The LysR-Type Nitrogen Assimilation Control Protein Forms Complexes with Both Long and Short DNA Binding Sites in the Absence of Cofactors[▽]

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Most LysR-type transcriptional regulators (LTTRs) function as tetramers when regulating gene expression. The nitrogen assimilation control protein (NAC) generally functions as a dimer when binding to DNA and activating transcription. However, at some sites, NAC binds as a tetramer. Like many LTTRs, NAC tetramers can recognize sites with long footprints (74 bp for the site at *nac*) with a substantial DNA bend or short footprints (56 bp for the site at *cod*) with less DNA bending. However, unlike other LTTRs, NAC can recognize both types of sites in the absence of physiologically relevant cofactors, suggesting that the two conformers of the NAC tetramer (extended and compact) are interchangeable without the need for any modification to induce or stabilize the change. In order for NAC to bind as a tetramer, three interactions must exist: an interaction between the two NAC dimers and an interaction between each NAC dimer and its corresponding binding site. The interaction between one dimer and its DNA site can be weak (recognizing a half-site rather than a full dimer-binding site), but the other two interactions must be strong. Since the conformation of the NAC tetramer (extended or compact) is determined by the nature of the DNA site without the intervention of a small molecule, we argue that the cofactor that determines the conformation of the NAC tetramer is the DNA site to which it binds.

The nitrogen assimilation control protein (NAC) of *Klebsiella pneumoniae* is a LysR-type transcriptional regulator (LTTR) that regulates a variety of genes and operons whose products allow the cell to utilize alternative nitrogen sources in place of ammonium, the preferred source (2, 3, 24). In particular, NAC activates expression of operons whose products catabolize histidine (*hut*), proline (*put*), urea (*ure*), alanine (*dad*), and cytosine (*cod*) as nitrogen sources (3, 11, 14, 19). Electrophoretic mobility shift analysis (EMSA) has shown that NAC binds to the promoter region of the *hutUH* operon (*hutUp*) and to the promoter region of the *ureDABCEFG* operon (*ureDp*) as a dimer (22). Binding of a dimer at these sites generates a DNase I footprint of about 28 bp (9). NAC also generates a DNase I footprint of 25 bp at *putPp*, again consistent with a dimer of NAC bound at this promoter (9). *codBp* from *Escherichia coli* is different in that EMSA shows that NAC binds as a tetramer, and the DNase I footprint of about 56 bp confirms this (19). However, a shorter (and still functional) version of *codBp* showed a dimer of NAC bound (by EMSA) and a footprint of about 28 bp (19). Centered within the 28-bp region defined by these DNase I footprints, we see a consensus sequence of ATA-N₉-TAT (9). Genetic analysis of this sequence within *hutUp* confirms that these nucleotides are important for NAC binding and function (21).

Like all LTTRs (15, 23), NAC assembles as a dimer of dimers (22). Although wild-type NAC is a tetramer in solution, we have characterized a mutant NAC (NAC^{L111K}) that cannot form the tetramer and remains a simple homodimer under all conditions tested (22). The L111K amino acid change lies in the C-terminal (regulatory) domain of NAC and suggests that the C-terminal domain is important for tetramer formation. We also found that NAC mutants containing only the N-terminal, DNA-binding domain (NAC^{100ter} and NAC^{86ter}) are dimeric under the conditions tested, arguing that the C-terminal domain is essential for tetramerization (18). All of these dimeric mutants are able to activate transcription from *hutUp* and other promoters (18, 22). Thus, the dimer of NAC is the functional form of NAC that activates transcription at many promoters.

In contrast, both EMSA and DNase I footprinting have shown that NAC binds as a tetramer at the promoter of the *nac* gene (8) and at the *codBp* promoter of the *codBA* operon from *E. coli* (19). EMSA and DNase I footprinting shows that NAC binds to each of two sites in the promoter region of the *gdhA* gene as a dimer (22). However, tetramerization of NAC is essential for the NAC-mediated repression of *gdhA* that uses these two sites, presumably by a DNA looping mechanism (22). Virtually all LTTRs give tetramer footprints at most of their binding sites (recently reviewed in reference 15). Also, most LTTRs give two different footprints, depending on the presence or absence of a regulatory cofactor. This cofactor is usually a small molecule related to the function being regulated, but in the case of OxyR, it is a disulfide bond formed within the C-terminal domain (5, 28). In the absence of a cofactor, most LTTRs give a relatively long footprint and elicit some degree of DNA bending; in the presence of the

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coeffector, the footprint is shorter and the DNA bend angle is shallower. As a result, in the absence of the coeffector, the LTTR recognizes a DNA region that contains two dimer-binding sites with some space between them. In the presence of the coeffector, the LTTR recognizes a region that contains two dimer-binding sites with less space between them. Thus, the DNA sites recognized by LTTRs with and without a coeffector are different, and the presence of the coeffector alters which sites are recognized. In some cases, the longer and shorter sites overlap so that it appears the coeffector shortens the footprint, but in others, the sites are distinct and the coeffector appears to increase the affinity of the LTTR for a shorter site while weakening the affinity for the longer sites.

NAC bound to the *nac* promoter produces a DNase I footprint of about 74 bp and induces a significant DNA bend with significant DNase I hypersensitivity at the site of the bend (8, 22). NAC bound to *codBp* produces a DNase I footprint of about 56 bp and very little DNase I hypersensitivity, perhaps suggesting less DNA bending (19). In other words, NAC appears to be able to produce footprints characteristic of both the modified and unmodified forms of LTTRs. However, there is no physiologically relevant coeffector for NAC (25). At every DNA site tested, NAC binds equally well in the presence or absence of any small molecule tested and regardless of whether the NAC was purified from cells grown under nitrogen excess or nitrogen limitation (9). Similarly, activation of *hut* and *ure*, as well as repression of *gdh*, occurs equally in strains with excess or limiting nitrogen if NAC is produced from an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter (25). If no coeffector exists and if NAC can function as either a dimer or a tetramer, two questions are raised. What determines whether NAC will bind as a tetramer? And what determines whether a NAC tetramer recognizes a longer site (typical of a coeffector-free LTTR) or a shorter site (typical of a coeffector-bound LTTR) if NAC has no coeffector?

Experiments with the NAC^{L111K} mutant suggested that the single tetramer-binding site at *nac* might actually be two adjacent dimer-binding sites (22). The data from *gdhA* expression also suggest that two dimer-binding sites, even when separated by 146 bp, might be able to interact in a way that mediates tetramer formation (10, 22). However, we also noted that neither *nac* nor *gdhAp* contained the consensus ATA-N₉-TAT (10, 22). Each of the three DNase I footprints did, however, include an appropriately located sequence, ATA-N₉-GAT (or its equivalent, ATC-N₉-TAT). So we set out to determine what is required for NAC to bind as a tetramer. We were also interested in determining whether there are two fundamentally different kinds of NAC tetramers, one able to bind to the longer DNA sites and one able to bind to the shorter sites, or whether NAC was a more flexible tetramer than other LTTRs and could assume either or both conformations without the need for a coeffector to stabilize one conformer or switch from one to another.

MATERIALS AND METHODS

PCR. PCR was carried out with plasmid DNA containing either the *nac* promoter (pJF200), the *cod* promoter (pCB816), or the *hut* promoter (pOS1) as a template (8, 19, 20). Reactions were carried out using ca. 10 ng of template DNA and a final concentration of 0.8 μ M for each primer in 50 μ l of Platinum PCR super mix (Invitrogen Corp.). PCR amplification was performed using a

standard PCR program: initial template denaturation (94°C for 5 min) was followed by 35 cycles of amplification (template denaturation at 94°C for 1 min, primer annealing at 51°C for 1 min, and primer extension at 72°C for 1 min), and finally, the program was terminated with a final extension at 72°C for 2 min. For mutations that could not be generated by a single primer pair, a two-step PCR was performed as described elsewhere to obtain the desired double-stranded fragment (1). Briefly, the first step was performed by generating two fragments with 10 to 20 bp of overlapping complementary strands at the 3' ends of the DNA. These fragments were isolated by Qiaquick gel extraction (Qiagen Inc.). These fragments were used as a template in a second PCR with primers that annealed to the 3' ends most distant from the overlapping region to generate a single fragment composed of both initial fragments.

Protein purification. C-terminally His-tagged versions of NAC^{WT} and NAC^{L111K} (henceforth referred to as NAC^{WT} and NAC^{L111K}) were purified as previously described (12). Purity was monitored by SDS-PAGE followed by Coomassie blue staining, using the method of Fairbanks et al. (6). Using band densitometry of the Coomassie-stained gels, the average purity of NAC^{WT} was 95%, and that of NAC^{L111K} was 89%. The activity of NAC^{WT} was approximately 24%, and the activity of NAC^{L111K} was approximately 4%. The protein concentration was determined using the method of Lowry et al. and a bovine serum albumin (BSA) standard (13). Purified protein was stored in 50% (vol/vol) glycerol at -20°C.

EMSA. An electrophoretic mobility shift assay (EMSA) was performed as previously described (9). Briefly, protein was diluted in buffer 6 (50% [vol/vol] glycerol, 125 mM NaCl, 50 mM NaH₂PO₄ [pH 7.0], 1.25 mM MgCl₂, 0.5 mM 2-mercaptoethanol, 1 mg/ml BSA), and DNA fragments were diluted in water. The concentration of protein and DNA added to each reaction is listed in the figure legends. These reactions did not contain nonspecific competitor DNA for two reasons: (i) nonspecific competitor DNA is incompatible with ethidium bromide staining, and (ii) the relatively large amount of DNA that was present in these reactions diminished the need for competitor DNA. Twelve μ l of the desired DNA dilution was mixed with either 8 μ l of buffer 6 alone or 8 μ l of the desired protein dilution and incubated at room temperature for 20 min. A 1.5- μ l volume of loading buffer (40 mM Tris-HCl [pH 8.4], 4 mM EDTA, 0.2% [wt/vol] bromophenol blue, 0.2% [wt/vol] xylene cyanol, 25% [vol/vol] glycerol) was added to each sample, and the sample was loaded onto a 6% gel (polyacrylamide buffered with 0.5 $\times$ Tris-borate-EDTA [TBE]) that had been prerun at 20 V/cm for 30 min (Invitrogen). NAC-DNA complexes were separated by electrophoresis at 20 V/cm for 1 h at room temperature. Gels were stained in ethidium bromide solution (40 μ g/ml) for 10 min at room temperature and then destained in water for 10 min with agitation. DNA was visualized by UV fluorescence.

Competition assay. The PCR product from the *codB* promoter of *E. coli* was end labeled with [³²P]dATP by end filling with Klenow fragment as described previously (16). Labeled DNA was separated from unincorporated nucleotide using Qiagen nucleotide removal columns (Qiagen corp.). For each reaction, 0.16 pmol of NAC in 4 μ l of buffer 6 was mixed with 0.08 pmol of labeled *codBp* DNA and 0, 0.08, 0.16, or 0.32 pmol of unlabeled competitor DNA in 6 μ l of double-distilled water (ddH₂O) for a total reaction volume of 10 μ l. Reaction mixtures were incubated at room temperature for 20 min. A 1- μ l volume of loading buffer was added to each sample, and the samples were loaded onto a prerun 5% acrylamide gel buffered with 0.5 $\times$ TBE. Unbound DNA and NAC-DNA complexes were separated by electrophoresis at 10 V/cm for 60 min. Gels were transferred to filter paper and exposed to RX-B X-ray film (Marsh Bio-products Inc.) for 60 min. Quantitation was performed using the densitometry feature of the Photoshop software program (Adobe Inc.).

DNA bending assay. The DNA bending assay was performed as previously described (27). DNA fragments (150 bp in length) were amplified as described above using the two-step PCR process. Fragments for "*codWT*" and "*nacWT*" had the binding site at 43 bp, 64 bp, or 75 bp from one end of the fragment. Fragments for "*cod*+10" had the binding site at 48 bp, 69 bp, or 80 bp from one end of the fragment. Fragments for "*nac*-10" had the binding site at 39 bp, 59 bp, or 70 bp from one end of the fragment. EMSA on these fragments was performed as described above except the concentration of NAC^{WT} used was adjusted to shift ca. 50% of the DNA for each fragment.

RESULTS

For most LTTRs, the ability to recognize binding sites of two different lengths depends on the presence or absence of a coeffector (15). In general, the free LTTR binds the longer site but not the shorter site. In the presence of the coeffector, the

FIG. 1. Sequence of the *hutU*, *codB*, and *nac* promoters. The matches to the consensus binding sites are boxed. For *hut*, the boxed region is centered at -64 relative to the start of transcription, for *cod*, it is centered at -59 , and for *nac*, it is centered at -75 . Regions protected from DNase I digest by NAC at each promoter are indicated by a bold line over the sequence and represent the farthest edges of the NAC footprint at these promoters. Arrowheads above the sequence indicate regions of DNase I hypersensitivity in the presence of NAC. The ATA half-site at the *codB* promoter and the secondary site of the *nac* promoter (the “*nac** site”) are underlined. Mutants made for analysis are indicated underneath the appropriate sequence and indicate the nucleotides changed, deleted, or inserted. The numbers under the *cod* and *nac* promoter changes correspond to the labels in Fig. 5. “*hutATA(42)*” and “*hutATA(32)*” correspond to labels in Fig. 6. The insertion of 10 bp in the *cod* promoter and the removal of 10 bp from the *nac* promoter refer to labels in Fig. 7.

LTTR is altered such that the shorter site is recognized, but not the longer site. In contrast, NAC recognizes both a long (74-bp) site and a short (56-bp) site in cells growing under the same condition, namely, nitrogen limitation (summarized in Fig. 1). This might suggest that there exist two different isoforms of NAC, one of which is assembled as an extended tetramer that recognizes the longer binding sites and the other of which is assembled as a compact tetramer that recognizes the shorter binding sites. Or it might suggest that a single pool of NAC tetramers is capable of freely converting from one conformation to the other without the need for a coeffector to drive the transition. Such a conversion might be direct or might involve dissociation to dimers as an intermediate step.

The EMSA experiment in Fig. 2 (lanes 1 and 2) shows that NAC binds to a radiolabeled DNA fragment containing the short (56 bp) NAC-binding site from the *cod* promoter region. If cold competitor DNA carrying a long (74-bp) NAC-binding site from the *nac* promoter was present in the binding reaction, it was fully able to compete for the NAC (lanes 3 to 5). When

a fragment known to contain no NAC-binding site (lanes 6 to 8) was used, no competition was seen. The ability of the long site to compete with the short site argues strongly that there is only one pool of NAC that can freely exchange between the forms that bind as a tetramer to the long and short sites.

At some sites (e.g., the *hut* promoter), NAC has been shown to bind as a dimer and to protect a 26-bp region of the DNA, as illustrated in Fig. 1 (9, 22). This raises the question of what features of the binding site determine whether a dimer or tetramer will bind. At sites where NAC activates expression (*viz.* *hut*, *put*, *ure*, *cod*, and *dad*), we have identified a consensus sequence of ATA-N₉-TAT that includes many of the most important determinants for binding (9). At sites where NAC represses expression, the corresponding sequence is ATC-N₉-TAT or its equivalent, ATA-N₉-GAT (10, 22). The NAC-protected region in the *nac* promoter includes one match to the repression consensus (boxed in Fig. 1 and hereinafter called the *nac* site) and a second region, ATA-N₉-GCT (underlined in Fig. 1 and hereinafter called the *nac** site), that differs by only one nucleotide from the repression consensus. This suggested that the *nac* promoter might contain two dimer-binding sites (22). To test this, we compared the binding of wild-type (tetrameric) NAC with the binding of a mutant, NAC^{L111K}, that is unable to form tetramers but retains all known activities associated with dimeric NAC (22). Wild-type NAC has been shown to bind to the site at *nac* primarily as a tetramer that induces a significant bend in the DNA (8), resulting in a greatly retarded mobility of the bound fragment in a gel shift assay (Fig. 3, lanes 1 to 3). When NAC^{L111K} was used in the assay, two shifted species were detected, one corresponding to a bound dimer and one with a mobility corresponding to two bound dimers (22), as seen in Fig. 3, lanes 4 to 6. This is consistent with our previous suggestion that the NAC-binding site at *nac* contains two dimer-binding sites.

In contrast, when the shorter NAC-binding site from the

FIG. 2. Competition between long and short sites for NAC binding. A radiolabeled DNA fragment containing the short NAC-binding site from the *codB* promoter (lane 1) was mixed with purified NAC in a 1:1 ratio (lanes 2 to 8) and analyzed by electrophoretic mobility shift assay. In lanes 3 to 8, unlabeled competitor DNA was added at the ratio indicated. In lanes 3 to 5, the competitor contained the long NAC-binding site from the *nac* promoter. In lanes 6 to 8, the competitor contained a promoter which had a scrambled NAC binding site. The band corresponding to unbound *codB* is indicated by a U, and the band representing a tetramer of NAC bound to *codB* is indicated by a T.

FIG. 3. Interaction of NAC^{WT} and NAC^{L111K} with the *nac* and *cod* promoters. DNA fragments (ca. 0.3 to 0.45 pmol) containing either the *nac* (lanes 1 to 6) or *cod* (lanes 7 to 12) promoter NAC binding regions were incubated with 0.25, 0.5, or 1.1 pmol purified NAC^{WT} (lanes 1 to 3 and lanes 7 to 9, respectively) or 6, 12, or 18 pmol purified NAC^{L111K} (lanes 3 to 6 and 10 to 12, respectively). The bands representing the unbound promoter fragments are indicated by a U, bands representing promoters bound by a dimer of NAC are indicated by a D, and bands representing promoters bound by a tetramer of NAC are indicated by a T.

codBA promoter (19) was used in this experiment, two differences were immediately apparent. First, wild-type NAC gave a mobility consistent with either two dimers of NAC or one tetramer that did not bend the DNA significantly (Fig. 3, lanes 7 to 9). When the dimeric mutant NAC^{L111K} was used, the only species detected had the mobility of a bound dimer (lanes 10 to 12). Thus, the site at *codBp* does not appear to have a second dimer-binding site. This is also reflected in the DNA sequence. Within one side of the *codBp* footprint, we see an activation consensus “the *cod* site” (boxed in Fig. 1), but the other side, the *cod** site, has only a half-consensus sequence, ATA-N₉-cca (underlined in Fig. 1).

To confirm the existence of a separate dimer-binding site at *nac* but not at *cod*, we generated DNA fragments corresponding to the regions upstream from the consensus dimer-binding sites from *hut*, *cod*, and *nac*. It is known that this region from *hut*, *hut**, does not bind NAC (9). The *nac** site did bind NAC as a dimer, but *cod** did not (Fig. 4, lanes 1 to 7 and 8 to 14). Thus, the *cod* promoter does not contain a second independent dimer-binding site. Taken together, these data suggest that NAC binds to the *codBp* region as a tetramer with little or no bending of the DNA.

Nevertheless, the full-length *cod* site can bind a tetramer (or two dimers) of wild-type NAC, while the corresponding region from *hut* cannot (Fig. 4, lanes 8 to 14 and 22 to 28). So there must be something in the *cod** site that allows a second dimer to bind. To identify the elements that allow *codBp* to bind a tetramer (or two dimers), we mutated sets of trinucleotides in the *cod** site to GGG, as indicated in Fig. 1, and asked whether the mutant fragments could still bind NAC tetramers. As a control, we also generated GGG replacements in the *nac** site. The alterations focused on the two key triplets of the near-consensus ATA-N₉-GCT sequence, as well as several nearby. These are indicated in Fig. 1 by the numbers underneath the various GGG elements.

The data in Fig. 5 show that changing ATA in the *nac** site to GGG (no. 1 in Fig. 1) reduced the relative amount of tetramer-bound DNA and yielded relatively more dimer-bound material (Fig. 5A, lanes 4 to 6). Not surprisingly, changing the other key triplet (no. 4 in Fig. 1) also reduced the fraction of tetramer-bound DNA in favor of dimer-bound DNA (Fig. 5A, lanes 16 to 18). Replacement of the triplet preceding this (no. 5 in Fig. 1) also reduced tetramer formation (Fig. 5A, lanes 19 to 21). Thus, within the *nac** site, the two key triplets in the region suspected to be a near-match to consensus, ATA-N₉-GCT, were both important, as was the triplet preceding GCT. Other triplets in the region had little or no effect on tetramer formation (Fig. 5A).

Within the *cod** site, only the first triplet of a consensus was evident by observation (ATA-N₉-cca). Changing the first triplet of this pair to GGG (no. 1 in Fig. 1) reduced tetramer formation (Fig. 5B, lanes 4 to 6), but changing the other triplet (no. 6 in Fig. 1) had little or no effect (Fig. 5B, lanes 22 to 24). Most other alterations in the *cod** site had little or no effect on tetramer formation except for the triplet in the center, between the position of the 2 key triplets (no. 4 in Fig. 1). Changing TTT (within a stretch of five T residues) to GGG led to a slight increase in dimer-bound DNA relative to tetramer-bound DNA (Fig. 5B, lanes 16 to 18).

The data from the mutagenesis of the *cod** site suggested that the ATA triplet was very important for tetramer binding, though other elements of the site might also play some role. To test whether an ATA half-site is sufficient to generate a tetramer-binding site, we placed an ATA sequence upstream from the known dimer-binding site from *hut*. We placed the

FIG. 4. Binding of NAC^{WT} or NAC^{L111K} to the *nac** site and *cod** site. Duplex DNA (1.5 pmol) containing the upstream DNA sequences from NAC-binding sites at *nac* (lanes 1 to 7 and 15 to 21), *cod* (lanes 8 to 14), or *hut* (lanes 22 to 28) was mixed with 0, 1.5, 3, or 6 pmol purified NAC^{WT} (lanes 1 to 4, 8 to 11, 15 to 18, or 22 to 25, respectively) and 4.5, 9, or 18 pmol purified NAC^{L111K} (lanes 5 to 7, 12 to 14, 19 to 21, or 26 to 28, respectively). Unbound and shifted bands are marked as described for earlier figures.

FIG. 5. Interaction of NAC with mutant forms of the NAC-binding sites from *nac* and *cod*. (A) One-hundred-fifty-base-pair DNA fragments (ca. 0.3 to 0.45 pmol) of the wild-type *nac* promoter or the *nac* promoter containing the mutations indicated by the numbers in Fig. 1 were mixed with 0.25, 0.5, or 1.0 pmol of purified NAC^{WT}. The asterisk indicates the mobility of a PCR-amplified DNA fragment that did not interact with NAC^{WT}. (B) One-hundred-fifty-base-pair DNA fragments (ca. 0.3 to 0.45 pmol) of the wild-type *cod* promoter or the *cod* promoter containing the mutations indicated by the numbers in Fig. 1 were mixed with 0.25, 0.5, or 1.0 pmol of purified NAC^{WT}. The asterisk indicates the mobility of a PCR-amplified DNA fragment that did not interact with NAC. The positions of bands corresponding to unbound (U), dimer-bound (D), and tetramer-bound (T) fragments are indicated.

ATA sequence at 32 bp upstream from the *hut* consensus (where the ATA sequence in the *cod** site lies) and at 42 bp upstream (where the ATA sequence in the *nac** site lies). The sites of these changes are illustrated as “*hut*ATA(32)” and “*hut*ATA(42)” in Fig. 1. The data in Fig. 6 show that the ATA at the *cod* distance of 32 bp generated a tetramer-binding sequence but the ATA at the *nac* distance of 42 bp did not.

To test whether the intact *cod** site would function at a distance of 42 bp where a simple ATA triplet did not, we inserted 10 bp (CGAGATCTGC) between the *cod** site and the *cod* site as described in Materials and Methods and depicted in Fig. 1. If such an arrangement allowed a tetramer to bind, we expected that the bound tetramer would have to induce a DNA bend similar to that seen when a tetramer binds at the *nac* site (8, 22). We tested both binding and bending using an EMSA assay involving fragments where the NAC-binding site was located near one end of the fragment, near the middle of the fragment, or halfway in between. As can be seen in Fig. 7, the retardation of the bound fragment containing the wild-type *nac* site increased as the location of the binding site moved from the edge to the middle of the fragment (lanes 7 to 9). This is diagnostic for bending of the DNA by the bound protein (27). In contrast, the retardation of the bound fragment containing the wild-type *cod* site did not change (lanes 1 to 3), consistent with the observation that the DNase I footprint of NAC at *cod* shows much less DNase I hypersensitivity than that at *nac* (8, 19, 22). However, the retardation of the bound fragment containing the *cod* site with the extra 10 bp

FIG. 6. An upstream ATA sequence is sufficient to bind a NAC tetramer at *hut*. One-hundred-fifty-base-pair DNA fragments (ca. 0.3 to 0.45 pmol) containing the *hut* promoter (lanes 1 to 4), the *hut* promoter with an ATA half-site positioned 3 helical turns upstream of the NAC-binding site [“*hut*ATA(32)” in Fig. 1] (lanes 5 to 8), and the *hut* promoter with an ATA half-site positioned 4 helical turns upstream of the NAC-binding site [“*hut*ATA(42)” in Fig. 1] (lanes 9 to 12) were incubated with 0, 0.5, 1.0, or 2.0 pmol purified NAC^{WT} (lanes 1 to 12). The asterisk indicates a PCR-amplified DNA fragment that did not interact with NAC. The positions of bands corresponding to unbound (U), dimer-bound (D), and tetramer bound (T) fragments are indicated.

(*cod*+10 in Fig. 7, lanes 4 to 6) did increase as the location of the binding site moved from the edge to the middle of the fragment. This suggests that NAC binding to the *cod*+10 site results in a significant DNA bend. To complete the set, we also tested a construct where 10 bp from the wild-type *nac* site was deleted, generating a “*nac*–10” site (as depicted in Fig. 1). NAC binding to the *nac*–10 site did not result in a significant bend (Fig. 7, lanes 10 to 12). These data suggest that the *cod** site is stronger than a simple ATA sequence in that it allows NAC to bind to a long site and bend the DNA whereas the ATA half-site can function only at the spacing of the short site.

DISCUSSION

It is common that an LTTR in cells grown under a particular growth condition recognizes one set of binding sites and that same protein in cells grown under a different set of growth conditions recognizes a different set of binding sites (15, 23). The choice of binding site is usually determined by the ar-

FIG. 7. NAC tetramers bend DNA at long sites but not short sites. One-hundred-fifty-base-pair PCR-amplified DNA fragments (ca. 0.7 to 0.8 pmol) that position the NAC-binding region of “*cod*WT” (lanes 1 to 3) or “*nac*WT” (lanes 7 to 9) either 43 bp (lanes 1 and 7), 64 bp (lanes 2 and 8), or 75 bp (lanes 3 and 9) from one end of the DNA fragment are analyzed. The “*cod*+10” NAC-binding region was positioned 48, 69, or 80 bp from one end of the fragment (lanes 4 to 6, respectively), and the “*nac*–10” NAC-binding region was positioned 39, 59, or 70 bp from one end of the fragment (lanes 10 to 12, respectively). Each fragment was incubated with 0.5 pmol of NAC^{WT}. The positions of bands corresponding to unbound (U) and tetramer bound (T) fragments are indicated.

rangement of dimers within the tetramer. Often, in the absence of a coeffector (or a covalent modification), the LTTR binds to a site with a longer footprint and a high-angle bend. In the presence of a coeffector or modification, the footprint is shorter and the bend is reduced (15, 23). NAC tetramers also recognize two kinds of site with these characteristics, but NAC recognizes both kinds of site under a single growth condition. This is not surprising, because NAC responds to its signal (nitrogen limitation) at the transcriptional level and appears not to respond to any physiological signal after its formation (7, 25). Both the short and long NAC-binding sites can titrate all the free NAC, arguing that both conformers of tetrameric NAC must be in equilibrium with each other. This equilibrium might be indirect, with the tetramers of one conformer dissociating into dimers and reassembling into tetramers of the other conformer, either free in solution or when bound to DNA. Or the equilibrium might be direct, with a tetramer of one conformer changing its conformation without dissociating into dimers. Our data are not conclusive on this question; however, we favor the model of direct conformational change. Both the *nac* site and the *cod* site are capable of binding dimers of NAC (Fig. 3). So if dissociation were necessary, we might expect that as the concentration of binding sites increased, we would have seen more DNA-dimer complexes and fewer DNA-tetramer complexes. However, we saw no increase in DNA-dimer complexes as the total concentration of sites increased (Fig. 3). In further support of this assumption, we have isolated mutant forms of NAC that can bind only long sites and others that can bind only short sites (21a). By analogy to other LTTRs for which structural data are available (5, 17, 26), these mutations affect regions that should lock NAC in an extended or compact conformation. Put simply, the molecule that determines the tetrameric conformation of NAC is the DNA molecule to which NAC is bound.

The data presented here argue that in order for a long site to be fully occupied by two dimers of NAC, three interactions are involved: interaction of one dimer with its binding site on the DNA (e.g., the *nac* site), interaction of the other dimer with its site (e.g., the *nac*^{*} site), and interaction between the two dimers. If the two binding sites are strong enough, then even NAC^{L111K}, the mutant of NAC that cannot tetramerize, can fill the complete site (Fig. 2). If one of the sites is weak (e.g., the half-site in the *cod*^{*} site), then the dimer-dimer interaction is required (NAC^{L111K} binds as a single dimer). Binding to the complete site requires that two of the three interactions be strong, but bending the DNA seems to require that all three be strong. When the *cod*^{*} site was positioned with its ATA half-site at -42, the binding of tetrameric NAC was weaker than that with wild-type *cod* where the ATA is at -32. When an ATA was placed at -42 from the *hut* site, the effect was even more pronounced (Fig. 6).

In our studies of sites where NAC dimers bind and activate transcription, we defined a consensus sequence of ATA-N₆-TNGTAT (9). Mutagenesis studies of the sites at *put* and *hut* confirmed the importance of the ATA-N₉-TAT consensus and suggested some role for the TNG triplet (4, 21). However, *nac*, *cod*, and *cod*^{*} lack a TNG triplet (Fig. 1), as does the downstream repression site at *gdhA* (10). Nevertheless, the data presented here argue that a triplet adjacent to the TAT half-

site is important for binding. The features that make this triplet region important remain obscure.

When a NAC dimer activates transcription (as it does at *hut*), the TNG triplet is important for a conformational change that is necessary for NAC's activating ability. Even changes to this trinucleotide that increase NAC's affinity for the *hut* site reduce *hut* activation (21). This raises an interesting question: does the conformational change caused when a dimer binds to an asymmetric site (ATAA-N₅-TNGTAT in *hut*) have any effect in determining the tetrameric conformers of NAC or vice versa? Inverting the *nac*^{*} site had no effect on the binding and bending ability of tetrameric NAC at the *nac* site (data not shown), so we may be able to exclude gross effects. But our data neither support nor exclude a connection between the effect on the dimeric conformers of NAC caused by the sequence of the DNA in the dimer-binding site and the effect on tetrameric conformers of NAC caused by the spacing between the two dimer-binding sites on the DNA. What is clear is that NAC has evolved to use DNA sequences and spacing (rather than small molecules) to signal conformational changes in both the dimers and the tetramers of the protein.

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