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Properties of the NAC (Nitrogen Assimilation Control Protein)-Binding Site within the *ureD* Promoter of *Klebsiella pneumoniae*▽

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The nitrogen assimilation control protein (NAC) of *Klebsiella pneumoniae* is a LysR-type transcriptional regulator that activates transcription when bound to a DNA site (ATAA-N5-TnGTAT) centered at a variety of distances from the start of transcription. The NAC-binding site from the *hutU* promoter (NBS^{hutU}) is centered at −64 relative to the start of transcription but can activate the *lacZ* promoter from sites at −64, −54, −52, and −42 but not from sites at −47 or −59. However, the NBSs from the *ureD* promoter (*ureDp*) and *codB* promoter (*codBp*) are centered at −47 and −59, respectively, and NAC is fully functional at these promoters. Therefore, we compared the activities of the NBS^{hutU} and NBS^{ureD} within the context of *ureDp* as well as within *codBp*. The NBS^{hutU} functioned at both of these sites. The NBS^{ureD} has the same asymmetric core as the NBS^{hutU}. Inverting the NBS^{ureD} abolished more than 99% of NAC's ability to activate *ureDp*. The key to the activation lies in the TnG segment of the TnGTAT half of the NBS^{ureD}. Changing TnG to GnT, TnT, or GnG drastically reduced *ureDp* activation (to 0.5%, 6%, or 15% of wild-type activation, respectively). The function of the NBS^{ureD}, like that of the NBS^{hutU}, requires that the TnGTAT half of the NBS be on the promoter-proximal (downstream) side of the NBS. Taken together, our data suggest that the positional specificity of an NBS is dependent on the promoter in question and is more flexible than previously thought, allowing considerable latitude both in distance and on the face of the DNA helix for the NBS relative to that of RNA polymerase.

The LysR-type transcriptional regulators (LTTRs) represent the largest family of regulatory proteins in all of the bacterial world (29). The nitrogen assimilation control protein (NAC) is unusual among the LTTRs in several important ways: it regulates scores of genes with diverse functions rather than regulating just a few genes with a specific function (14, 25), it functions as a dimer rather than as a tetramer at many of the genes whose expression it activates (8, 9, 24), and it requires no coeffector to assume its active conformation (22, 26), using the DNA sequence of the binding site to determine activity (21). The features of the dimer-binding sites that allow a NAC dimer to bind and activate are not well understood. The NAC-binding site at the *hutU* promoter (NBS^{hutU}) is the best characterized of the NBSs (21). The NBS^{hutU} is centered on the sequence T-N11-A, typical of most LTTRs (25). A more specific 15-bp "core sequence," ATA-N9-TAT, has been recognized as a consensus for sites where NAC dimers activate transcription (7). A comparison of the four well-studied promoters where a dimer of NAC activates expression (*hutUp*, *putPp*, *ureDp*, and *dadAp*) suggested an "activation consensus" of ATAA-N5-TnGTAT (1, 7, 8). Genetic analysis of the NBS^{hutU} showed that its 15-bp core contained all the information needed for NAC to bind and activate *hut* expression (20). The promoter-proximal half of the activation consensus (TnGTAT) was crucial for the NBS^{hutU} to function in activating *hutUp* (21). The promoter-distal half of the NBS^{hutU} appeared to be much less effective in allowing activation by NAC but contributed to the binding of NAC. Thus, the asymmetry in the activation consensus was important for NAC's function at *hutUp*.

Within the *hutUp* region, the NBS^{hutU} is centered at −64 and situated such that a NAC dimer bound there could potentially interact with the C-terminal domain of the α subunit (αCTD) of RNA polymerase. However, the NBS^{hutU} is also quite versatile, and its ability to activate transcription is not specific to the *hutU* promoter. The NBS^{hutU} was able to activate all three promoters in the *lacZ* promoter region and was able to do so from a variety of distances, including from positions −64, −54, −52, and −42 relative to the start of transcription (20). But this flexibility was limited. It did not activate expression when the distance was −74 from the start of transcription, nor when the NBS^{hutU} was on the opposite face of the helix at position −69, −59, −49, or −47 (20). Thus, it was surprising when we discovered that NAC not only activates *ureDp* from an NBS centered at −47 but does so very effectively (9). Moreover, NAC activates the *Escherichia coli codB* promoter from an NBS centered at −59, though less effectively (19). This was even more surprising given a positive-control mutant (NAC^{H26D}), which by analogy to other LTTRs (10, 30) was initially thought to define the interaction region of LTTRs with the α subunit of RNA polymerase. However, NAC^{H26D} fails to activate both *hutUp* and *ureDp* (23), and it seems unlikely that amino acid H26 makes the same contact with α from such very different positions on the DNA. That led us to examine whether there was something unique to the NBS^{ureD} or the *ureDp* that differed from their *hut* counterparts. Therefore, we tested whether the NBS^{hutU} could replace the NBS^{ureD}, whether the asymmetry of the NBS^{ureD} was important at *ureDp*, and, if so, whether it was the promoter-proximal half of the NBS (the TnGTAT portion) that was needed for

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NAC to be able to activate *ure* operon expression. We also tested whether the NBS^{hutU} would function in the context of the *codBp*. The binding of NAC at the wild-type *codBp* is complex, with an NBS^{codB} (ATAT-N5-AnATAT) that differs significantly from the activation consensus defined above and a second half-site located just upstream of the NBS^{codB}. As a result, NAC binds to wild-type *codBp* as a tetramer, but deletion of the upstream half-site results in binding of a dimer of NAC to the NBS^{codB} and a somewhat weaker activation of *codBp*.

MATERIALS AND METHODS

Strains and bacterial growth. *Klebsiella pneumoniae* strains used in this study were derived from strain W70 (13). Cells cultured under nitrogen-limiting conditions were grown in W4 salts containing 0.4% (wt/vol) glucose and 0.2% (wt/vol) monosodium glutamate (12). For general use as rich medium for cloning and propagation of cells, L broth was used (16). For selection of alleles and plasmids, the following antibiotics were used at the indicated concentrations: ampicillin (100 μg/ml), kanamycin sulfate (50 μg/ml), streptomycin sulfate (50 μg/ml), and tetracycline hydrochloride (25 μg/ml). Solid media were supplemented with 1.5% Bacto agar.

Genetic techniques. DNA manipulation was carried out as described by Maniatis et al. (15). Mutant promoters were amplified via PCR using wild-type promoter templates and primers containing the desired mutations. In this study, the 15-bp core NAC-binding site plus 6 bp upstream and downstream were used as the NAC-binding site. The sequences of the sites in the study (with the 15-bp core underlined) are as follows: for NBS^{ureD}, GATGACATAAGCGTTTCGTATGACCGG, for NBS^{codB}, CTCATTCATATAAAAAATATATTTCCCC, and for NBS^{hutU}, CGCAATATAACAAAATTGTATCATTTC. In each case, the 27-bp sequence was inserted such that the 15-bp core was in the same position in the mutant promoter as the native 15-bp core was in the wild-type promoter. The architecture of the transcriptional *ureDp-lacZ* fusion has been described previously (9). This fusion contains a segment of *ure* DNA from −77 to +107 relative to the start of transcription and fuses the promoterless *lacZ* gene after 64 bp of the *ureD* sequence (9). The transcriptional *codBp* fusion (19) contains a segment of *cod* DNA from −120 to +67 and fuses the promoterless *lacZ* gene after 76 bp of the *codB* coding sequence (19). The DNA sequences of primers used in this study are available upon request. PCR products were cloned into the EcoRI and BamHI sites of pRS415 (27). The DNA sequence of each cloned fragment was determined to ensure that no unwanted mutations had been introduced. In order to integrate the fusions into the chromosome of *K. pneumoniae*, the fusions were subcloned into a *pir*-dependent vector, pCB1583, based on pKAS46 (6, 28). Fusions were integrated into the *rbs* landing pad as previously described (6). Transduction of the *nac-2* allele, an in-frame deletion of the NAC open reading frame, with the *aph-1* cassette replacing the deleted material (2) was performed utilizing P1$_{vir}$ grown on KC5447 as previously described (5).

NAC purification. *K. pneumoniae* NAC was purified from *E. coli* cells as described previously (7). Purity was monitored via SDS-PAGE and staining by the method of Fairbanks et al. (3). Purified protein was quantified by the method of Lowry et al. and compared to a bovine serum albumin (BSA) standard (11). Protein was diluted 1:1 in glycerol and stored at −20°C.

Electrophoretic mobility shift assay (EMSA). Gel mobility shifts were performed with purified NAC as described previously (7). Briefly, 6 μl (0.08 pmol) of purified PCR-amplified fragments of *ureDp*, *codBp*, or mutant promoter regions was mixed with 4 μl of buffer 6 (50% glycerol, 125 mM NaCl, 50 mM NaH_2PO_4 [pH 7.0], 1.25 mM $MgCl_2$, 0.5 mM 2-mercaptoethanol, 1 mg/ml BSA) containing 0, 0.07, 0.14, or 0.28 pmol of purified NAC. Reaction mixtures were incubated at room temperature for 20 min, and then 1 μl of 10× loading buffer (25% Ficoll, 100 mM Tris-HCl, 10 mM EDTA, 0.05% [wt/vol] cresol red, and 0.05% [wt/vol] orange G, pH 7.4) was added to each reaction mixture. A 10-μl portion of each reaction mixture was loaded on a prerun gel (5% polyacrylamide buffered with 0.5× Tris-borate-EDTA [TBE]). Bound and unbound species were separated by electrophoresis at 10 V/cm for 60 min. Gels were stained with ethidium bromide (40 μg/ml) and then destained with water. The mobilities of the dimer-bound and tetramer-bound promoter fragments were determined by comparison to known shifts of *ureDp* (dimer) and *codBp* (tetramer), as shown previously (19, 24).

β-Galactosidase assay. Liquid cultures of 10 ml were grown in 125-ml sidearm flasks at 30°C and 250 rpm to mid-log phase (50 Klett units, ca. 1.2×10^8 CFU/ml). Cells were washed once with one culture volume of cold 1% KCl. Cells were resuspended in 1/10 culture volume of cold 1% KCl. Assays were performed on whole cells permeabilized by detergent as previously described (12). Three different volumes of 10× cells were assayed for each culture, and each strain was cultured at least three independent times. Values for specific activity are reported as nanomoles of product formed per minute per milligram of total protein (as determined by the method of Lowry) at 30°C (11).

RESULTS

NBS^{hutU} functions in the *ureD* and *codB* promoters. Both the *ureD* and *codB* promoters are activated by NAC, even though the NBS^{ureD} is centered at −47 and the NBS^{codB} is centered at −59, positions where the NBS^{hutU} appeared to be ineffective in the context of the *lac* promoters (20). The 27-bp NBS^{hutU} used here has been defined by DNase I footprinting (7) and contains the 15-bp activation consensus (ATAA-N5-TnGTAT) and 6 bp of DNA sequence on either side of that 15-bp core. The NBS^{hutU} was used to replace the native NBS within the context of the *ureD* or *codB* promoters. In both cases, the 27-bp replacement was aligned such that the 15-bp core of the inserted NBS^{hutU} was in the same location as the cores of the native NBS^{ureD} and NBS^{codB}, centered at −47 and −59 bp, respectively, relative to the start of transcription of the reporter. A transcriptional fusion of *ureDp* to a promoterless *lacZ* gene was used to measure *ureDp* activity. This fusion, described previously (9), carries a segment of *ure* DNA from −77 to +107 relative to the start of *ureDp* transcription. Thus, the *lacZ* fusion occurred after 64 bp of *ureD* sequence. The comparable reporter for *codBp* described previously (19) carries *cod* DNA from −120 to +67, and thus the *lacZ* fusion occurred after 76 bp of *codB* sequence. Each of these fusions was inserted into the *K. pneumoniae* chromosome in single copy at the *rbs* locus using "landing pad technology" (6). The 15-bp cores of the NBS^{hutU} and the NBS^{ureD} are prefect matches to the activation consensus, but the core of the NBS^{codB} differs in three positions from the consensus (Fig. 1A). Nevertheless, the NBS^{codB} still contains the critical ATA-N9-TAT motif.

NAC formed a stable interaction, as assayed by electrophoretic mobility shift assay (EMSA), with *ureDp* containing the NBS^{hutU} and *codBp* containing the NBS^{hutU} (Fig. 1B). NAC interacted with the NBS^{hutU} as a dimer within the context of *ureDp* and as a tetramer within the context of *codBp*, determined as described in Materials and Methods. This is not surprising, since NAC associates with the native *ureDp* as a dimer and the native *codBp* as a tetramer (19, 22, 24). Since NAC was able to associate with the NBS^{hutU} in both the *ureDp* and *codBp* contexts, the ability of NAC to activate transcription from these constructs was tested.

Nac$^+$ and Nac$^-$ derivatives of the reporter strains were grown under nitrogen-limiting conditions and assayed for β-galactosidase activity (Fig. 1C). Transcription of all four promoters was dependent on NAC. The NAC-mediated activation of the *ureD* promoter containing the NBS^{hutU} was similar to the 8-fold activation of the native *hutUp* by NAC (21). The NBS^{hutU} was also able to activate transcription when positioned at −59 in the context of the *codB* promoter. In this case, the maximal activation was similar to the maximal activation seen for the wild-type *codB* promoter rather than the wild-type *hutUp*. Clearly, the positions of −47 and −59 are not forbidden for the NBS^{hutU}, at least in the context of the NAC-activated promoters, *ureDp* and *codBp*, respectively. The basal level of

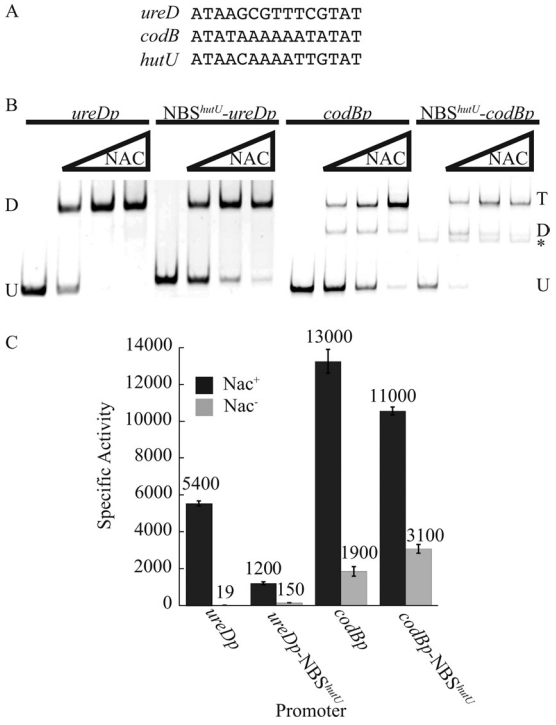

FIG. 1. Functional equivalence of the NAC-binding sites. (A) Alignment of the core, 15-nucleotide NAC-binding sites from the *ureD*, *codB*, and *hutU* promoters. (B) Electrophoretic mobility shift assay (EMSA) of the *ureDp*, NBS^{hutU}-*ureDp*, *codBp*, and NBS^{hutU}-*codBp* fragments mixed with buffer 6 and increasing concentrations of NAC. U indicates the mobility of the unbound DNA, D indicates the band corresponding to a dimer of NAC associated with the DNA, T indicates the band corresponding to a tetramer of NAC associated with the DNA, and * indicates a PCR artifact in the NBS^{hutU}-*codBp* mobility shifts that is not NAC reactive. (C) β-Galactosidase activities of *ureDp*, NBS^{hutU}-*ureDp*, *codBp*, and NBS^{hutU}-*codBp* as *lacZ* fusions integrated into the Nac$^+$ or Nac$^-$ background and grown under nitrogen-limiting conditions. Bars indicate standard errors.

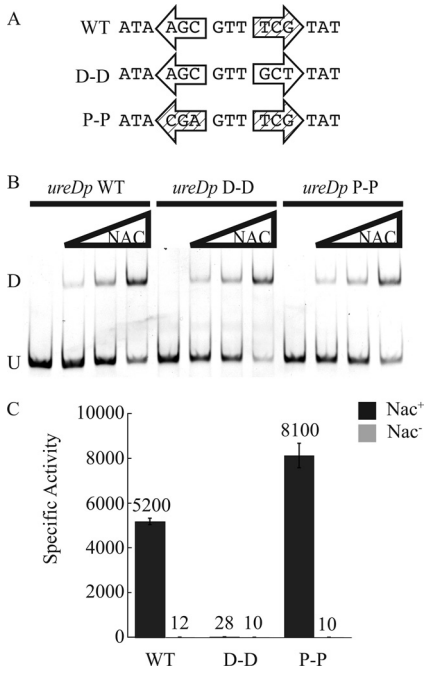

FIG. 2. Asymmetry of the *ureD* promoter NAC-binding site. (A) Alignment of the core, 15-nucleotide NAC-binding sites of wild-type *ureD* NAC (WT) and the proximal-proximal (P-P) and distal-distal (D-D) mutants. The proximal half-site is shown as a cross-hatched arrow, and the distal half-site is an open arrow. (B) EMSA of the *ureDp*, D-D, and P-P fragments mixed with buffer 6 and increasing concentrations of NAC. U indicates the mobility of the unbound DNA, and D indicates the band corresponding to a dimer of NAC associated with the DNA. (C) β-Galactosidase activities of *ureDp* and the D-D and P-P mutants as *lacZ* fusions integrated into the Nac$^+$ or Nac$^-$ background and grown under nitrogen-limiting conditions. Bars indicate standard errors.

activity from both the *ureD* and *codB* promoters containing the NBS^{hutU} was higher than that of the wild-type *ureD* or *codB* promoter, and this will be addressed in Discussion.

The two halves of the NBS^{ureD} are functionally different. Despite the presence of some symmetry within the NBS^{hutU} (ATA-N9-TAT), the more complete activation sequence of the NBS^{hutU} is asymmetric (ATAA-N5-TnGTAT) and this asymmetry is important for NAC activation of the *hutU* promoter (21). Within the context of *hutUp*, the promoter-distal half of the NBS^{hutU} (containing the hexanucleotide ATAAnn) is important for strong binding of NAC and the promoter-proximal half of the site (the hexanucleotide TnGTAT) is important for activation of transcription by NAC (21). We reasoned that the NBS^{ureD}, located closer to the promoter and on the opposite face of the DNA helix, might display different requirements. The role of each half of the NBS^{ureD} was examined by creating mutant constructs with either two distal or two proximal half-sites (D-D and P-P, respectively) (Fig. 2A). The promoter-distal half of the site (shown in the open arrow) was used to replace the promoter-proximal half of the site (shown in the cross-hatched arrow) to create the construct referred to as distal-distal (D-D). The opposite replacement was made to create the proximal-proximal construct (P-P). NAC bound to promoters containing the D-D and P-P NBS mutants in a manner similar to that of the wild-type *ureD* promoter (Fig. 2B). This suggested that unlike the NBS^{hutU}, both halves of the NBS^{ureD} were sufficient for strong NAC binding, perhaps reflecting a role for the 6 bp of flanking sequence.

Since both the D-D and P-P constructs bound NAC well, we next tested their ability to function in NAC-mediated activation of *ureDp*. Fusions of *ureD* promoters containing the P-P or D-D mutant NBSs to *lacZ* were integrated in single copy into the *K. pneumoniae* chromosome in Nac$^+$ and Nac$^-$ strain backgrounds. NAC was severely defective in activating transcription from the promoter containing the D-D mutant NBS (Fig. 2C) even though NAC bound well to this site (Fig. 2B). In contrast, the NAC-mediated activation of the promoter containing the P-P mutant was even stronger than the wild-type *ureD* promoter (Fig. 2C). These data suggested that at least one TnGTAT-containing half-site is required for NAC transcriptional activation from the NBS^{ureD}.

NBS^{ureD} is directional. We next asked if the orientation of the TnGTAT half of the NBS^{ureD} was important. To test this, we constructed *ureD* promoters containing an inverted NBS^{ureD} or a scrambled NBS^{ureD} (Fig. 3A). As expected, both the inverted NBS and the wild-type NBS bound NAC well and the scrambled NBS failed to bind NAC (Fig. 3B). Single-copy

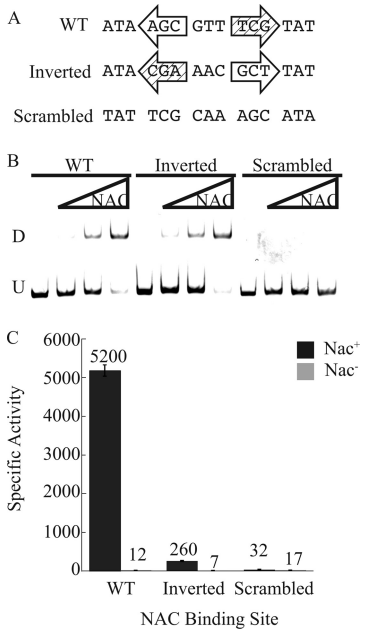

FIG. 3. Directionality of the *ureD* promoter NBS. (A) Alignment of the wild type and the inverted and scrambled mutants containing *ureD* promoter NAC-binding sites. The distal half-site is shown as an open arrow, and the proximal half-site is a cross-hatched arrow. (B) EMSA of the wild-type and the inverted and scrambled *ureDp* fragments. U indicates the mobility of the unbound DNA, and D indicates the band corresponding to a dimer of NAC associated with the DNA. (C) β-Galactosidase activities of the wild type and the inverted and scrambled mutants containing *ureDp* as *lacZ* fusions integrated into the Nac^+ or Nac^- background and grown under nitrogen-limiting conditions. Bars indicate standard errors.

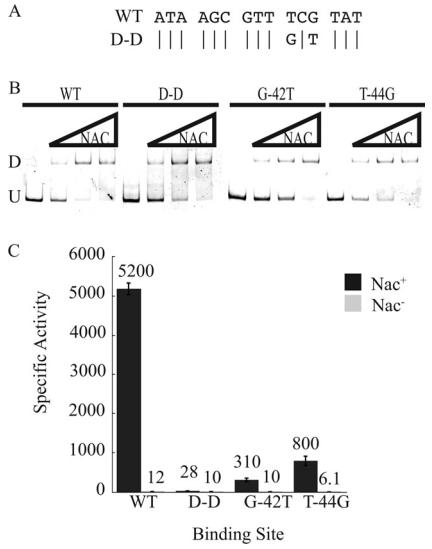

FIG. 4. Role of T−44 and G−42 in $NBS^{ureD.}$ (A) Alignment of the wild-type and D-D *ureDp* NAC-binding sites. (B) EMSA of the wild-type and the D-D-, G−42T-, and T−44G-containing *ureD* promoter fragments. U indicates the mobility of the unbound DNA, and D indicates the band corresponding to a dimer of NAC associated with the DNA. (C) β-Galactosidase activities of the wild-type and the D-D-, G−42T-, and T−44G-containing *ureDp* as *lacZ* fusions integrated into the Nac^+ or Nac^- background and grown under nitrogen-limiting conditions. Bars indicate standard errors.

promoter-*lacZ* fusions with either the inverted or scrambled NAC-binding site were integrated into the *K. pneumoniae* chromosome. Not surprisingly, the scrambled site, to which NAC did not bind *in vitro*, demonstrated little or no NAC-mediated transcriptional activation (Fig. 3C). The inverted site, to which NAC bound well *in vitro*, exhibited only 5% as much activation as the wild-type NBS (Fig. 3C). This suggested that NBS^{ureD} requires the TnGTAT-containing half of the NBS to be on the promoter-proximal side of the NBS in order for NAC to activate transcription effectively.

The TnG sequence is critical for NAC activation. The D-D mutant, which failed in activation despite strong binding, contained only two changes from the wild-type NBS^{ureD}. The two changes occurred in the 10th and 12th nucleotides of the 15-bp core. These nucleotides correspond to nucleotides −44 and −42 with respect to the start of transcription (Fig. 4A). Since the D-D construct had lost virtually all NAC-mediated transcriptional control of the *ureD* promoter, the role of each of these changes, T−44G and G−42T, was assessed individually.

EMSA was performed with *ureD* promoters containing the D-D, T−44G, and G−42T NBS^{ureD} mutants. NAC stably interacted with both single mutants (Fig. 4B). Single-copy promoter-*lacZ* fusions of the mutant promoters integrated into the *K. pneumoniae* chromosome were assayed in Nac^+ and Nac^- backgrounds (Fig. 4C). The promoter that contained the G−42T mutation had NAC-mediated activation that was only 6% of that of wild-type *ureDp* (Fig. 4C). The promoter that contained the T−44G mutation was slightly better, with 15% of the NAC-mediated activation of wild-type *ureDp* (Fig. 4C). These data suggest that nucleotides T−44 and G−42 work synergistically to elicit NAC-mediated activation of the *ureD* promoter.

The 10th and 12th nucleotides of the NBS^{ureD} core were critical for NAC-mediated activation of the promoter but were nonessential for NAC binding to the promoter. This raised the question of whether the 11th nucleotide of the core, C−43, which remained unchanged in both the P-P and D-D constructs, plays a role in NAC-mediated transcriptional activation of the *ureD* promoter. The role of C−43 in the NAC-mediated transcriptional activation of the *ureD* promoter was examined by changing C−43 to the other three nucleotides, creating C−43A, C−43T, and C−43G constructs (Fig. 5A). The ability of these three constructs to interact with NAC *in vitro* and *in vivo* was examined to determine the role of C−43 in both NAC binding and NAC-mediated transcriptional activation under nitrogen-limiting conditions.

NAC bound all three of the C−43 substitution mutants *in vitro* (Fig. 5B). Single-copy integrants of the mutant promoters fused to *lacZ* were assayed in the Nac^+ and Nac^- backgrounds (Fig. 5C). All three of the promoters containing NBSs with C−43 substitution mutations showed less activation in response to NAC than the wild-type *ureD* promoter. The C−43A and C−43T mutants had slightly less NAC-mediated activation, 65% and 61% of the wild-type *ureDp* activity, respectively (Fig. 5C). The promoter containing the C−43G mutant had activation that was 30% of that of the wild-type *ureD* promoter.

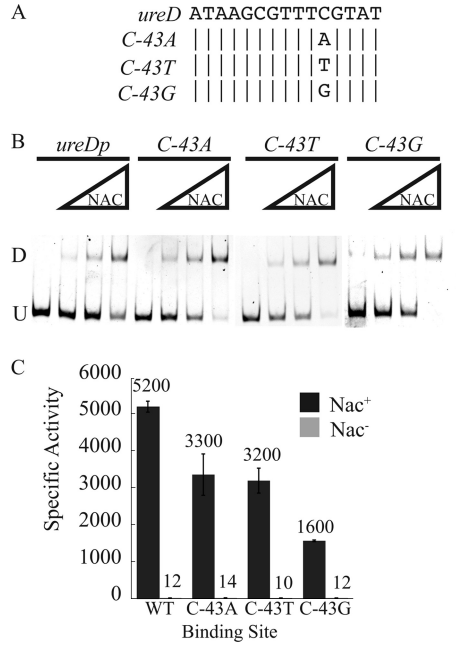

FIG. 5. Role of C−43 in NBS$^{ureD.}$ (A) Alignment of the wild-type and the C−43A, C−43T, and C−43G *ureDp* NAC-binding sites. (B) EMSA of the wild-type and the C−43A-, C−43T-, and C−43G-containing *ureD* promoter fragments. U indicates the mobility of the unbound DNA, and D indicates the band corresponding to a dimer of NAC associated with the DNA. (C) β-Galactosidase activities of the wild-type and the C−43A-, C−43T-, and C−43G-containing *ureDp* as *lacZ* fusions integrated into the Nac$^+$ or Nac$^-$ background and grown under nitrogen-limiting conditions. Bars indicate standard errors.

DISCUSSION

Previous data had suggested that NAC-mediated activation of transcription required that NAC be located on the same face of the DNA helix as RNA polymerase but that the distance from the RNA polymerase was somewhat flexible (20). The NBS^{hutU} brought the *lac* promoters of *E. coli* under the control of NAC when placed at −64, −54, and −42, all positions on the same face of the helix. Transcriptional activation by NAC at the closer distances was weaker than that seen from −64, suggesting that NBSs located close to the promoter are less effective (20). The NBS^{hutU} failed to bring the *lac* promoters under the control of the nitrogen regulon when located at −69, −59, and −47, all positions on the opposite face of the helix (20). Interestingly, the NBS^{ureD} is located at the "weaker" spacing and on the "forbidden" face of the helix and demonstrates strong NAC-dependent transcriptional activation (9). The data presented here show that the NBS^{hutU} was able to activate the *ureD* promoter even at −47, a position where it failed to activate the *lac* promoter. In addition, the NBS^{hutU} activated the *codB* promoter even when it was at −59, a position from which it failed to activate at the *lac* promoter. Thus, there appears to be considerable flexibility in how NAC activates transcription.

We noted that many constructs with altered NBSs had elevated levels of basal activity compared to those of the wild-type promoters. The *ureDp* is an extremely poor promoter in the absence of NAC. Visual inspection of the −10 and −35 regions fails to detect any good match to the consensus sequences. The replacement of the NBS^{ureD} with the NBS^{hutU} changed the nucleotide composition as close to the promoter as position −34, and these changes, increasing the A/T richness of the −35 region, might improve the overall strength of an extremely poor promoter. Likewise, replacement of the NBS^{codB} with the NBS^{hutU} increased the A/T richness of the −60 region of the *codB* promoter, perhaps improving the binding of the αCTD, which again might improve the overall strength of a promoter. These changes appear to have increased the basal level of expression from these promoters. Nevertheless, NAC was still able to activate expression from these promoters.

The NBS^{hutU} contains two functionally important regions: the promoter-distal half of the site (containing the sequence ATAA), which is important for NAC binding affinity, and the promoter-proximal half of the site (TnGTAT), which is important for NAC transcriptional activation of the *hutU* promoter (21). In order for NAC to yield strong activation, the TnGTAT half of the site must be located in the proper orientation (facing the promoter), suggesting that for an NBS positioned at −64, the activation of transcription by NAC occurs in a directional manner (21). The NBS^{ureD} shows the same nonequivalent halves of the NBS as does the NBS^{hutU}. Only the TnGTAT half of the site allows activation, and this half must be present on the side nearest the start of transcription. So it appears that the NBS^{ureD}, like the NBS^{hutU}, induces a conformation change in the bound NAC that allows NAC to activate transcription by RNA polymerase.

Studies with the positive-control (PC) mutant of CysB, CysB^{Y27A}, have been interpreted to suggest that amino acid Y27 of CysB interacts with a region of RNA polymerase near amino acid E273 of the α subunit (10). An analogous PC mutant of NAC, NAC^{H26D}, has been isolated. NAC^{H26D} is able to bind DNA and repress transcription but cannot activate either *hutUp* or *ureDp* (23). NAC can activate *hutUp* from an NBS at −64 and *ureDp* from an NBS at −47, on the face of the DNA helix opposite RNA polymerase. Thus, it seems unlikely that amino acid H26 of NAC could contact similar regions of RNA polymerase (e.g., the E273 region of the α subunit) in both cases. Moreover, the crystal structure of a related LTTR (17) suggests that Y27 of CysB and H26 of NAC lie at the edge of the first helix in the DNA-binding helix-turn-helix domain and are likely to lie close to the DNA. Thus, it appears that H26 of NAC may play a role other than making direct contact with the α subunit of RNA polymerase. We suggest that H26 may interfere with the conformational change in NAC that is induced by the TnGTAT sequence and that is required for NAC's ability to activate transcription. But this raises the broader question of how NAC activates transcription. Perhaps NAC interacts with RNA polymerase at *hutUp* and *ureDp* in fundamentally different ways. For example, NAC might interact with α at *hutUp* and with a different subunit of RNA polymerase at *ureDp*. This might help to explain how NAC can activate so many different promoters (4) and do so from so many different distances relative to the start of transcription (9, 19, 20). Alternatively, NAC might distort the DNA at *hutUp* and *ureDp* in ways that allow rather poor promoter sequences to function more effectively. It is worth noting that *hutUp* has a rather poor −10 region (TATATAT). The −10 region of the NAC-dependent *ureDp* is even worse, and it has no recogniz-

able −35 sequence at all. NAC dimers are known to bend the NBS^{nac} about 42° (24), and the NBS^{hutU} an estimated 23° (18). So a model involving DNA distortion is certainly plausible. The answers to these questions will require a structure for a cocrystal of NAC bound to its NBS. What is clear, however, is that NAC is an unusually versatile regulator that can function as a dimer or tetramer, on the same face of the DNA helix as RNA polymerase or on the opposite face, and as an activator or a repressor and whose ability to activate transcription depends on the sequence of the NBS that it uses for binding.

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