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Genetic Analysis of the Nitrogen Assimilation Control Protein from *Klebsiella pneumoniae*▽

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The nitrogen assimilation control protein (NAC) from *Klebsiella pneumoniae* is a typical LysR-type transcriptional regulator (LTTR) in many ways. However, the lack of a physiologically relevant coeffector for NAC and the fact that NAC can carry out many of its functions as a dimer make NAC unusual among the LTTRs. In the absence of a crystal structure for NAC, we analyzed the effects of amino acid substitutions with a variety of phenotypes in an attempt to identify functionally important features of NAC. A substitution that changed the glutamine at amino acid 29 to alanine (Q29A) resulted in a NAC that was seriously defective in binding to DNA. The H26D substitution resulted in a NAC that could bind and repress transcription but not activate transcription. The I71A substitution resulted in a NAC polypeptide that remained monomeric. NAC tetramers can bind to both long and shorter binding sites (like other LTTRs). However, the absence of a coeffector to induce the conformational change needed for the switch from the former to the latter raised a question. Are there two conformations of NAC, analogous to the other LTTRs? The G217R substitution resulted in a NAC that could bind to the longer sites but had difficulty in binding to the shorter sites, and the I222R and A230R substitutions resulted in a NAC that could bind to the shorter sites but had difficulty in binding properly to the longer sites. Thus, there appear to be two conformations of NAC that can freely interconvert in the absence of a coeffector.

The LysR-type transcriptional regulator (LTTR) family contains the highest number of regulatory proteins in the bacterial world, with more than 23,000 identified in the completed bacterial genome sequences as of March 2009 (43). They are assembled as tetramers, which are in fact dimers of dimers (27, 39). Like many transcriptional regulators, they contain two well-defined domains, an N-terminal DNA-binding domain and a C-terminal regulatory domain responsible for dimer-dimer interactions and for binding a regulatory coeffector. This coeffector is usually a small molecule that, when bound, moves the DNA-binding domains of the two dimers closer together such that a shorter DNA-binding site is recognized in the presence of the coeffector than in its absence (29, 42, 45, 47). Despite their abundance, few LTTRs have been crystallized and even fewer structures are available. Complete structures of CbnR, and more recently TsaR and ArgP, have been solved at high resolution (28, 29, 48), and a complete DntR structure has also been solved, although its DNA-binding domain was less well resolved (42). The C-terminal domains of several other LTTRs have been crystallized and their structures solved, but only after the DNA-binding domain was removed (6, 7, 44, 46). Unfortunately, no full-length LTTR structure has been solved in both the coeffector-bound and coeffector-free conformations, leaving us to extrapolate the effects seen from the C-terminal domain structures, where both forms have been solved in several cases (see references 6, 7, and 42 for examples).

The nitrogen assimilation control protein, NAC, from *Klebsiella pneumoniae* is in many ways a typical LTTR. It shows sequence similarity to the family (40), it is a dimer of dimers (38), it activates some genes and represses others (25, 41), it has the typical two domains (31, 40), and it has proved recalcitrant to our attempts at crystallization. But in other ways, NAC is a special case among the LTTRs. At most of the sites where NAC activates transcription, it functions as a dimer (18, 38). Other functionally dimeric LTTRs are known, e.g., MetR (23); however, even these LTTRs usually function in their tetrameric state to activate transcription. Truncated versions of NAC with as few as 86 amino acids (of the 305 total) are able to activate transcription at many, if not all, sites (31, 38). The NAC regulon is unusually large, with scores of genes binding and responding to NAC-mediated activation or repression (11). NAC has no physiologically relevant coeffector (13, 41). The decision to activate or repress gene expression is determined solely by whether NAC is made and not by a differential activity of the NAC once made (41). Although NAC tetramers can recognize both longer and shorter binding sites (like other LTTRs), it does so without the intervention of any coeffector or modification (37). This suggested that NAC tetramers can undergo a conformational change from a more compact to a more extended form in response to the DNA site presented to them, a conformational change that corresponds to the change induced by the binding of coeffectors in other LTTRs (37).

In addition, we have postulated a conformational change that occurs when a NAC dimer interacts with its binding site to activate transcription (35). The NAC-binding sites (NBS) at the *hutU* promoter (NBS^{hutU}) and the *ureD* promoter (NBS^{ureD}) are functionally asymmetric (10, 35). Inverting the

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site (from ATAA-N5-TnGTAT to ATACnA-N5-TTAT) leads to loss of activation. Making the sites symmetric for the half-site nearest the start of transcription (ATACnA-N3-TnGTAT) results in a functional site. However, making the sites symmetric for the other half-site (ATAA-N7-TTAT) allows NAC to bind well, but the binding is ineffective and transcriptional activation is lost (10, 35). It is not known whether the conformational change that occurs when a dimer binds has any effect on the conformational change that involves reorientation of the dimers within the tetramer to allow recognition of the longer or shorter tetramer-binding sites.

In the absence of a crystal structure for NAC, we embarked on a genetic analysis to identify functionally important elements of NAC and to determine whether these features were consistent with the structural elements defined for other LTTRs.

MATERIALS AND METHODS

Strains and plasmids. All *K. pneumoniae* strains used here were derived from strain W70, which was originally called *K. aerogenes* (26). Strains KC4598 (*hutC515* Δ[*bla*]-*2 dadA1 nac-204*::λp*lac*Mu53 *srl-7012*::Tn*5-132*) and KC4727 (*hutC515* Δ[*bla*]-*2 dadA1 nac-204*::λp*lac*Mu53) were derived by P1-mediated transductions of strain KC2668 (16) with phage grown on strains carrying the *srl-7012*::Tn*5-132* (unpublished) and *nac-204* (25) alleles.

Plasmid pCB1041 (38) is a low-copy-number vector (six to eight copies in *Escherichia coli*) that confers ampicillin resistance and allows expression of cloned material from a *lacZ* promoter (ultimately derived from plasmid pUC8). Plasmid pCB1051 is pCB1041 with an EcoRI-SalI fragment that puts a wild-type *nac* gene under the control of the *lacZ* promoter. Plasmid pCB1026 (38) is the expression vector pQE70 (Qiagen) with an EcoRI-BamHI fragment containing a *nac* gene with six histidine codons fused to the 3′ end of the gene. Plasmid pCB797 is pBCKS+ (Stratagene) with an EcoRI-HindIII fragment that carries a wild-type *nac* gene with six histidine codons added to the 3′ end of the gene. Plasmid pCB1560 is plasmid pKSM13(+) (Stratagene) with NBS^{hutU} cloned as the EcoRI-HindIII fragment from pOS1 (33).

Enzyme assays. Cultures of *K. pneumoniae* were grown in W4 minimal medium (25) supplemented with glucose (0.4%, wt/vol), ammonium sulfate (0.2%, wt/vol), L-glutamine (0.2%, wt/vol), nickel sulfate (2 μM), and ampicillin (100 μg/ml). L-Lysine (0.01%, wt/vol) was also added to some of the cultures, as indicated below in Table 3. Histidase, glutamate dehydrogenase, urease, and β-galactosidase were assayed in cells made permeable with detergent as described previously (25). Specific activities are reported as nmol of product formed or of substrate consumed per min per mg of cellular protein, as measured by the Lowry assay (24). The values reported are the averages from three or more independent cultures, with standard errors of the means (SEM) that were less than 20% of the mean in all cases (as noted in the table footnotes).

Site-directed mutagenesis. Alanine scanning mutagenesis was performed using the GeneEditor kit (Promega) according to the manufacturer's directions. Each mutation was introduced in plasmid pCB1051. *E. coli* strain DH5α was transformed with the mutagenized plasmids, and candidate colonies were saved. Plasmid DNA from these candidates was submitted for DNA sequence analysis at the University of Michigan DNA Sequencing Core. Plasmid DNA containing the desired mutation was used to transform strain DH5α, and these secondary transformants were saved. Minipreps of the plasmid DNA from these secondary transformants were used to transform the Nac$^-$ *K. pneumoniae* strain KC4598. Plasmid DNA from the transformed KC4598 strains was submitted for DNA sequence analysis to confirm that the *nac* gene was present as expected, and these transformants were used for the assays reported below in Table 1. Plasmid DNA was prepared from each strain that showed a phenotype other than wild type (see Table 1). An EcoRI-Eco47III fragment, which carried the N-terminal region of the *nac* gene, was then subcloned into plasmid pCB797. This yielded a C-terminally His-tagged version of the mutant *nac* gene. When mutant NAC protein was to be isolated, the N-terminal region of the *nac* coding sequence was cloned as an EcoRI-HindIII fragment from the pCB797-derived clones into plasmid pQE70 (Qiagen). All clones in pQE70 were submitted for DNA sequencing to confirm the integrity of the (mutant) *nac* gene sequence.

Arginine scanning mutagenesis was performed similarly using plasmid pCB1051 as starting material. Secondary transformants were saved in *E. coli* strain DH5α, and DNA from these strains was used to transform *K. pneumoniae* strain KC4727 for the assays reported below in Table 3.

Protein purification. His-tagged wild-type and mutant NAC proteins were purified by nickel affinity chromatography as described previously (38). After elution from the nickel resin, the eluate was dialyzed overnight against buffer 4 (250 mM NaCl, 100 mM sodium phosphate [pH 7.0], 2.5 mM $MgCl_2$, 1 mM β-mercaptoethanol) to remove the imidazole used for elution from the column. The resulting protein was concentrated using Centricon filters according to the manufacturer's directions and either used immediately or stored at 20°C in buffer 4 containing 50% glycerol. The protein concentration was measured by using the method of Lowry (24).

Gel filtration. A 250-ml sample of His-tagged wild-type NAC (NAC^{WT}) or of a His-tagged mutant NAC (NAC^{L111K} or NAC^{I71A}) was applied to a Sephadex S200 fast-performance liquid chromatography (FPLC) gel filtration column (Amersham Pharmacia Biotech) equilibrated with buffer 4. Protein eluting from the column was detected by monitoring absorbance at 280 nm.

Radioactive electrophoretic mobility shift assay (EMSA). For the experiments shown below in Fig. 1 and 5, DNA fragments were isolated and labeled with [α-^{32}P]dATP by using Klenow fragment (Roche) according to the manufacturer's instructions. Labeled DNA fragments were incubated with His-tagged NAC in a 10-μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 10 μg of bovine serum albumin (BSA), and 20 nM poly(dI · dC). After 20 min of incubation at 30°C, 1 μl of loading buffer (40 mM Tris-HCl [pH 8.4], 4 mM EDTA, 0.2% bromophenol blue, 0.2% xylene cyanol, 25% glycerol) was added. Bound and unbound fragments were separated by electrophoresis on a Tris-borate-EDTA–5% polyacrylamide gel at 13 V/cm for 3 h. The gel was transferred to Whatman 3MM filter paper and dried. Autoradiograms were obtained by exposing the dried gels to X-ray film at −70°C with an intensifying screen.

Nonradioactive EMSA. When the use of higher concentrations of protein and DNA would not interfere with the interpretation of the results (e.g., the experiments shown below in Fig. 3 and 7), a nonradioactive EMSA was used. DNA was prepared by PCR amplification using primers that generated a 150-bp fragment with the NBS at the approximate center of the fragment. Plasmid pOS1 (33) was used as template for NBS^{hutU}, plasmid pJF200 (8) was used as template for NBS^{nac}, and pCB816 (32) was used as template for NBS^{codB}. Histidine-tagged wild-type or mutant NAC protein was mixed with DNA in buffer 4 in the presence of 10 μg of BSA. The reaction volume was 10 μl, the DNA amounts were generally in the range of 30 to 45 ng, and the protein amounts were generally adjusted to allow shifting of about half of the input DNA. After 20 min of incubation at room temperature, 1.5 μl of loading buffer (40 mM Tris-HCl [pH 8.4], 4 mM EDTA, 0.2% bromophenol blue, 0.2% xylene cyanol, 25% glycerol) was added to the samples, which were then loaded onto a 4% acrylamide–Tris-acetate-EDTA gel. The bound and unbound fragments were separated by electrophoresis at 10 or 15 V/cm for 1 h at 4°C. Gels were stained with ethidium bromide, and the DNA was detected by UV fluorescence.

The identity of the band resulting from the binding of a dimer of NAC was determined from the mobility of a shift induced by NAC^{L111K}, which is known to bind as a dimer (38). The identity of the band resulting from the binding of either two dimers or a tetramer that does not bend the DNA was determined from the mobility of the shift of a *nac* fragment induced by NAC^{L111K}, where two dimers bind (37, 38), or from the mobility of a *cod* fragment bound by NAC^{WT}, which binds as a tetramer with little bending of the DNA (37). The identity of the band resulting from the binding of a tetramer that bends the DNA was determined from the mobility of the shift of a *nac* fragment induced by the binding of NAC^{WT}, which causes a significant bend in the DNA (9, 38).

DNase I footprint assay. DNase I footprint analysis was performed as described previously (13). In brief, 10 μg of plasmid pCB1560 was digested with BamHI and labeled with [α-^{32}P]dATP in the presence of Klenow fragment, followed by a second digestion with XbaI. This generated two DNA fragments, a long fragment containing the NBS^{hutU} and a short DNA fragment of less than 25 bp, which ran off the bottom of the gel. Approximately 0.3 pmol of labeled DNA was incubated with His-tagged NAC for 20 min, and then 1.7×10^{-4} U of DNase I (Promega) was added and the incubation was continued for another 3 min at room temperature. Digestion was stopped with a loading buffer containing 40 mM Tris-HCl (pH 8.4), 4 mM EDTA, 0.2% bromophenol blue, 0.2% xylene cyanol, 25% glycerol, and 50% formamide. Digestion products were separated by electrophoresis on a 6% urea–Tris-borate-EDTA sequencing gel at 1,200 V for about 4 h. The gel was dried on Whatman 3MM paper and exposed to X-ray film. A "G ladder" was prepared with 0.3 pmol of labeled DNA as described previously (1).

RESULTS

Alanine scanning mutagenesis of the N-terminal domain of NAC. At many of the sites where NAC activates transcription, NAC functions as a dimer; at many of those sites, an N-terminal fragment of NAC containing as few as 86 amino acids is able to bind and activate transcription (31, 38). Our goal was to identify features of NAC required for activation. Therefore, we performed alanine scanning mutagenesis of the amino acids 2 through 100, generating a set of 89 substitution mutants, each with a single amino acid changed to alanine (there are 10 alanines native to this region of NAC). The DNA sequence of each mutant *nac* gene was determined and the mutant gene was cloned into a plasmid under the control of the *lac* promoter as described in Materials and Methods. The expression of NAC^{WT} from this plasmid is sufficient to activate all known NAC-dependent genes. The ability of NAC to activate gene expression was initially determined by measuring the activity of histidase formed (from the *hut* operon), and the ability of NAC to repress gene expression was determined by measuring the activity of glutamate dehydrogenase formed (from the *gdhA* gene), as shown in Table 1. About one-third of the substitutions in the first 86 amino acids were essentially wild type, activating *hut* and repressing *gdhA* about as well as the wild-type NAC protein did. All of the substitutions in amino acids 87 to 100 were essentially wild type. This was expected, since this region functions as a linker between the two independent domains of LTTRs. Moreover, NAC mutants with only the N-terminal 86 amino acids are able to activate *hutUp* and *ureDp* almost as well as wild-type NAC.

Substitutions that inactivate NAC. It requires more NAC to activate *hut* operon expression than to regulate other genes (41), and so we were concerned that mutants showing less histidase activity than the wild type might result from a partially defective NAC or a more unstable NAC that accumulated to lower levels. Therefore, for every substitution with lower-than-wild-type levels of histidase (specific activity of less than 220 nmol/min/mg of protein), we also assayed urease from the *ure* operon, which has been shown to be activated by very low levels of NAC, less than or equal to the amount required to repress *gdhA* (41). This allowed us to distinguish between mutants with the phenotype expected of an inactive NAC (urease activity less than 125 and GDH greater than 600 nmol/min/mg of protein) from those with a partial phenotype (urease activity greater than 125 or GDH less than 600 nmol/min/mg of protein). By this definition, only 19 of the substitution mutants showed the phenotype of a completely inactive NAC (Table 1).

A simple explanation for a lack of function in a mutant protein is that the protein is degraded and does not accumulate in the cell. NAC^{WT} is known to be rather unstable (30) and is degraded in chloramphenicol-treated cells with a half-life of about 10 min (data not shown). To eliminate the trivial case where there was no NAC function because no NAC polypeptide was present in the cells, we performed a qualitative Western blot assay on the 19 mutant strains that showed neither significant activation nor significant repression by NAC (Table 1). Six of those, with alanine substitutions of amino acids N2, L3, L6, F9, D14, and L32, gave no signal in the Western blot probed with polyclonal anti-NAC antibody. Five of these were clustered in an N-terminal region that is highly conserved among LTTRs and corresponds to an important α-helix in the known structure of CbnR (29). We had noted previously that modifications of the N terminus of NAC led to inactivation of NAC's function (31). Taken together, these data suggest that the N-terminal region of NAC (corresponding to the α1-helix of CbnR and other LTTRs) is important for structural integrity.

The DNA-binding domain of NAC (amino acids 1 to 86) contains 11 leucine residues. Leucine-to-alanine substitution of seven of them (residues 3, 6, 18, 32, 39, 47, and 48) led to a complete loss of NAC function. (NAC^{L80A} had wild-type function; NAC^{L25A}, NAC^{L64A}, and NAC^{L72A} retained partial activity.) Of the seven substitutions that eliminated NAC function, three of them (L3A, L6A, and L32A) were not detectable in our Western blot assays, and the remaining four, as well as the three with partial activity, showed a significant reduction in the NAC signal (Table 1), suggesting that these substitutions led to a NAC that was even more unstable than NAC^{WT}. Whatever the cause of the reduced signal in our Western blotting results may have been, it is clear that the leucine residues in NAC's DNA-binding (and activation) domain play an important structural role.

Two of the alanine substitutions that lacked NAC activity but showed at least some signal in our Western blot assays were chosen for further study: Q29A, a highly conserved residue that was expected to lie in the helix-turn-helix (HTH) region, and I71A, which was expected to lie within the dimerization helix region. Many mutant NAC proteins cannot be purified by our standard purification procedure. Therefore, all *in vitro* experiments were performed with His-tagged variants of the NAC protein under study. We have shown elsewhere (36) that NAC and His-tagged NAC give the same gel shifts and DNase I footprints. *In vivo*, His-tagged NAC gives stronger activation of *hutUp* and stronger repression of *gdhAp* under conditions of moderate nitrogen limitation. The reason for this increased activity is unknown, but it may reflect the fact that purified His-tagged NAC is more resistant to at least one protease *in vitro* than untagged NAC (30). Therefore, *in vivo* experiments were performed with proteins lacking the His tag.

Effect of the Q29A substitution on NAC binding. The glutamine at position 29 is conserved among many LTTRs and is located at the beginning of the second helix of the HTH motif (21, 29, 39), despite the presence of a conserved proline in position 30. Western blotting results suggested that NAC^{Q29A} is present in cells carrying the substitution (Table 1) and at levels comparable to NAC^{WT} (data not shown). We cloned the Q29A mutant gene encoding a C-terminal His tag, purified the resulting protein by nickel affinity chromatography, and compared its ability to bind the NBS^{hutU} in an EMSA. As shown in Fig. 1A, binding of NAC^{WT} resulted in a single shifted band whose mobility has been shown to represent a dimer of NAC bound to DNA (37, 38). In contrast, only in a significant excess of NAC^{Q29A} was a shifted band observed, and that shifted band had a mobility consistent with a tetramer of NAC bound to the DNA. Such a "tetramer shift" is also seen with NAC^{WT} when NAC is in significant excess (Fig. 1A) and represents the ability of NAC to bind to almost any AT-rich DNA ("pseudosites") if the concentration of NAC is high enough (see below). Such a shift has a mobility expected of a tetramer

TABLE 1. Alanine scanning mutants of NAC

nac allele[a]	Sp act[b] (nmol/min/mg of protein)			NAC function[c]	Western blotting result[d]
	Histidase	GDH	Urease		
None	65	668	30	None	ND
Wild type	360	43	798	Wild type	+
N2A	38	791	29	None	−
L3A	36	709	30	None	−
R4A	93	132	209	Partial	+
R5A	87	487	233	Partial	++
L6A	43	725	35	None	−
K7A	165	91	342	Partial	+
Y8A	68	476	501	Partial	+
F9A	44	694	32	None	−
V10A	162	110	528	Partial	ND
K11A	368	48	ND	Wild type	ND
I12A	438	74	ND	Wild type	ND
V13A	139	86	634	Partial	ND
D14A	47	601	115	None	−
I15A	115	258	119	Partial	+/−
G16A	141	166	854	Partial	+
S17A	151	127	535	Partial	+
L18A	51	695	61	None	+/−
T19A	91	688	120	None	+
Q20A	470	26	ND	Wild type	ND
E23A	625	83	ND	Wild type	+
V24	360	65	ND	Wild type	ND
L25A	47	406	276	Partial	+/−
H26A	282	30	ND	Wild type	+
I27A	82	209	201	Partial	+/−
Q29A	69	731	43	None	+
P30A	82	76	486	Partial	+
L32A	54	529	28	None	−
S33A	52	566	37	None	+
Q34A	294	63	ND	Wild type	ND
Q35A	560	80	351	Partial	+/−
V36A	51	259	316	Partial	+/−
T38	272	44	ND	Wild type	ND
L39A	55	717	44	None	+/−
E40A	53	604	45	None	+
G41A	317	72	ND	Wild type	ND
E42A	87	280	464	Partial	+/−
M43A	92	157	576	Partial	+/−
D44A	354	53	ND	Wild type	ND
Q45A	256	69	ND	Wild type	ND
Q46A	270	42	ND	Wild type	ND
L47A	50	564	41	None	+/−
L48A	46	534	36	None	+/−
I49A	126	97	862	Partial	+
R50A	42	547	38	None	+
T51A	190	136	845	Partial	+/−
K52A	383	82	ND	Wild type	ND
R53A	117	112	774	Partial	+
G54A	57	566	38	None	+
V55A	141	161	523	Partial	+
T56A	264	66	ND	Wild type	ND
P57A	347	64	ND	Wild type	ND
T58A	53	721	58	None	+/−
E59A	363	57	ND	Wild type	ND
G61A	46	704	61	None	+/−
L62A	297	71	ND	Wild type	ND
I63A	224	94	536	Partial	ND
L64A	68	485	372	Partial	+/−
Y65A	211	64	328	Partial	+
T66A	468	52	ND	Wild type	ND
H67A	171	115	903	Partial	+/−
R69A	362	94	443	Partial	ND
T70A	364	86	ND	Wild type	ND
I71A	46	841	26	None	+/−

Continued on following page

TABLE 1—*Continued*

nac allele[a]	Sp act[b] (nmol/min/mg of protein)			NAC function[c]	Western blotting result[d]
	Histidase	GDH	Urease		
L72A	96	115	622	Partial	ND
R73A	356	78	ND	Wild type	ND
Q74A	286	55	698	Wild type	ND
C75A	198	98	748	Partial	ND
E76A	113	114	1128	Partial	+
Q77A	391	81	ND	Wild type	ND
Q79A	359	30	ND	Wild type	ND
L80A	364	60	ND	Wild type	ND
V82A	60	514	452	Partial	+
N83A	470	51	ND	Wild type	ND
N84A	347	53	ND	Wild type	ND
V85A	348	54	ND	Wild type	ND
G86A	335	49	ND	Wild type	ND
Q87A	329	69	ND	Wild type	ND
T88A	382	56	ND	Wild type	ND
L89A	329	53	ND	Wild type	ND
R90A	284	113	681	Partial	ND
G91A	221	84	ND	Wild type	ND
Q92A	301	58	ND	Wild type	ND
V93A	347	48	ND	Wild type	ND
S94A	335	59	ND	Wild type	ND
I95A	177	59	ND	Wild type	ND
G96A	274	52	ND	Wild type	ND
L97A	296	60	ND	Wild type	ND
P99A	323	71	ND	Wild type	ND
G100A	363	53	ND	Wild type	ND

[a] Each allele designation indicates the amino acid change. Thus, N2A indicates that asparagine in position 2 of the polypeptide was changed to alanine. Note that amino acid numbers 21, 22, 28, 31, 37, 60, 68, 78, 81, and 98 in wild-type NAC are alanine, so they do not appear in the table. Each mutant *nac* gene was cloned in the low-copy-number vector pCB1041 and transferred to the Nac$^-$ strain KC4599. Cells were grown as described in Materials and Methods.

[b] The specific activities of histidase, GDH, and urease are the means of assays from three independent cultures. In every case the SEM was less than 20%.

[c] The apparent activity of NAC in cells bearing the indicated substitution. "Wild type" indicates that the activation levels of histidase and urease and the repression of GDH expression were similar to those seen in the strain carrying pCB1051 (which carried a wild-type copy of *nac* cloned in pCB1041). "Partial" indicates that activation levels of urease, repression of GDH, or both were less than wild type. "None" indicates that the strain resembled the negative control bearing the empty vector pCB1041.

[d] Western blots were performed on samples from most strains with defects in NAC regulation. +, approximately as much signal as wild type; +/−, a signal was easily visible but less intense than that seen with wild-type cells on the same blot; −, no signal was detected. For reasons that are unclear, the strain expressing the R5A substitution gave a noticeably stronger signal in the Western blot than the wild type, and this is indicated by ++ in the table. ND, not determined.

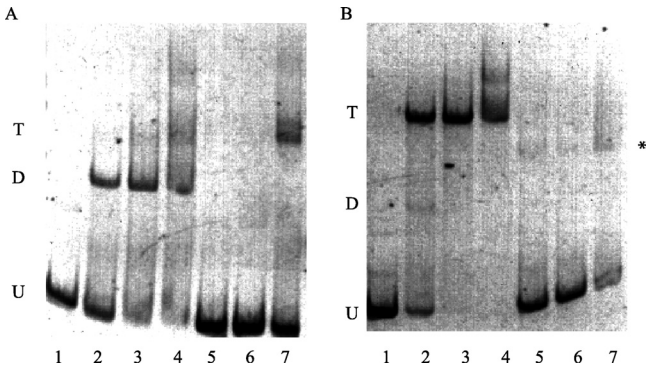

FIG. 1. Electrophoretic mobility shift analysis of the binding of NAC^{WT} and NAC^{Q29A} to the NBSs from *hut* and *nac*. A radioactive EMSA was performed as described in Materials and Methods. All reaction mixtures contained DNA fragments at a concentration of 300 nM in a 10-μl reaction volume. Lanes 2 to 4, 2.6, 5.1, and 10.2 pmol of His-tagged NAC^{WT}, respectively; lanes 5 to 7, 2.6, 5.1, and 10.2 pmol of His-tagged NAC^{Q29A}, respectively. (A) The DNA fragment was a ca. 250-bp fragment from plasmid pOS1 containing the NBS^{hutU}. (B) The DNA fragment was a ca. 280-bp fragment from plasmid pJF200 carrying the NBS^{nac}. Letters at the left of the gels indicate the positions of the unshifted DNA (U), the band corresponding to a dimer bound to DNA (D), and the band corresponding to a tetramer bound to DNA (T). The asterisk to the right of the gel in panel B indicates a band whose mobility corresponds to none of the above.

of NAC bound to DNA (38). Binding of NAC^{Q29A} was also tested with NBS^{nac}, where the presence of two dimer-binding sites allows NAC^{WT} to bind as a tetramer but also to bend the DNA significantly (8, 37, 38). Figure 1B shows that NAC^{WT} bound almost exclusively as a tetramer in this experiment with only a trace band representing dimeric NAC bound to DNA. In contrast, NAC^{Q29A} yielded almost no shifted material, and such shifted material as was seen had a mobility characteristic of a tetramer of NAC bound to DNA without bending the DNA (38).

Dimers of NAC^{Q29A} did not bind tightly enough to the site at *hut* to be detected by EMSA. However, a band with the mobility expected of a tetramer was detected at higher concentrations of NAC^{Q29A}, suggesting that NAC^{Q29A} does retain some specific binding affinity. The DNase I footprint in Fig. 2 (lanes 3 to 6) shows that when NAC^{WT} was present in excess, it strongly protected the known NBS^{hutU} (indicated by the thick line in Fig. 2) as well as a nonconsensus site with no regulatory significance (indicated by the thin line in Fig. 2). The latter site (approximately −18 to +18 in Fig. 2) appears to bind a NAC^{WT} tetramer even in the absence of the strong site if NAC^{WT} is in excess (Fig. 3). NAC^{Q29A} also showed protection of the known NBS^{hutU} but only at a higher concentration of NAC (Fig. 2, lanes 7 to 10). Thus, the Q29A substitution

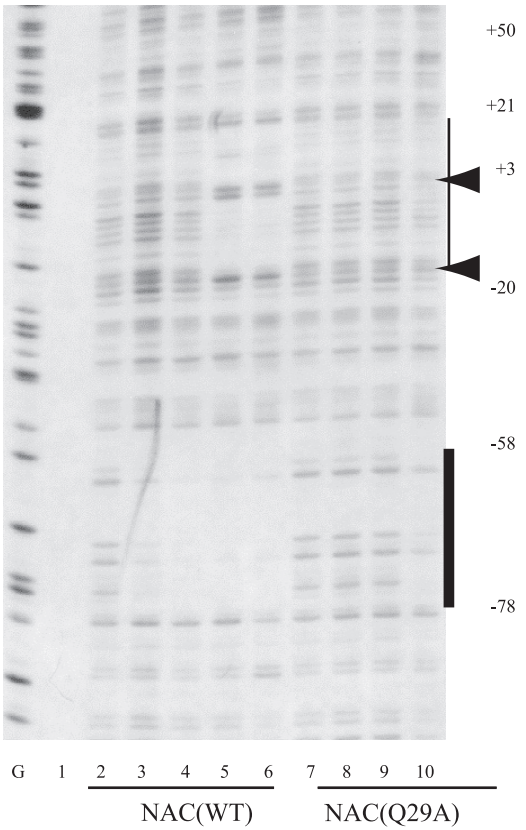

FIG. 2. DNase I footprint of NAC^{WT} and NAC^{Q29A} binding to the NBS^{hutU}. A radioactively labeled fragment from plasmid pCB1560 was digested with DNase I in the presence of NAC^{WT} (lanes 3 to 6, containing 0.6, 1.1, 2.7, and 5.6 pmol, respectively) or NAC^{Q29A} (lanes 7 to 10, containing 0.6, 1.2, 2.3, and 4.7 pmol, respectively). Lane 1, no DNase I; lane 2, no NAC; lane G, a G ladder of the same fragment of DNA. The thick line on the right of the footprint is the protection shared by NAC^{WT} and NAC^{Q29A}. The thin line is the protection of a nonconsensus DNA region by NAC^{WT} (see Fig. 3). The numbers represent the position of the nucleotides relative to the start of transcription. Arrowheads indicate sites of DNase I hypersensitivity.

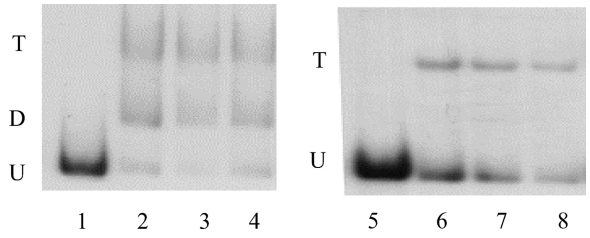

FIG. 3. Binding of NAC^{WT} to a nonconsensus site on DNA. Nonradioactive EMSA of DNA fragments with (lanes 1 to 4) or without (lanes 5 to 8) an NBS. DNA for lanes 1 to 4 was PCR amplified from plasmid pCB648 (34), which carries the NBS from *hut*. DNA for lanes 5 to 8 was PCR amplified from plasmid pCB903 (35), in which the NBS in pCB648 was "scrambled" by reversing the sequence without reversing the 5′-to-3′ orientation. Thus, the NAC-binding sequence ATAA CAAAATTGTAT in pCB648 is replaced by TATGTTAAAACA ATA, which does not bind NAC (35). Both of the amplified fragments contain the region referred to as nonconsensus in Fig. 2. A nonradioactive EMSA was performed as described in Materials and Methods. Lanes 2 to 4 and 6 to 8 contained 4.1, 4.9, and 5.7 pmol of active NAC^{WT}. Activity was determined based on the ability to shift a *hut* fragment under saturating conditions. Each lane contained about 35 ng of the 150-bp PCR-derived fragment.

appears to significantly reduce the affinity of NAC for its normal binding sites, consistent with its presumed position in the HTH region of the DNA-binding domain of NAC.

A NAC dimer can interact weakly with almost any AT-rich DNA, but this interaction is too weak to be detected under our EMSA conditions unless NAC is present in considerable excess. We have shown that the specific binding of a NAC tetramer at a true NBS involves three sets of interactions: two of them involve the interaction of each dimer with its specific NBS and the third involves the interaction between the two dimers within the tetramer (37). If one of those three interactions is weak, a NAC tetramer can still bind, but the binding is not strong enough to bend the DNA (37). Thus, it is not surprising that the NAC bound to weak sites (AT rich but lacking specific sequences) appears as a bound tetramer, where each dimer contributes a small amount of binding energy. The binding of either NAC^{WT} or NAC^{Q29A} to the fragment lacking a specific NBS most likely represents such a nonspecific affinity for AT-rich DNA.

While it is not surprising that a substitution in the helix-turn-helix (DNA-binding) region of the protein would affect the ability of NAC to recognize its specific sites, it does not necessarily mean that Q29 interacts directly with DNA. In fact, the crystal structure of the related LTTR, CbnR, suggests that the side chain of Q29 is directed away from the DNA and into the body of the N-terminal domain of the protein (29). So, it is more likely that the Q29A substitution distorts the general structure of the DNA-binding domain in ways that reduce specific binding more than nonspecific binding.

NAC^{I71A} is monomeric. NAC^{I71A} was also cloned with a C-terminal His tag and purified by nickel affinity chromatography. The structure of CbnR suggests that the amino acid corresponding to I71 should lie solidly within the long helix responsible for holding the monomers of LTTRs together in the dimer (42). Therefore, we used FPLC to compare the apparent size of the His-tagged NAC^{I71A} to His-tagged versions of NAC^{WT} and NAC^{L111K} (Fig. 4). NAC^{WT} is known to elute in FPLC as a tetramer, and NAC^{L111K} is known to elute

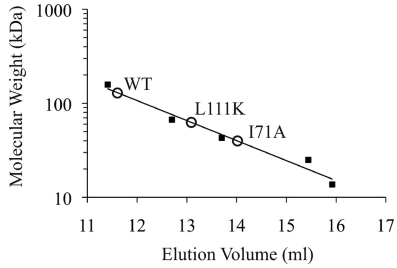

FIG. 4. FPLC analysis of NAC^{I71A}. His-tagged NAC^{I71A} was applied to a Sephadex S200 column and eluted in buffer 4. The elution profile of His-tagged NAC^{I71A} was compared to that of His-tagged NAC^{L111K} (known to be dimeric) and His-tagged NAC^{WT} (known to be tetrameric). The elution volumes of these three NAC proteins are indicated (open circles). Protein was detected based on absorbance at 280 nm. Other size standards (solid squares) were aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), chymotrypsin (25 kDa), and RNase (16 kDa).

as a dimer (38). NAC^{WT} eluted with an apparent molecular weight of 128,100 (predicted tetramer molecular weight, 134,307.2), NAC^{L111K} eluted with an apparent molecular weight of 63,900 (predicted dimer molecular weight, 67,153.6), and NAC^{I71A} eluted with an apparent molecular weight of 39,800 (predicted monomer molecular weight, 33,576.8). Thus, the I71A substitution appears to interfere with NAC's ability to form dimers and hence the tetrameric "dimer of dimers" characteristic of LTTRs.

NAC^{H26D} can bind and repress but not activate transcription. Mutants of the carbon catabolite activator protein (CAP, also known as CRP) have been isolated that bind DNA normally (and repress transcription) but cannot activate transcription (15). Since these mutants are defective in positive control of transcription but not in negative control, they have been called "positive control" or PC mutants (15). Many of the PC mutants of CAP are altered in the amino acids that make direct contact with RNA polymerase (5). Mutants of two other LTTRs, GcvA and CysB, have been reported to bind DNA normally but fail to activate transcription (19, 21). By analogy to CAP, these have been assumed to affect amino acids involved in contacting RNA polymerase, but there is no direct evidence for this in any LTTR.

Somewhat surprisingly, our alanine scan of the entire N-terminal domain of NAC failed to identify any substitution that resulted in a PC phenotype, leaving us with no candidates for amino acids that might be required for contact with RNA polymerase. The reported PC mutants of GcvA and CysB are altered at the amino acid corresponding to H26 in NAC. Therefore, we isolated a collection of *nac* mutants that changed H26 to each of the other 19 possible amino acids. Each of these mutant *nac* genes was cloned and tested in a Nac^- strain for the ability to activate *hut* expression, measured as histidase formation, and repress *gdhA* expression, measured as glutamate dehydrogenase (GDH) formation (Table 2). Seven of the mutants (the bottom seven in Table 2) failed to repress *gdhA* significantly or activate *hut*. These were assumed to yield inactive or unstable NAC and were not studied further. Another nine showed at least a 2-fold activation of *hut* (relative to the empty vector control) and were also not studied further (the nine listed below the vector and wild type in Table 2). However, three of the mutants showed strong repression of *gdhA* with weak or no activation of *hut* and were candidates for PC mutants.

In vivo, more NAC is required to activate *hut* than to repress *gdh* (41). Thus, the NAC in the putative PC mutant strains might merely be less stable or less active than wild type, resulting in enough active NAC to repress *gdh* but not enough to activate *hut*. Alternatively, a PC candidate might be generally defective in its ability to activate transcription. To distinguish between these possibilities, we assayed two candidates, H26W and H26D, for activation of *ure* operon expression. *In vivo*, at least as much NAC is required to repress *gdh* as to activate *ure* (41). Thus, repression of *gdh* shows that there is sufficient active NAC to activate *ure*, and failure to activate *ure* suggests a defect in the ability to activate rather than a deficiency of active NAC caused by an unstable or less active NAC. The Nac^- strain with an empty vector had a urease specific activity of 51 nmol/min/mg of protein. With NAC^{WT} on the plasmid, the specific activity was 1,600 nmol/min/mg of protein. With NAC^{H26W} on the plasmid, the specific activity of urease was 1,900 nmol/min/mg of protein, and with NAC^{H26D} the specific activity was 68 nmol/min/mg of protein. Thus, NAC^{H26D} met our criteria for a PC mutant in that it failed to activate *ure* expression even though there was sufficient active NAC present to repress *gdh* expression. However, the fact that the H26A substitution was indistinguishable from wild type suggests that the role of H26 may be something other than to provide a required contact with RNA polymerase.

TABLE 2. Effects of amino acid substitutions at position H26

Substitution[a]	Sp act[b] (nmol/min/mg of protein)	
	Histidase	GDH
Vector	30	733
Wild type	215	29
H26A	215	40
H26K	157	23
H26R	133	35
H26M	128	39
H26N	108	23
H26Y	93	42
H26L	84	63
H26F	78	110
H26Q	72	19
H26W	57	86
H26D	54	64
H26E	32	36
H26P	30	252
H26I	30	202
H26T	29	768
H26C	28	668
H26G	25	556
H26V	20	593
H26S	42	754

[a] The mutant *nac* alleles were cloned in the low-copy-number vector pCB1041 and transferred into the Nac^- strain KC4598.

[b] Cells were grown and assayed for histidase and GDH activities as described in Materials and Methods. Specific activities reported are the means of at least three independent experiments, with SEM of less than 15% of the means.

EMSA confirmed that NAC^{H26D} binds to NBSs even though it fails to activate *ure* or *hut* expression *in vivo*. Figure 5A shows that both NAC^{WT} and NAC^{H26D} gave the same shifted species of *ure* DNA and that all the *ure* DNA was shifted except at the lowest concentration used here. The situation with *hut* was more complex. Although NAC^{H26D} bound to *hut* DNA (shown by the disappearance of the unshifted band in Fig. 5A), it did not yield a discrete band. The shifted band was diffuse and did not account for all the DNA missing from the unshifted material, suggesting that the complex was not stable during electrophoresis. However, the DNase I footprint of NAC^{H26D} on *hut* DNA is very similar to that of NAC^{WT} (Fig. 6). Thus, NAC^{H26D} fits the formal definition of a PC mutant in the sense that it binds to DNA and represses transcription but fails to activate transcription, although the binding may not be the same as wild type.

Substitutions affecting NAC tetramer formation. LTTR tetramers generally assemble in an extended conformation that binds to a longer DNA-binding site, where the two dimer-binding sites are separated by some distance and formation of the complex results in a significant bend in the DNA. In the presence of their coeffectors, they assume a more compact conformation that binds to a shorter DNA site, where there is

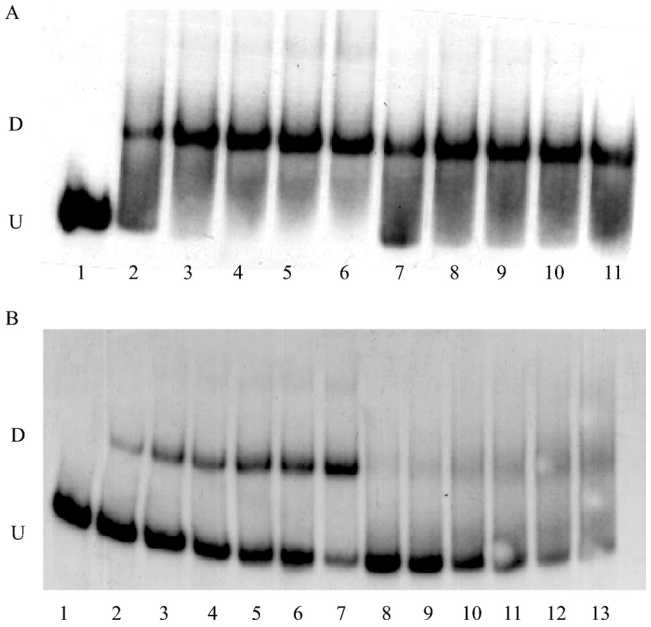

FIG. 5. Binding of NAC^{WT} and NAC^{H26D} to NBS^{hutU} and NBS^{ureD}. A radioactive EMSA was performed as described in Materials and Methods. All reaction mixtures contained DNA fragments at a concentration of 128 nM in a 10-μl reaction volume. (A) The DNA was a ca. 320-bp fragment of plasmid pCB1242 containing NBS^{ureD}. Lanes 2 to 6 contained 1.6, 3.2, 4.8, 5.4, and 7.0 pmol of NAC^{WT}. Lanes 7 to 11 contained 1, 2, 3, 4.1, and 5.1 pmol of NAC^{H26D}. (B) The DNA was a ca. 250-bp fragment of plasmid pOS1 containing NBS^{hutU}. Lanes 2 to 7 contained 1.6, 3.2, 4.8, 5.4, 7.0, and 9.7 pmol of His-tagged NAC^{WT}, respectively; lanes 8 to 13 contained 1, 2, 3, 4.2, 5.2, and 6.1 pmol of His-tagged NAC^{H26D}, respectively.

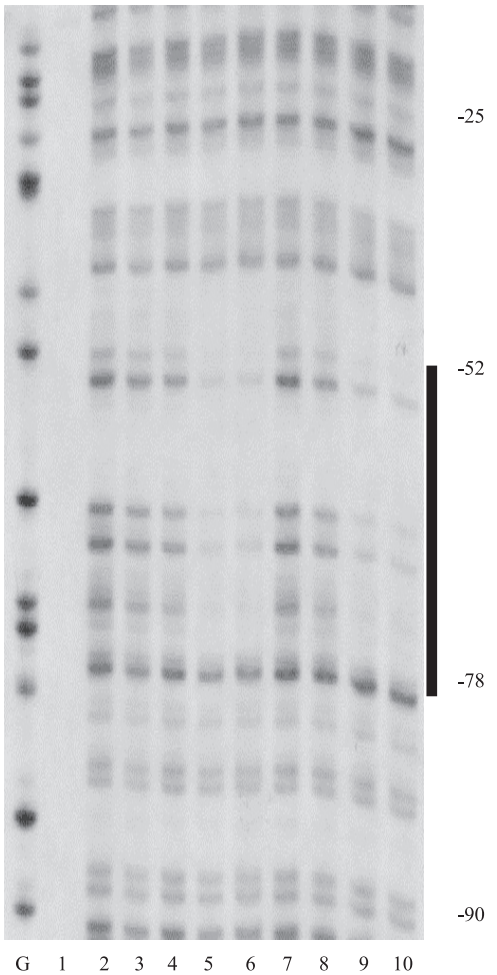

FIG. 6. DNase I footprint of NAC^{WT} and NAC^{H26D} bound to NBS^{hutU}. A radioactively labeled fragment from plasmid pCB1560 was digested with DNase I in the presence of NAC^{WT} (0.55, 1.1, 2.2, and 2.2 pmol of protein in lanes 3 to 6, respectively) or NAC^{H26D} (0.9, 1.8, 2.7, and 3.6 pmol of protein in lanes 7 to 10, respectively). Lane 1, no DNase I or NAC; lane 2, DNase I but no NAC; lane G, a G ladder of the same fragment of DNA. The numbers represent the positions of the nucleotides relative to the start of transcription.

less distance between the dimer-binding sites and the DNA bend angle is reduced (39). We have shown that NAC can bind to both a longer and a shorter DNA site, and we have suggested that this means that NAC can assume both the extended and compact conformations typical of other LTTRs (29, 39, 42). In order to support the argument that the conformations that NAC can assume are indeed different and correspond to those of other LTTRs, we sought mutants that might distinguish one conformation from the other. We had already shown that substitutions affecting amino acids 111 and 125 (NAC^{L111K} and NAC^{L125R}) prevent NAC dimers from assembling into tetramers (38). Biochemical and structural studies of OxyR suggested that amino acids I110 and L124 (corresponding to L111 and L125 in NAC) and the region containing amino acids 219 to 233 make up a tetramerization domain of OxyR (6). The crystal structures of OxyR show that I110 and L124 in one subunit of OxyR interact with A233 in the other subunit in the reduced form (the extended form that recognizes the longer site and bends the DNA) and with F219 in the other subunit in the oxidized form (the compact form that recognizes the shorter site and bends the DNA less dramatically). Lochowska et al. showed that the A227D substitution of CysB (corresponding to amino acid I222 of NAC) also fails to bend the DNA to which it binds (21), suggesting a defect in one conformation of CysB.

Substitutions that changed L111 or L125 in NAC to a basic amino acid prevented NAC from forming tetramers of any kind (38). We reasoned that introducing a basic amino acid into one of the regions that can interact with the L111/L125 region might also prevent NAC from forming one or both forms of the tetramer. Therefore, we changed amino acids in the region from 217 through 233 to arginine to test for effects on tetramer conformation. The resulting mutant *nac* genes were cloned in plasmid pCB1026 (pQE70 with an EcoRI-BamHI fragment carrying *nac*), which allowed expression of the cloned gene under the control of an isopropyl-β-D-thiogalactopyranoside-inducible promoter and adds a C-terminal His tag to each protein to allow for protein isolation. Two of the mutants, NAC^{L225R} and NAC^{A230R}, were not stably expressed in *E. coli* and were not studied further.

We then tested each mutant protein for its ability to bind and bend a longer DNA site (*nac*) and a shorter site (*cod*) as described in reference 37, which also appears in this journal issue. The NAC-binding region on each DNA fragment was

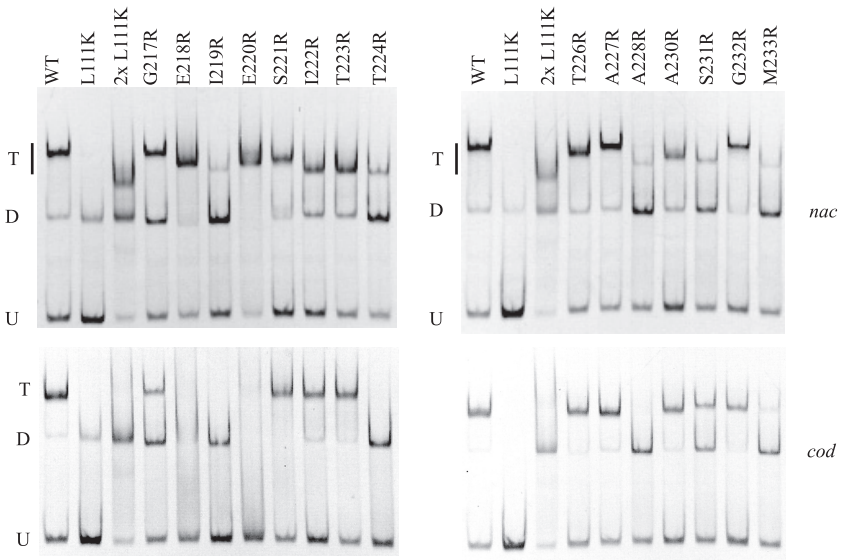

FIG. 7. Binding of NAC mutants to the (longer) NBS^{nac} and the (shorter) NBS^{codB}. The specific activities of the mutant proteins are unknown, and so approximately 30 to 45 ng of the 150-bp fragments amplified from the *nac* and *cod* promoter regions was mixed with enough mutant protein to shift about 50% of the DNA. NAC^{WT} was included to indicate the mobility of the complex with the longer site from *nac*, which is significantly bent, and NAC^{L111K} is included to indicate the mobility of a dimer bound at *nac* or *cod*. Lanes marked 2xL111K have twice as much NAC^{L111K}, to indicate the mobility of a fragment containing two dimers of NAC. The letters to the left of the gels indicate the mobilities associated with the unshifted DNA (U), the DNA bound by a dimer (D), and the DNA bound by a tetramer (T). For the upper (*nac*) panels, the tetramer-associated mobility covers a range between that represented by two dimers of NAC^{L111K} (minimum bending of the DNA) and that represented by NAC^{WT} (significant bending of the DNA).

positioned at the center of the fragment to maximize the effect of DNA bending. Two of the mutants, NAC^{A227R} and NAC^{G232R}, gave the same mobility shift as wild type in this test (Fig. 7). These mobilities have been shown to represent NAC bound as a tetramer at the longer (*nac*) site with a significant bend introduced into the DNA (9, 38), and NAC bound as a tetramer to the shorter (*cod*) site without significant bending of the DNA (32, 37). Five other mutants, NAC^{I219R}, NAC^{T224R}, NAC^{A228R}, NAC^{M233R}, and possibly NAC^{S231R}, appeared to be severely defective in tetramer formation and bound to both *nac* and *cod* almost exclusively as dimers. The remaining mutants showed wild-type tetramer binding to one of the sites but with some sort of defect in binding to the other. For example, NAC^{I222R}, NAC^{T223R}, and NAC^{A230R} showed normal tetrameric binding to *cod*, but the binding to *nac* was aberrant in that the mobility of the shifted band did not correspond to that seen with the wild type. It was more retarded than that expected of two independent dimers of NAC^{L111K} (seen in the lane marked 2xL111K) but less retarded than that seen with the wild type (WT lane). Apparently, NAC^{I222R} and NAC^{T223R} are not able to properly bend the *nac* DNA fragment, suggesting that they cannot assume the extended form of an LTTR. In contrast, NAC^{G217R} shows a normal tetrameric shift of *nac* DNA with the same mobility as the (bent) wild-type complex, but binding to *cod* DNA left much of the material with a mobility characteristic of a dimer/DNA complex, suggesting that it cannot assume the compact form of an LTTR that binds to shorter DNA sites.

***In vivo* effects of tetramerization mutants.** The simplest explanation for these data is that some of these mutants (e.g., NAC^{G217R}) might be defective in forming the compact form of a NAC tetramer that recognizes the short footprint sites, like NBS^{codB}. Other mutants (e.g., NAC^{I222R} and NAC^{A230R}) might be defective in forming the extended form of the tetramer that recognizes the long footprint sites, like NBS^{nac}, where NAC bends the DNA to which it binds. To test whether these mutants were able to form functional tetramers of any kind *in vivo*, we examined the abilities of single and double mutants to repress *gdhAp* expression *in vivo*.

Each of the mutant *nac* genes was cloned without a His tag in the low-copy-number vector pCB1051, which allows expression of *nac* from the *lacZ* promoter. In each case, activation of *hut* expression was monitored by assaying histidase formation to guarantee that defects in *gdhAp* repression did not result from a less stable or less active NAC. (Recall that more NAC is required to activate *hutUp in vivo* than to repress *gdhAp* and that activation of *hutUp* does not require tetramer formation.) There are two different forms of repression exerted by NAC at *gdhA*. In the absence of NAC, ArgP binds to a site upstream from the *gdhA* promoter and activates *gdhA* expression about 2.5- to 3-fold (12, 17). The binding of a dimer of NAC to this same site prevents ArgP binding and activation and thus reduces GDH expression about 2.5- to 3-fold. We refer to this as "weak repression." The effect of this weak repression can be mimicked by the addition of lysine to the growth medium, since lysine prevents ArgP from binding and activating (12). An independent effect occurs when a second dimer of NAC binds to a site downstream from the promoter. If the NAC is able to form a tetramer, then a much stronger, 10-fold repression of *gdhA* expression is observed (14). Mutants that cannot form tetramers do not exert this "strong repression" (38). Thus, the *in vivo* tests monitor two functions of NAC: binding to the site upstream from *gdhA* and tetramerization of NAC. These data are presented in Table 3.

TABLE 3. Substitutions affecting the tetramerization domain of NAC

Substitution(s)[a]	Lysine[b]	Sp act[c] (nmol/min/mg of protein)	
		Histidase	GDH
Vector (Nac$^-$)	−	52	585
	+		211
Wild type	−	248	29
	+		19
G217R	−	310	33
	+		20
I219R	−	322	174
	+		105
I222R	−	287	27
	+		23
I222S	−	246	47
	+		ND
T223R	−	97	292
	+		91
T224R	−	176	244
	+		145
T226R	−	177	52
	+		33
A227R	−	96	218
	+		80
A228R	−	180	213
	+		131
A230R	−	178	37
	+		15
A230S	−	196	22
	+		ND
G217R, I222R	−	248	193
	+		168
G217R, I222S	−	207	141
	+		98
G217R, T223R	−	163	235
	+		109
G217R, T224R	−	185	192
	+		97
G217R, T226R	−	165	254
	+		133
G217R, A228R	−	148	178
	+		172
G217R, A230R	−	249	205
	+		169
G217R, A230S	−	115	185
	+		115

[a] All *nac* genes were cloned in the low-copy-number vector and transferred into the Nac$^-$ strain KC4727 (*hutC515* [Δ*bla-2*] *nac-204*::λ*plac* Mu53). Cells were grown and assayed for histidase and GDH activities as described in Materials and Methods.

[b] The presence or absence of L-lysine (0.01%, wt/vol) in the growth medium. The presence of lysine mimics the weak repression caused when NAC binds to the *gdh* promoter by preventing ArgP-mediated activation of *gdh* expression (12, 17).

[c] Specific activities reported are the means of at least three independent experiments, with SEM of less than 15% of the means. ND, not determined.

Most of the mutants were relatively good at activating histidase formation. Only two of the mutants, those carrying the T223R and A227R substitutions, showed less than half the wild-type level of histidase activity. We did not consider these two mutants further. Three of the substitutions shown in Table 3 (I219R, T224R, and A228R) appeared to be unable to form tetramers of either type effectively (Fig. 7). These showed the weak repression of GDH expression but not the strong repression. The remaining substitutions showed the strong repression of GDH repression, which we have shown to require tetramer formation (38). Note that this group included NAC^{G217R}, which had difficulty assuming the compact form of the tetramer, which recognizes shorter DNA sites. It also included NAC^{I222R} and NAC^{A230R}, which had difficulty forming the fully bent extended form of the tetramer, which recognizes longer DNA sites. We also generated two additional substitutions, I222S and A230S, which have less drastic substitutions than their counterparts I222R and A230R. These mutants also showed strong repression of GDH expression, suggesting tetramer formation. We interpret these data as indicating that the DNA loop presumed to be required for strong *gdhAp* repression can be formed by either the compact form of the tetramer (using an interaction between the L111/L125 region of one subunit and the G217 region of the other) or by the extended form of the tetramer (using an interaction between the L111/L125 region and the I222/A230 region). If this interpretation is correct, then a double mutant carrying a G217R substitution and either an I222R or an A230R substitution should be unable to form either type of tetramer and thus should be as defective in *gdhAp* repression as NAC^{L111K} (38).

Double mutants carrying the G217R substitution and either I222R or A230R were tested, as were the equivalent double mutants with G217R and I222S or A230S. All four of the double mutants showed activation of *hut*, although G217R A230S seemed less effective in this activity. However, all four of the double mutants failed to show strong repression of GDH expression (suggesting lack of tetramer formation), even though weak repression was intact (showing binding of dimers to be intact).

DISCUSSION

When this work was initiated, the CbnR protein was the only LTTR for which a high-resolution structure of the entire tetrameric protein existed (29). Since then, two additional structures of typical LTTRs have been solved (28, 48). Although subtle differences exist in the structures, the general principles identified by the CbnR structure and discussed here are mirrored in these two newer structures as well. Therefore, we have used the CbnR structure as our model here. Unfortunately, there are no examples where a high-resolution structure of an intact tetramer exists in both the effector-bound and the effector-free state. However, there are high-resolution structures of the C-terminal domains of several other LTTRs, and for some of these, e.g., OxyR (6) and DntR (42), the orientation of the monomers at the tetrameric interface has been determined under conditions that correspond to the extended and compact forms of the tetramer. It is clear that the structural conservation among the LTTRs is even higher than the conservation of primary sequence. For example, the C-terminal domain of

BenM shares only 12% and 21% amino acid identity with the C-terminal domains CysB and OxyR, respectively. However, the α-carbon backbone of the C-terminal domain of BenR, when aligned with the C-terminal domains of CysB and OxyR, shows average root mean square deviations of only 2.6Å and 2.5Å, respectively (7). This surprising conservation of structure makes it possible to use the structures of CbnR and OxyR to suggest interpretations for the phenotypes of the NAC substitutions described here.

The Q29A substitution, which greatly reduces the affinity of NAC for its specific binding site (though not for nonspecific binding) lies in the predicted helix-turn-helix region. The I71A substitution, which prevents dimerization of NAC monomers, lies in the middle of the predicted dimerization helix. The H26D substitution, which fails to activate *hut* expression but still binds at *hut* and both binds and represses at *gdhA*, affects the same amino acid as the PC mutants of GcvA and CysB (19, 21). All of these findings suggest that, at least to a first approximation, the structure of NAC is typical of other LTTRs.

We were initially surprised that the alanine scanning mutagenesis did not yield any PC mutants. Therefore, we, like others (21), followed the example of GcvA and looked at amino acid H26, equivalent to the F31 of GcvA, where the PC mutant was found (19). Of all the amino acid changes possible at H26, only the replacement of histidine with an acidic amino acid (H26D or H26E) resulted in a PC phenotype. The other candidate for PC in Table 1, H26W, was fully able to activate urease. We have not determined whether H26W is specifically defective in its ability to activate *hut* expression or whether it simply produces too little NAC to activate *hut* but still produces enough to activate *ure* and repress *gdhA*. We also noted that the binding of NAC^{H26D} was less stable in the EMSA than NAC^{WT}, even though the DNase I footprint appeared normal. The location of H26, at the edge of the first helix of the HTH motif, just before the turn, is curious. It seems an unusual location for an amino acid located so close to the DNA in the HTH motif to be in contact with RNA polymerase. It is also curious that so many substitutions at H26, including H26A, were essentially wild type for activation. This is not the behavior expected of an amino acid residue that makes an essential contact with RNA polymerase. Moreover, it is difficult to reconcile the fact that NAC activates *hutUp* from a site centered at −64 and *ureDp* from a site centered at −47 with the notion that the same H26 residue could make the essential contact with RNA polymerase (20). Lochowska et al. (22) have shown that CysB interacts with the α-CTD of RNA polymerase in a two-hybrid assay and that a Y27A substitution eliminates this interaction. It remains to be seen, though, if the Y27A substitution of CysB (or the H26D substitution of NAC) defines the site of the interaction with RNA polymerase or some more general conformational defect.

We have suggested elsewhere (37) that NAC can assume two different tetrameric conformations, one corresponding to a coeffector-free LTTR and one corresponding to a coeffector-bound LTTR. This was based on the observation that NAC could bind to two different kinds of DNA sites, a shorter site with two dimer-binding sites adjacent to each other and a longer site with the two dimer-binding sites separated by about 10 bp. These would be the two kinds of sites normally recognized by the coeffector-bound and coeffector-free forms of an LTTR, respectively. However, there is no coeffector for NAC. Therefore, in order to strengthen our argument about two distinct but interconvertable conformations of NAC, we sought substitutions that would prevent NAC from assuming one or the other of the conformations. Our data suggest that G217R and I222R are just such types of a substitution. *In vitro* EMSAs showed that NAC^{G217R} binds to a longer site (*nac*) and yields the mobility shift typical of strongly bent DNA, exactly as is seen with NAC^{WT}. However, it has difficulty in recognizing the shorter site (*cod*), and much of the shifted material has the mobility of a dimer rather than of a tetramer. In contrast, NAC^{I222R} binds normally to *cod*, but the binding at *nac* is aberrant in that the mobility of the band representing tetramers does not show the normal bend angle seen with NAC^{WT}. One might expect that NAC^{G217R} and NAC^{I222R} would be defective *in vivo* for regulating *cod* and *nac*, respectively. However, both the activation of *cod* and the repression of *nac* can be accomplished almost as well by the dimeric form of NAC as by the tetrameric form (18, 32), so those defects are in fact not observed.

Although the *in vivo* data for *gdhA* expression are not directly comparable to the *in vitro* EMSA data for *nac* and *cod*, the *gdhA* data argue strongly that the double mutant $NAC^{G217R,I222R}$ cannot form the tetramers required for the strong repression of *gdh* expression. However, the double mutant still retains its ability bind at *gdh* (presumably as a dimer) in that $NAC^{G217R,I222R}$ still exerts the "weak repression" that results from its ability to prevent binding of ArgP. $NAC^{G217R,I222R}$ can also bind and activate *hut* expression, again presumably as a dimer. In contrast, each of the single mutants, NAC^{G217R} and NAC^{I222R}, gave full repression of *gdh*, suggesting that tetramers were formed and were strong enough to generate the interaction between the two NBSs at *gdhA* that is needed for strong repression.

The effects of the G217R and I222R substitutions on *gdh* expression also lend support to the model that repression of *gdhA* requires the formation of a loop between the two separate NBSs in the *gdhA* region, one centered at −89 relative to the start of transcription and the other centered at +57 (14). The cartoon in Fig. 8 shows how one conformation of NAC would bind to a shorter site with little bending and to a longer site with considerable bending. If the two dimer-binding sites within a tetramer site are near each other, as in *cod* or *nac*, then the single mutants, NAC^{G217R} or NAC^{I222R}, should each function at one of the sites but not both. On the other hand, if the two dimer-binding sites are distant from each other as in *gdh* and if a loop is formed by the tetramer as part of the mechanism of repression, then either of the single mutants would be functional and it would require elimination of both conformers, as in the double mutant, to eliminate functional binding.

One final point to note is that we have shown here that at sufficiently high concentrations of NAC, binding of NAC tetramers, but not dimers, can be detected that has no physiological relevance. Such binding sites, with a range of affinities for NAC, have been seen before in places as unexpected, as with the *bla* gene of pUC19 (13), and have even been footprinted at several sites (36), showing that they are not entirely nonspecific. Nevertheless, they are unlikely to function under normal conditions, since autorepression of *nac* expression keeps the

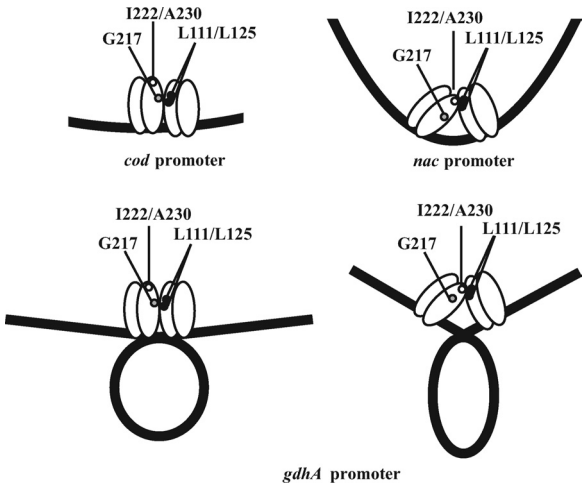

FIG. 8. Interaction of NAC tetramers with DNA containing two dimer-binding sites. This cartoon illustrates how substitutions affecting one form of the NAC tetramer but not the other can affect NAC binding. If two dimer-binding sites were close together (as at *cod*), then only the compact form of NAC tetramer would be able to recognize the site. NAC^{G217R} would not be able to make the stable interactions between the L111/L125 region of one dimer with the G217 region of the other dimer, and thus the ability to form an compact tetramer would be impeded. If two dimer-binding sites were separated by 10 bp (as at *nac*), then only the extended form of NAC tetramer would be able to recognize the site. $NAC^{I222R}$and NAC^{A230R} would not be able to make the stable interactions between the L111/L125 region of one dimer with the I222/A230 region of the other dimer, and thus the ability to form an extended tetramer would be impeded. However, if the two dimer-binding sites were separated by enough DNA to allow a loop to form, then either the compact or the extended form of NAC tetramer would be able to recognize the sites and form a loop. Substitutions affecting both the G217 region and the I222/A230 region would have difficulty forming stable tetramers of either configuration, similar to the mutants affecting L111 and L125, which have been shown to be dimeric.

concentration of NAC at physiologically relevant levels *in vivo* (9, 25). On the other hand, we and others have noted that artificial overproduction of NAC is quite toxic to cells (2–4), suggesting that occupancy of some of these sites may be deleterious. This atypical binding of NAC seems to require a tetramer; we have never seen dimers binding to such sites. We assume that four monomer HTH motifs interacting weakly with DNA are sufficient to hold a tetramer, whereas two monomers interacting weakly are not sufficient to hold a dimer. In fact, this may explain why NAC functions as a dimer at many of the sites where NAC activates gene expression. Such dimer-binding sites would have to have a higher affinity for NAC and would thus be more likely to be the most specific and the first to bind NAC. In trying to identify the nucleotides important for binding of NAC, we have been surprised at the number of different sequences that can serve as a half-site for dimer binding within either a dimer-binding site or a tetramer-binding site (37). The rules are far from clear. Although this may simply be a defect in the design of NAC, it is also possible that this is a potent evolutionary strategy, allowing NAC to bind weakly to a large number of potential promoters and then to evolve more effective binding under selective pressure. This might explain how NAC has been able to bring so many different genes into the NAC regulon of *K. pneumoniae* (11).

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