, W. B., C. J. Rosario, and R. A. Bender.** 2003. Nitrogen regulation of the *codBA* (cytosine deaminase) operon from *Escherichia coli* by the nitrogen assimilation control protein, NAC. J. Bacteriol. **185:**2920–2926.
51. **Nagatani, H., M. Shimizu, and R. C. Valentine.** 1971. The mechanism of ammonia assimilation in nitrogen fixing bacteria. Arch. Mikrobiol. **79:**164–175.
52. **Neidhardt, F. C., and B. Magasanik.** 1957. Effect of mixtures of substrates on the biosynthesis of inducible enzymes in *Aerobacter aerogenes*. J. Bacteriol. **73:**260–263.
53. **Neidhardt, F. C., and B. Magasanik.** 1957. Reversal of the glucose inhibition of histidase biosynthesis in *Aerobacter aerogenes*. J. Bacteriol. **73:**253–259.
54. **Nieuwkoop, A. J., S. A. Baldauf, M. E. Hudspeth, and R. A. Bender.** 1988. Bidirectional promoter in the *hut*(*P*) region of the histidine utilization (*hut*) operons from *Klebsiella aerogenes*. J. Bacteriol. **170:**2240–2246.
55. **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.
56. **Ninfa, A. J., and B. Magasanik.** 1986. Covalent modification of the *glnG* product, NRI, by the *glnL* product, NRII, regulates the transcription of the *glnALG* operon in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **83:**5909–5913.
57. **Oliver, G., G. Gosset, R. Sanchez-Pescador, E. Lozoya, L. M. Ku, N. Flores, B. Becerril, F. Valle, and F. Bolivar.** 1987. Determination of the nucleotide sequence for the glutamate synthase structural genes of *Escherichia coli* K-12. Gene **60:**1–11.
58. **Osuna, R., S. A. Boylan, and R. A. Bender.** 1991. In vitro transcription of the histidine utilization (*hutUH*) operon from *Klebsiella aerogenes*. J. Bacteriol. **173:**116–123.
59. **Pomposiello, P. J., and R. A. Bender.** 1995. Activation of the *Escherichia coli lacZ* promoter by the *Klebsiella aerogenes* nitrogen assimilation control protein (NAC), a LysR family transcription factor. J. Bacteriol. **177:**4820–4824.
60. **Pomposiello, P. J., B. K. Janes, and R. A. Bender.** 1998. Two roles for the DNA recognition site of the *Klebsiella aerogenes* nitrogen assimilation control protein. J. Bacteriol. **180:**578–585.
61. **Porter, R. I., A. K. North, and S. Kustu.** 1995. Mechanism of transcriptional activation by NtrC, p. 147–158. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-component signal transduction. American Society for Microbiology, Washington, DC.
62. **Prival, M. J., J. E. Brenchley, and B. Magasanik.** 1973. Glutamine synthetase and the regulation of histidase formation in *Klebsiella aerogenes*. J. Biol. Chem. **248:**4334–4344.
63. **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.
64. **Revel, H. R., and B. Magasanik.** 1958. The enzymatic degradation of urocanic acid. J. Biol. Chem. **233:**930–935.
65. **Rosario, C. J.** 2005. The importance of tetramer formation by the nitrogen assimilation control protein (NAC) for DNA binding and repression at the *gdhA* promoter in *Klebsiella pneumoniae.* Ph.D. thesis. University of Michigan, Ann Arbor, MI.
66. **Rosario, C. J., R. L. Frisch, and R. A. Bender.** 2010. The LysR-type nitrogen assimilation control protein forms complexes with both long and short DNA binding sites in the absence of coeffectors. J. Bacteriol. **192:**4827–4833.
67. **Rosario, C. J., B. K. Janes, and R. A. Bender.** 2010. Genetic analysis of the nitrogen assimilation control protein from *Klebsiella pneumoniae*. J. Bacteriol. **192:**4834–4846.
68. **Rosario, C. J., and R. A. Bender.** 2005. Importance of tetramer formation by the nitrogen assimilation control protein for strong repression of glutamate dehydrogenase formation in *Klebsiella pneumoniae*. J. Bacteriol. **187:**8291–8299.
69. **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.
70. **Schell, M. A.** 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. **47:**597–626.
71. **Schwacha, A., and R. A. Bender.** 1993. The *nac* (nitrogen assimilation control) gene from *Klebsiella aerogenes*. J. Bacteriol. **175:**2107–2115.
72. **Schwacha, A., and R. A. Bender.** 1993. The product of the *Klebsiella aerogenes nac* (nitrogen assimilation control) gene is sufficient for activation of the *hut* operons and repression of the *gdh* operon. J. Bacteriol. **175:**2116–2124.
73. **Streicher, S. L., R. A. Bender, and B. Magasanik.** 1975. Genetic control of glutamine synthetase in *Klebsiella aerogenes*. J. Bacteriol. **121:**320–331.
74. **Tempest, D. W., J. L. Meers, and C. M. Brown.** 1970. Synthesis of glutamate in *Aerobacter aerogenes* by a hitherto unknown route. Biochem. J. **117:**405–407.
75. **Tyler, B.** 1978. Regulation of the assimilation of nitrogen compounds. Annu. Rev. Biochem. **47:**1127–1162.
76. **Ushiba, D., and B. Magasanik.** 1952. Effects of auxotrophic mutations on the adaptation to inositol degradation in *Aerobacter aerogenes*. Proc. Soc. Exp. Biol. Med. **80:**626–632.
77. **Zhang, X. X., and P. B. Rainey.** 2008. Dual involvement of CbrAB and NtrBC in the regulation of histidine utilization in *Pseudomonas fluorescens* SBW25. Genetics **178:**185–195.
78. **Zimmer, D. P., E. Soupene, H. L. Lee, V. F. Wendisch, A. B. Khodursky, B. J. Peter, R. A. Bender, and S. Kustu.** 2000. Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: scavenging as a defense against nitrogen limitation. Proc. Natl. Acad. Sci. U. S. A. **97:**14674–14679.