

The Amino-Terminal 100 Residues of the Nitrogen Assimilation Control Protein (NAC) Encode All Known Properties of NAC from *Klebsiella aerogenes* and *Escherichia coli*

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The nitrogen assimilation control protein (NAC) from *Klebsiella aerogenes* or *Escherichia coli* (NAC_K or NAC_E, respectively) is a transcriptional regulator that is both necessary and sufficient to activate transcription of the histidine utilization (*hut*) operon and to repress transcription of the glutamate dehydrogenase (*gdh*) operon in *K. aerogenes*. Truncated NAC polypeptides, generated by the introduction of stop codons within the *nac* open reading frame, were tested for the ability to activate *hut* and repress *gdh* in vivo. Most of the NAC_K and NAC_E fragments with 100 or more amino acids (wild-type NAC_K and NAC_E both have 305 amino acids) were functional in activating *hut* and repressing *gdh* expression in vivo. Full-length NAC_K and NAC_E were isolated as chimeric proteins with the maltose-binding protein (MBP). NAC_K and NAC_E released from such chimeras were able to activate *hut* transcription in a purified system in vitro, as were NAC_K129 and NAC_E100 (a NAC_K fragment of 129 amino acids and a NAC_E fragment of 100 amino acids) released from comparable chimeras. A set of NAC_E and NAC_K fragments carrying nickel-binding histidine tags (*his*₆) at their C termini were also generated. All such constructs derived from NAC_E were insoluble, as was NAC_E itself. Of the *his*₆-tagged constructs derived from NAC_K, NAC_K100 was inactive, but NAC_K120 was active. Several NAC fragments were tested for dimerization. NAC_K120-*his*₆ and NAC_K100-*his*₆ were dimers in solution. MBP-NAC_K and MBP-NAC_K129 were monomers in solution but dimerized when the MBP was released by cleavage with factor Xa. MBP-NAC_E was readily cleaved by factor Xa, but the resulting NAC_E was also degraded by the protease. However, MBP-NAC_E-*his*₆ was completely resistant to cleavage by factor Xa, suggesting an interaction between the C and N termini of this protein.

The nitrogen regulatory (Ntr) system of enteric bacteria allows *Klebsiella aerogenes*, *Escherichia coli*, and other enteric bacteria to respond rapidly and effectively to changes in the quality of the nitrogen source provided (13). Under conditions of nitrogen limitation, a complex cascade of regulatory events involving uridylylation and phosphorylation reactions leads to the accumulation of the phosphorylated (active) form of the transcriptional activator NtrC. NtrC-phosphate can activate the σ^{54} -dependent expression of a variety of genes involved in utilization of organic and inorganic nitrogen sources. NtrC-phosphate also activates transcription of the *nac* gene in *K. aerogenes* and *E. coli* (6).

The nitrogen assimilation control protein (NAC) is a regulatory protein responsible for activating the transcription of operons such as *hutUH*, *putP*, and *ureDABCEFG*, whose products supply the cell with ammonia or glutamate from histidine, proline, and urea, respectively (2). NAC is also responsible for repressing transcription of the *gdhA* and *gltBD* operons, whose products are involved in assimilating ammonia under conditions of nitrogen excess or limitation (2). NAC is also responsible for down-regulating its own transcription (3, 7). The expression of the *nac* gene is entirely dependent on the Ntr system such that under conditions of nitrogen-limited growth, the Ntr system is active and *nac* is actively transcribed by RNA polymerase charged with the minor sigma factor σ^{54} (6, 12). There are no known cofactors involved in the regulation of transcription by NAC (17), so if *nac* is expressed, then NAC is

produced and activates transcription of *hut*, *put*, and *ure* by RNA polymerase charged with the major sigma factor σ^{70} . Thus, NAC serves as a coupling factor between the σ^{54} -dependent Ntr system and σ^{70} -dependent genes like *hut* (2).

NAC is a member of the large family of LysR-type transcriptional regulators (LTTRs; 15, 16). This group of over 50 proteins are identified on the basis of amino acid sequence similarity. Most of the members of this family have between 300 and 350 amino acids and show a surprisingly high degree of sequence similarity. For example, NAC from *K. aerogenes* shows about 40% sequence identity with OxyR from *E. coli* (16). Within the LTTRs, the DNA-binding domain occurs near the N terminus of the protein, where a predicted helix-turn-helix motif (approximately amino acids 20 to 40 in NAC) is located (15). In several LTTRs, the binding site for a required cofactor has been identified. These binding sites are generally in the C-terminal third of the polypeptide, suggesting that the DNA-binding and cofactor-binding domains are physically separated (15).

Very little structural information about LTTRs is available. A fragment of CysB containing the C-terminal 233 amino acids has been crystallized, and a structure was derived from X-ray diffraction data (18). However, this fragment lacks the N-terminal 87 amino acids and thus the DNA-binding domain. As a result, this structure says little about how the DNA-protein interactions occur, are altered by the presence of inducer, or result in the activation of transcription.

Comparison of NAC from *E. coli* (NAC_E) with NAC from *K. aerogenes* (NAC_K) revealed a surprising lack of sequence similarity (14). NAC_E was only about 75% identical to NAC_K, in contrast to other *E. coli* and *K. aerogenes* regulatory proteins, which are >95% identical. Most of the sequence divergence

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
KC2668	<i>hutC515</i> Δ[<i>bla</i>]-2 <i>nac</i> ⁺	10
KC2725	<i>hutC515</i> Δ[<i>bla</i>]-2 <i>nac</i> -203::Tn5-131	10
KC2972	KC2941/pCB511	Transformation
KC3220	KC2725/pCB594	Transformation
KC3555	KC2725/pCB771	Transformation
KC3568	KC2725/pCB774	Transformation
KC3572	KC2725/pCB778	Transformation
KC3578	KC2725/pCB781	Transformation
KC3580	KC2725/pCB782	Transformation
KC3591	KC2725/pCB788	Transformation
KC3615	KC2725/pCB800/pCB648	Transformation
KC3973	KC2725/pCB941	Transformation
KC3977	KC2725/pCB529	Transformation
KC3981	KC2725/pCB606	Transformation
KC4510	KC2725/pCB547	Transformation
Plasmids		
pCB511	pGB2; wild-type <i>nac</i> _E ^a	This laboratory
pCB529	pGB2; <i>nac</i> -10 allele of <i>E. coli</i>	This laboratory
pCB547	pGB2; <i>nac</i> -28 allele of <i>E. coli</i>	This laboratory
pCB594	pMalC2; MBP-NAC _E fusion protein	This laboratory
pCB606	pMalC2; MBP-NAC _K fusion protein	This laboratory
pCB771	pGD103; NAC _E -his ₆	This laboratory
pCB774	pGD103; wild-type NAC _E	This laboratory
pCB778	pGD103; wild-type NAC _K	This laboratory
pCB781	pGD103; NAC _K -his ₆	This laboratory
pCB782	pGD103; NAC _K ⁻ (DFGRSGHTDSL)	This laboratory
pCB788	pGD103; NAC _K 100-his ₆	This laboratory
pCB800	pGD103; NAC _K 120-his ₆	This laboratory
pCB941	pGD103; NAC _E 100-his ₆	This laboratory
pGB2	Low-copy cloning vector; insert expressed from its own promoter	4
pGD103	Low-copy vector expressing inserted material from <i>lacP</i>	5
pMalC2	MBP fusion vector expressing inserted material from <i>tac</i> promoter	New England BioLabs

^a Vector; cloned insert or product.

between NAC_E and NAC_K occurs in the C-terminal two-thirds of the protein, consistent with the lack of a need to conserve a coeffector-binding site in the C-terminal domain. This raised the question of whether the C-terminal domain has any role at all, leading us to seek the smallest N-terminal fragment that would retain the biological activities associated with NAC.

Two *nac* mutations also suggested that a C-terminal portion of NAC might be dispensable, further encouraging us to search for a small active fragment of NAC. The *nac*-112 allele of *K. aerogenes* is an insertion of *Mudlac*, *amp* at an unknown site within *nac*_K, which retains considerable NAC activity (12). The *nac*-10 allele of *E. coli*, an insertion of a stop codon and a drug resistance cartridge in the center of the *nac*_E gene, was constructed as an intermediate in constructing the *nac*-28 null allele (14). Strains carrying *nac*-10 also retained considerable NAC activity. Thus, we attempted to determine whether the C-terminal domain of NAC from *E. coli* and *K. aerogenes* plays an essential role and to define the smallest N-terminal fragment of NAC that would retain the ability to function as a transcriptional regulator.

MATERIALS AND METHODS

Strains and plasmids. All of the *K. aerogenes* strains used in this study were derived from W70 and are listed in Table 1.

Generation of C-terminal deletions of NAC. Unidirectional exonuclease III deletions were performed to make 3' deletions in the *E. coli nac* gene by using the protocol described by Henikoff (9) with minor modifications. Plasmid pCB574 contains the *E. coli nac* gene inserted into the *EcoRI* site of plasmid pGB2 (4) such that the 3' end of the gene is proximal to the *PstI* site present in the multiple cloning site. Deletions were initiated by cutting with *Sall* and *PstI* to allow unidirectional deletions into *nac*. After digestion with exonuclease III and mung bean nuclease, the deleted DNA fragments were resuspended in 20 μl of ligation buffer (250 mM Tris-HCl [pH 7.6], 50 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM ATP) and 100 pmol of a dephosphorylated *NheI* linker (containing stop codons in three reading frames) to which T4 DNA ligase was added. The ligation mixture was used to transform *E. coli* DH5α to streptomycin (50 μg/ml) and spectinomycin (100 μg/ml) resistance. Deletions of interest were sequenced by using the pUC reverse primer to determine the precise endpoint of each deletion. These were brought under the control of the *lac* promoter by PCR amplification by using the primers CGAATTCAACTGGAGACTCATATGAAC (forward) and AGGATCCTCACACAGGAAACAGCTATGAC (reverse) and inserted into the *EcoRI* and *BamHI* sites of pGD103 (5). Specific truncations in the *K. aerogenes nac* gene were generated by PCR using a forward primer (CTGGAATTCCTTACAGGAGGCA) containing an *EcoRI* site and reverse primers complementary to the last 18 bases prior to the desired truncation point followed by an amber stop codon and GGATCC (*BamHI*). PCR products were introduced into the *EcoRI* and *BamHI* sites of plasmid pGD103, and the sequences were verified by dideoxy sequencing.

Purification of MBP-NAC fusions. The *nac* genes from *E. coli* and *K. aerogenes*, along with the C-terminal truncated derivatives of each, were inserted into vector pMalC2 (New England Biolabs) as a blunt-ended 5' end of the *nac* gene beginning at the initiating methionine ligated to the blunt-end *XmnI* site within the vector. The 3' ends were ligated into either the *EcoRI* or *BamHI* sites within the multiple cloning site. The initial construct was prepared by PCR and sequenced to verify that errors had not been introduced by PCR amplification. Later derivatives were made by replacing sequences within this clone by using internal sites (*XmnI*, *SspI*, and *BglII*) in the *nac* gene rather than reamplifying for each construct. Insertion of a blunt-ended DNA fragment into the *XmnI* site of the vector results in fusion of the NAC protein to the 42-kDa maltose-binding protein (MBP). The junction between the two proteins contains the recognition site for factor Xa protease (Ile Glu Gly Arg) positioned such that cleavage with this enzyme would result in separation of the intact NAC protein from the fusion.

Purification of the fusion protein was initiated by inducing mid-log-phase cultures (3 × 10⁸ CFU/ml) with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The induced cultures (500 ml in 2-liter flasks) were shifted from 37 to 28°C and grown for an additional 2 h. Cells were chilled on ice and harvested by centrifugation. The pellets were washed twice in column buffer (100 mM Tris-HCl [pH 7.5], 250 mM NaCl, 1 mM dithiothreitol, 1 mM 2-mercaptoethanol) and resuspended in a final volume of 15 ml. Cell disruption was accomplished by passage through a French pressure cell (twice at 12,000 lb/in²). The resulting lysate was clarified by centrifugation for 30 min at 30,000 × g at 4°C. The supernatant solution containing the fusion protein was increased in volume to 30 ml with column buffer containing 50 mM freshly prepared phenylmethylsulfonyl fluoride. The crude preparation was applied to a column (2.5 cm in diameter) containing a 10-ml bed volume of amylose-agarose resin (New England Biolabs no. 800-21L) at a flow rate of 1 ml/min. Bound fusion protein was washed with 300 ml of column buffer at a 2-ml/min flow rate and eluted with column buffer supplemented with 1 M maltose at a rate of 1.5 ml/min with 3-ml fractions collected over 50 ml. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine which fractions contained MBP-NAC. The MBP-NAC fusion generally eluted over a wide peak from fractions 3 to 7. Fractions were pooled if SDS-PAGE indicated sufficient purity. Protein concentrations were determined by Bio-Rad protein assay and generally indicated values between 0.7 and 2.5 mg/ml. Diafiltration was performed by using either an Amicon 10- or 25-ml stir cell apparatus with PM30 filters to concentrate samples. Fusions were stored at -20°C following the addition of an equal volume of glycerol. If the fusion was to be cleaved or used immediately, it was kept on ice at all times. Diafiltration was also used to change the buffer in which the MBP-NAC was suspended to buffer 4 (8), which contains 100 mM NaH₂PO₄ (pH 7.0), 250 mM NaCl, 2.5 mM MgCl₂, and 1 mM 2-mercaptoethanol. The NAC protein was not transcriptionally active as a fusion protein with MBP. Therefore, to retrieve active NAC from these fusions for in vitro transcription experiments, it was necessary to cleave NAC from the fusion by using factor Xa protease. This was accomplished by adding 1 μg of protease to 0.5 mg of the fusion protein in a 100-ml volume and then incubating it for 16 h at 22°C. Following cleavage, freshly prepared phenylmethylsulfonyl fluoride was added to 20 mM. The cleaved NAC fusion protein had to be used immediately because it degraded quickly over time.

Purification of his₆-tagged NAC. To overcome the problems associated with MBP-NAC fusions, various six-histidine (his₆)-tagged constructs were built. NAC N-terminal constructs were made by cloning *nac* genes into the *EcoRI/HindIII* sites of pET28 (Novagen). The N-terminal fusions added a leader to the NAC proteins consisting of 56 amino acids which include a stretch of six histidines in a row to facilitate nickel-nitrilotriacetic acid affinity purification (Qiagen). Constructs in which carboxy-terminal his₆ tags were added had the DNA sequence (CAT)₆ followed by an amber stop codon designed into the

primers used to amplify the *nac* gene. The C-terminal his₆-tagged genes were inserted into a derivative of the pET28 vector in which the material between the *Xho*I and *Eco*RI sites had been removed. Histidine-tagged NAC proteins were expressed as previously described for the MBP fusions and prepared for affinity purification in a similar fashion, except that the pH of the buffer was raised to 7.8 to facilitate nickel interaction. Clarified S30 French pressate was added to a column with an internal diameter of 1.25 cm containing a 10-ml bed volume of nickel-nitrilotriacetic acid resin (Qiagen). NAC was allowed to bind the column at a flow rate of 2 ml/min. The column was washed with 150 ml of column buffer supplemented with 5 mM imidazole to eliminate nonspecific binding. NAC was eluted with elution buffer (300 mM imidazole [pH 7.5], 250 NaCl, 1 mM MgCl₂, 1 mM 2-mercaptoethanol). Fractions of 3 ml were collected and screened by Bio-Rad protein assay. NAC generally eluted in a tight peak in fraction 3. Purity was monitored by SDS-PAGE.

Gel filtration analysis of purified proteins. Gel filtration chromatography was used to determine the size and oligomeric state of purified NAC proteins. Size determinations were made by one of two methods: fast protein liquid chromatography (FPLC)-mediated gel filtration or gravity-fed chromatography. For determinations by FPLC, a Pharmacia LCC-501 FPLC apparatus was used in conjunction with a Superdex 75 HR 10/30 prepacked column. The column was equilibrated with buffer 4 (described above) and loaded with 30 μ l (75 μ g) of NAC. Separation was allowed to continue at a flow rate of 1 ml/min for 40 min. Detection of eluting protein was done by monitoring *A*₂₈₀. Standardization of the column was done with gel filtration protein standards purchased from Sigma Chemical Co. resuspended in buffer 4. Generally, dimeric NAC (66-kDa dimer) eluted at a 15-ml volume at the same fraction as bovine serum albumin (66 kDa). Gravity flow gel filtration was performed by using an SR 25/100 column (25-mm internal diameter) packed with a 150-ml bed volume of Sephacryl S-100. Standardization of this column was done with the same buffers, globular weight standards, and concentrations as the FPLC, but the loading volume was increased to 1 ml. The flow rate was adjusted to 0.5 ml/min, and 150 ml was allowed to flow. Generally, dimeric NAC (66 kDa) eluted at a volume of 16 ml from this column.

In vitro transcription assays. In vitro transcriptions were carried out essentially as described previously (8). The template used was plasmid pCB695, which carries the *K. aerogenes hutUH* promoter inserted into vector pTE103, similar to pAM1202, which was described previously (8). Transcription mixtures contained 0.16 pmol of supercoiled plasmid as the template and 2 to 6 pmol of RNA polymerase in a total volume of 25 μ l.

Enzyme assays. Histidase and glutamate dehydrogenase (GDH) activities were measured as described previously (12). Specific activities are reported as nanomoles of urocanate formed (histidase) or NADPH₂ oxidized (GDH) per minute per milligram of cell protein. Cell protein was determined by the method of Lowry et al. (11).

Gel mobility shift assays. Gel mobility shift assays were performed by using purified NAC_E or NAC_K or fragments thereof. The ability of NAC to bind DNA was determined by using the 330-bp *Eco*RI-to-*Hind*III fragment of the *ureD* promoter, which contains a strong NAC-binding site. Binding reaction mixtures consisted of 1 μ l of DNA (0.05 pmol), 1 μ l of poly[d(I·C)] (50 ng/ml), 4 μ l of deionized distilled H₂O, and 1 μ l of a NAC dilution (0.35 to 1.7 pmol) in 50 mM NaH₂PO₄ (pH 7)–125 mM NaCl–0.5 mM MgCl₂–0.1 mM β -mercaptoethanol–50% glycerol, 1-mg/ml bovine serum albumin. NAC was incubated with the DNA for 30 min before addition of 1.5 μ l of loading buffer (40 mM Tris [pH 8.4], 4 mM EDTA, 0.2% bromothymol blue, 0.2% xylene cyanol, 15% Ficoll) and applied to a 2% Tris-borate-EDTA agarose gel. Electrophoresis was carried out at room temperature at 15 V/cm for approximately 1 h. Gels were stained with ethidium bromide, and the fragments were visualized by UV fluorescence.

RESULTS

Residual NAC activity in *nac-10* mutants. The *nac-10* allele of the *E. coli nac* gene has a stop codon and a kanamycin resistance gene inserted into the middle of *nac_E* after codon 165. The resulting NAC polypeptide has four amino acids encoded by the polylinker fused to the 165th amino acid of NAC; the C-terminal 140 amino acids of NAC are not present. The *nac* gene of *E. coli* was replaced with *nac-10* as described elsewhere (14), and the replacement was confirmed by Southern blotting (data not shown). To test for NAC activity, we measured the ability of a strain carrying *nac-10* to activate the NAC-dependent expression of the *hut* operon in response to nitrogen-limited growth. The *nac-10* mutant showed about half as much histidase as the wild type under NAC-dependent conditions (data not shown), suggesting that the NAC protein encoded by *nac-10* retained considerable activity. However, NAC-dependent activation of histidase expression in *E. coli* is

TABLE 2. Residual activity of *nac-10* mutation^a

Strain	Chromosomal allele	Plasmid allele	Presence of N in medium	Activity ^b of:	
				Histidase	GDH
KC2668	<i>nac</i> ⁺	None	+	28	410
			–	389	65
KC2725	<i>nac-203</i>	None	+	31	413
			–	54	436
KC2972	<i>nac-203</i>	<i>nac</i> ⁺	+	31	292
			–	460	41
KC3977	<i>nac-203</i>	<i>nac-10</i>	+	35	470
			–	265	141
KC4510	<i>nac-203</i>	<i>nac-28</i>	+	33	397
			–	59	425

^a Strains were grown to mid-log phase in glucose minimal medium with 0.2% (wt/vol) glutamine as the sole nitrogen source (–) or with a combination of 0.2% glutamine and 0.2% ammonium sulfate as the nitrogen source (+) and assayed for histidase and GDH (12).

^b Specific activity is reported as nanomoles per minute per milligram of cell protein.

weak, making it dangerous to draw strong conclusions based on small differences.

NAC-dependent activation of histidase expression is stronger in *K. aerogenes*, and repression of glutamate dehydrogenase formation by NAC serves as another measure of NAC activity. Therefore, we studied the effect of the *nac-10* allele in *K. aerogenes*. The *nac-10* allele was cloned in low-copy expression vector pGD103 and tested for the ability to complement a *nac-203* mutation in *K. aerogenes*. The wild-type *nac* gene from *E. coli* was able to complement *nac_{K-203}* fully, showing 15-fold activation of histidase formation and 7-fold repression of GDH expression in response to nitrogen starvation. This is similar to the effect seen in wild-type (*nac*⁺) *K. aerogenes* (Table 2, cf. KC2668 and KC2972). In contrast, a known *E. coli* null allele, *nac_{E-28}*, failed to complement *nac_{K-203}* with strain KC4510 (*nac-28/nac-203*), showing no more activation of histidase or repression of GDH expression than the *nac-203* mutant, KC2725. Strain KC3977 (*nac-10/nac-203*) showed about sevenfold activation of histidase formation and threefold repression of GDH formation in response to nitrogen starvation. Thus, the *nac-10* mutation, with an insertion of a stop codon and a kanamycin resistance cassette after amino acid 165, retained about half as much ability to activate and repress transcription as wild-type *nac*⁺ from *E. coli*, despite the absence of the entire C-terminal half of NAC.

This residual activity is particularly surprising compared to the *nac_{K-203}* mutation of *K. aerogenes*. *nac-203* is an insertion of Tn5 (or Tn5-131) after the 199th codon of *nac_K* and has one of the tightest Nac[–] phenotypes of all of the characterized *K. aerogenes nac* mutants (12). Therefore, we began a systematic search for the smallest NAC fragment with biological activity.

The N-terminal 100 amino acids of NAC are sufficient to activate and repress transcription. A collection of mutants with deletions of portions of the C-terminal part of the *K. aerogenes* and *E. coli nac* genes (*nac_K* and *nac_E*, respectively) was generated by exonuclease III digestion as described in Materials and Methods. These truncated *nac* genes were cloned into pGD103, where *nac* expression was under the control of the *lacZ* promoter. *K. aerogenes nac-203* mutant KC2725 was transformed with the resulting plasmids, and histidase and glutamate dehydrogenase activities were deter-

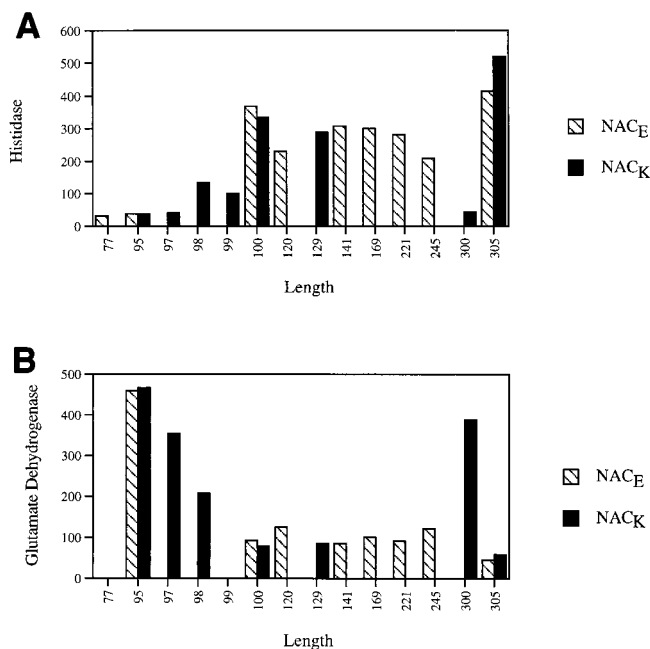


FIG. 1. *hut* activation (A) and *gdh* repression (B) by N-terminal fragments of NAC. *K. aerogenes* KC2725 (*nac-203::Tn5-131*) was transformed with derivatives of pGD103 containing an insert that coded for either full-length NAC or truncated versions of NAC. These transformants were assayed for NAC-dependent activation of histidase formation and repression of GDH formation. The values on the x axis represent the number of amino acids remaining in the NAC fragment. Thus, 305 represents the wild type and 100 represents the N-terminal 100 amino acids. Histidase and GDH values are reported as specific activities.

mined as a measure of NAC-dependent activation and repression. For convenience, the truncated proteins will be named by the number of the last remaining amino acid. Thus, wild-type *E. coli* NAC is NAC_E305 and a *K. aerogenes* NAC lacking the last five amino acids is NAC_K300.

Most of the truncated genes that retained 100 or more codons at the amino terminus were able to complement the *nac_K-203* allele for activation of histidase formation and repression of GDH formation (Fig. 1). The magnitude of the effect varied from about 50 to 90% as much activation or repression as the wild type, and the mutants retaining only 100 amino acids of NAC (NAC_K100 and NAC_E100) were among the most active of the truncations in vivo. This result was as true of the *E. coli nac* gene as of the *K. aerogenes nac* gene and appears to be a general property of NAC rather than a species-specific anomaly.

The single exception in this set was NAC_K300, which lacks only the five C-terminal amino acids of NAC_K and was totally inactive in complementation. Western blot assays of extracts prepared from cells carrying wild-type NAC_K or NAC_K300 were carried out by using anti-NAC serum. The wild type was readily seen, but NAC_K300 was not (data not shown), suggesting that the NAC_K300 product is unstable and degraded rapidly.

Although NAC_K100 has nearly full activity in vivo, NAC_K98 complements *nac_K-203* less well, and truncations with 77, 95, or 97 amino acids remaining failed to complement *nac_K-203*. We have not determined whether the failure of these very short NAC truncation products resulted from rapid degradation or an inactive protein. However, it is clear that a mutant *nac* gene that encodes only the first 100 of the 305 amino acids of NAC

is functional in activation and repression of transcription, at least in vivo.

Activity of N-terminal fragments of NAC purified using MBP tags. Fragments of NAC containing only the N-terminal 100, 120, or 129 amino acids were nearly as active as wild-type NAC in vivo. In order to prove that these fragments were directly responsible for the activity, we attempted to demonstrate activation of transcription in vitro by using purified components. To purify these fragments, we fused the MBP to the N terminus via a peptide linker that could be cleaved with factor Xa protease, leaving an N-terminal methionine, as is seen in wild-type NAC. MBP-tagged variants of wild-type NAC (NAC_K305 and NAC_E305) and two N-terminal fragments (NAC_K129 and NAC_E100) were constructed. All of these fusion proteins were inactive in vivo.

NAC fragments released from MBP-NAC fusions were unstable in the presence of factor Xa (or thrombin when a thrombin-sensitive linker was used); therefore, the factor Xa-mediated cleavage products of MBP-NAC were added immediately to an in vitro transcription reaction mixture without further purification. As expected, cleaved MBP-NAC_K305 was able to activate *hut* transcription (Fig. 2A). Cleaved MBP-NAC_E305 was also able to activate *hut* transcription, consistent with the observation that the *nac_E* gene was able to complement a *nac_K* mutant in vivo. This also provided the first direct evidence that NAC_E was active in vitro as well as in vivo, since we had been unable to purify NAC_E because of its insolubility under all of the conditions tested. Cleaved MBP-NAC_K129 and MBP-NAC_E100 were also able to activate *hut* transcription almost as well as cleaved MBP-NAC_K305 (Fig. 2B). Thus, NAC_K129 and NAC_E100 were able to activate *hut* operon expression in vitro as well as in vivo.

The instability of the NAC fragments in the presence of factor Xa precluded any attempt to quantify NAC in these reaction mixtures, as well as any attempt to isolate large quantities of NAC fragments. Thus, we attempted to characterize NAC fragments with a nickel-binding his₆ tag at the C terminus.

Activity of NAC fragments with his₆ tags. Full-length NAC_E305 and NAC_K305 with the nickel-binding his₆ tag at the C terminus were active in vivo. In fact, the activation of histidase and repression of GDH formation were even stronger with NAC305-his₆ than with the corresponding unmodified NAC305 (Table 3). This increased activation and repression appears not to be specific to the nature of the C-terminal tag, since a cloning artifact that fused an 11-amino-acid peptide (DFGRSGHTDSL) to the C terminus of NAC_K305 also resulted in more activation and repression than NAC_K305 (Table 3). Although the modified *E. coli* NAC (NAC_E305-his₆) was active in vivo, the modification did not prove useful as a means of purifying NAC_E, since NAC_E305-his₆ was insoluble in all of the buffers tested, just like NAC_E305.

Three N-terminal fragments with the his₆ tag at the C terminus were constructed as described in Materials and Methods: NAC_E100-his₆, NAC_K100-his₆, and NAC_K120-his₆. In contrast to their unmodified counterparts, both NAC_K100-his₆ and NAC_E100-his₆ were inactive in vivo (Table 3). Thus, we focused our attention on NAC_K120-his₆. NAC_K120-his₆ was purified by Ni affinity chromatography and shown to be able to activate *hut* operon transcription by purified RNA polymerase (data not shown). Unfortunately, we have had difficulty purifying NAC_K120-his₆ from strains carrying either high-copy or low-copy expression vectors on a reliable basis. We have no explanation for this difficulty, although it may represent either instability of the polypeptide or toxicity of the fragment.

In a final attempt to isolate an active form of NAC_E for

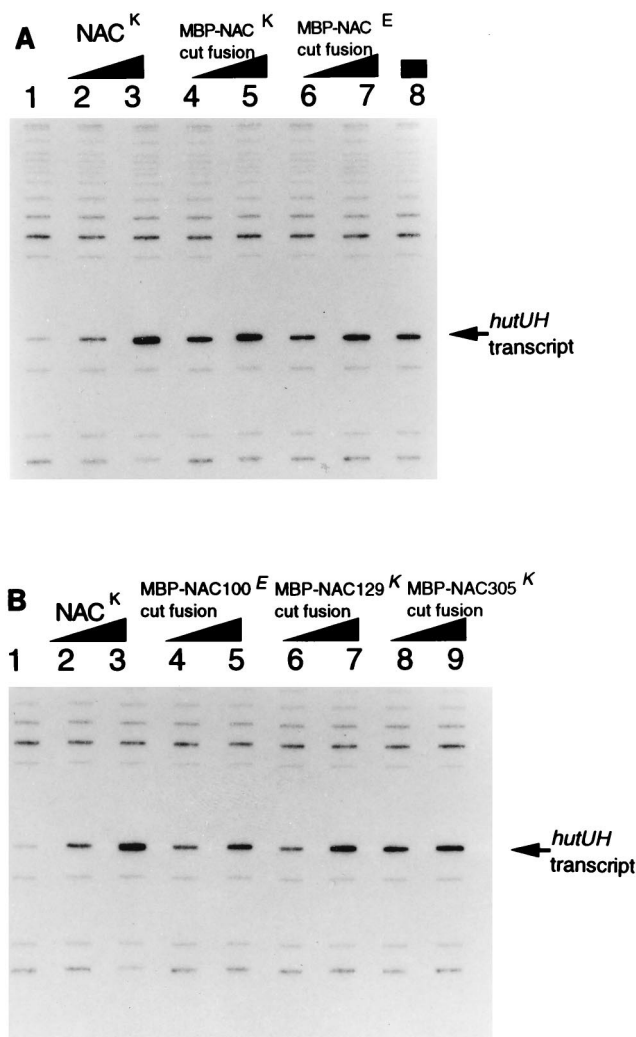


FIG. 2. Activation of *hutUH* transcription in vitro by NAC and N-terminal fragments of NAC. In vitro transcription was carried out by using purified components as described in Materials and Methods. The supercoiled template, pCB695, was similar to pAM1202 (8) and contained the *hutUH* promoter region cloned upstream of a strong transcriptional terminator such that transcription from this promoter would yield a transcript with the mobility on SDS-PAGE indicated at the right. All reactions contained RNA polymerase, nucleotides (including radioactive UTP), and buffers. NAC and NAC fragments were added as indicated. (A) Addition of full-length NAC. Lanes: 1, no NAC; 2 and 3, 0.04 and 0.4 μg of NAC (0.06 and 0.6 pmol of dimers) purified from *K. aerogenes* by salt precipitation (8); 4 and 5, 0.1 and 1 μg of MBP-NAC_K (1.3 and 13.3 pmol of MBP-NAC_K monomers) treated with factor Xa for 30 min immediately before addition to the transcription mixture; 6 and 7, 0.1 and 1 μg of MBP-NAC_E (1.3 and 13.3 pmol of monomers) treated with factor Xa for 30 min immediately before addition to the transcription mixture; 8, 3 μg of MBP-NAC_E (40 pmol of monomers) treated with factor Xa for 16 h before addition to the transcription mixture. (B) Addition of NAC fragments. Lanes: 1, no NAC; 2 and 3, 0.07 and 0.7 μg of NAC (1.1 and 10.5 pmol of dimers) purified from *K. aerogenes* by salt precipitation; 4 and 5, 0.1 and 1 μg of MBP-NAC_E100 (1.9 and 19 pmol of monomers) treated with factor Xa for 30 min immediately before addition to the transcription mixture; 6 and 7, 0.13 and 1.3 μg of MBP-NAC_K129 (1.9 and 19 pmol of monomers) treated with factor Xa for 30 min immediately before addition to the transcription mixture; 8 and 9, 0.14 and 1.4 μg of MBP-NAC_K305 (1.9 and 19 pmol of monomers) treated with factor Xa for 30 min immediately before addition to the transcription mixture.

comparison to NAC_K in vitro, we constructed a gene encoding NAC_E that was modified at the N terminus with MBP and at the C terminus with his₆ (MBP-NAC_E-his₆). We hoped to use the his₆ tag to separate the NAC_E fragment from factor Xa

TABLE 3. Activation of histidase formation and repression of GDH formation in vivo by modified forms of NAC

NAC source and strain ^a	Plasmid ^b	NAC product ^c	Sp act ^d of:	
			Histidase	GDH
<i>E. coli</i>				
KC2725	None	None	31	413
KC3568	pCB774	NAC _E	415	46
KC3210	pCB594	MBP-NAC _E	34	435
KC3555	pCB771	NAC _E -his ₆	890	21
KC3973	pCB941	NAC _E 100-his ₆	42	354
<i>K. aerogenes</i>				
KC3572	pCB778	NAC _K	520	59
KC3981	pCB606	MBP-NAC _K	37	417
KC3578	pCB781	NAC _K -his ₆	650	35
KC3580	pCB782	NAC _K -DFGRSGHDTSL	720	32
KC3615	pCB800	NAC _K 120-his ₆	295	ND ^e
KC3591	pCB788	NAC _K 100-his ₆	45	335

^a All strains are derived from *K. aerogenes* KC2725 (*nac-203::Tn5-131*), grown in glucose minimal medium to mid-log phase, and assayed as previously described (12). IPTG (0.5 mM) was included for strain KC3981 to guarantee expression from the pMalC2 vector.

^b All plasmids except pCB594 and PCB606 were derived from pGD103 as described in Materials and Methods. Plasmids pCB594 and pCB606 were derived from pMalC2.

^c The code is described in detail in the text. Subscripts indicate derivation from *E. coli* (E) or *K. aerogenes* (K). MBP indicates MBP fused to the N terminus of NAC. his₆ indicates six histidine residues added to the C terminus. DFGRSGHDTSL indicates fusion of 11 amino acids to the C terminus of NAC (amino acids are indicated by the one-letter code). NAC100 and NAC120 indicate that only the first 100 or 120 amino acids of NAC were present.

^d Specific activity is reported as nanomoles per minute per milligram of cell protein.

^e ND, not determined.

after cleavage of MBP-NAC_E-his₆. However, MBP-NAC_E-his₆ was completely resistant to cleavage by factor Xa, in marked contrast to MBP-NAC_E. Only the addition of strong denaturants allowed MBP to be removed, and this was accompanied by very rapid disappearance of NAC_E. No active NAC_E was recovered after attempts to renature the material. Apparently, the C-terminal his₆ tag had altered the factor Xa cleavage site in MBP-NAC_E-his₆ so as to make it inaccessible to cleavage by factor Xa, suggesting that the C terminus of the folded protein is close to the N terminus. Thus, the C-terminal his₆ tag would be able to prevent the protease from reaching the cleavage site at the N terminus of the NAC polypeptide.

Subunit structures. We next used the tagged NAC polypeptides to search for the dimerization site on NAC. These data (along with a summary of the in vitro activation data and DNA-binding data) are presented in Table 4. NAC_E and NAC_K with an N-terminal MBP tag eluted as monomers. If factor Xa was added, the elution profile was complex and included a peak of the size expected for MBP-NAC (uncleaved, monomer), MBP (monomer), NAC (dimer), and an unknown peak with an apparent size of 8.5 kDa (presumed to be a degradation product of the 33-kDa NAC monomer).

A smaller N-terminal modification was also tested by fusing a 56-amino-acid peptide that included a his₆ tag in its midst to the N terminus of NAC_K and NAC_E. Both of these fusions were inactive in vivo (data not shown). The N-terminally his₆-tagged NAC_E and NAC_K were purified by Ni affinity chromatography. The tagged NAC_K was found to be dimeric in solution even before cleavage (NAC_E was not tested). However, neither the NAC_K nor the NAC_E fusion protein was able to activate transcription in vitro (Table 4). Cleavage of the tagged NAC_E with thrombin removed the his₆ domain but left an

TABLE 4. In vitro properties of modified forms of NAC^a

Construct	Apparent molecular size in solution (kDa)	Form ^b	Gel mobility shift ^c	Transcription activation ^c
NAC _E		I		
MBP-NAC _E	75 (66)	M (D)	Y (Y)	(Y)
56his ₆ -NAC _E			Y (Y)	N (N)
NAC _E -his ₆		I		
NAC _E 100-his ₆		I		
MBP-NAC _E 100				(Y)
NAC _K	66	D	Y	Y
MBP-NAC _K	70 (66)	M (D)	Y (Y)	N (Y)
56his ₆ -NAC _K	77	D	Y	
NAC _K -his ₆		Y	Y	
MBP-NAC _K 129	56 (28)	M (D)		(Y)
NAC _K 120-his ₆	27	D	Y	Y
NAC _K 100-his ₆	22	D	Y	

^a NAC_E, NAC_K, and several modified forms thereof were purified as described in Materials and Methods. The apparent molecular size in solution was determined by gel filtration, and this size was used to infer whether the product was monomeric or dimeric in solution. The ability to bind DNA was determined in a gel mobility shift assay using the urease operon promoter (*ureDp*) as a target. The ability to activate transcription from the *hutUH* promoter (*hutUp*) was tested as described in the legend to Fig. 2. The values in parentheses were determined after cleavage with Factor Xa or thrombin.

^b M, monomer; I, insoluble; D, dimer.

^c Y, yes; N, no.

18-amino-acid peptide fused to the N terminus of NAC_E. This cleavage product was also unable to activate transcription in vitro (Table 4). In summary, fusions to the N terminus of NAC_E increase its solubility, but even small additions at the N terminus block its ability to activate transcription. Large additions (MBP) block the ability to dimerize in solution (Table 4).

Somewhat surprisingly, the C-terminally his₆-tagged NAC fragments of NAC_K (NAC_K100-his₆ and NAC_K120-his₆), as well as the product released from MBP-NAC_K129 by (factor Xa) cleavage, were all dimers in solution as measured by gel filtration. Thus, the N-terminal domain of 100 amino acids also appears to contain the signals required for dimer formation, as well as for activation of transcription.

DISCUSSION

Perhaps the most surprising finding in these experiments is that the N-terminal 100 amino acids of NAC contain the determinants for all of the known functions of NAC. NAC100 was able to activate histidase formation in vivo and *hutUp* transcription in vitro. NAC100 was able to repress GDH formation in vivo. NAC_K100-his₆ was able to bind a DNA fragment with a NAC-binding site, and NAC_K100-his₆ was present as a dimer in solution. In short, NAC_K100 (or NAC_K100-his₆) displayed all four known properties of NAC: activation of transcription, repression of transcription, site-specific DNA binding, and dimerization.

All of the truncations of NAC isolated in these experiments, except NAC_K300, showed considerable NAC activity. However, several tight *nac* mutants have been isolated before by using selection and screening. One of these, *nac-203::Tn5*, has been sequenced and is an insertion of Tn5 after the 199th codon of *nac_K*. The data in Fig. 1 suggest that this mutation should leave NAC partially active, but *nac-203* is one of the tightest *nac* mutants in our collection (although even *nac-203* retains some activity, particularly on the *ure* operon promoter). Thus, it seems likely that NAC_K300 and NAC_K199 are inactive

because of instability or misfolding rather than because of any lack of informative sequence.

The data presented here also provide some information about the function of the N and C termini of NAC. Additions to the N terminus block the ability of NAC to activate transcription, and small additions to the C terminus increase this activity, at least in vivo. The effect of the N-terminal additions appears to be intrinsic to the protein, since it is also seen in vitro with purified protein. Moreover, the ability to activate transcription can be recovered (in the case of MBP-NAC) by removing the N-terminal addition. The effect of the C-terminal additions may likewise be intrinsic to the protein, but it may also reflect an increased stability of the C-terminally modified NAC. We have been unable to raise a high-titer antibody with sufficient specificity for NAC to address this question directly, but we have previously suggested that wild-type NAC is rapidly turned over in vivo (14).

Two lines of evidence led us to suspect an interaction between the N and C termini of NAC and perhaps other LTTRs. First, MBP-NAC-his₆ was completely resistant to cleavage by factor Xa, while MBP-NAC was readily cleaved. Second, other work in this laboratory (1) has shown that replacement of the amino-terminal 20 amino acids of NAC with the corresponding amino acids from OxyR results in an inactive NAC unless the C-terminal domain of this NAC-OxyR chimera is also replaced with the C-terminal domain from OxyR.

The helix-turn-helix region thought to be the DNA-binding site is located very close to the N terminus of LTTRs (about amino acids 20 to 40 for NAC). The binding site for cofactors of LTTRs has generally been found in the C-terminal domain, between amino acids 100 and 200 (15). Thus, an effect of the C-terminal domain on the N-terminal domain must exist. The specificity requirement in the OxyR-NAC chimeras (1) and the ability of the C-terminal his₆ tag to prevent cleavage of MBP from the N terminus of MBP-NAC-his₆ seem more consistent with a hypothesis of direct interaction than with transmission of a signal through the middle part of the protein. However, in the absence of experiments to test a direct interaction, such arguments remain speculative at best.

It was exciting to discover that the four known regulatory properties of NAC are encoded within a NAC fragment containing only the N-terminal 100 amino acids, NAC100. It was disappointing to discover that a fifth property of NAC, the insolubility of NAC_E, was also encoded within this fragment. Most of the amino acid differences between NAC_E and NAC_K lie in the C-terminal domain; the N-terminal domains are more similar (14). Nevertheless, even NAC_E100-his₆ is much more insoluble than NAC_K100-his₆. Thus, future studies will continue to focus on NAC_K for in vitro studies.

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