JOURNAL OF BACTERIOLOGY, Nov. 1997, p. 6649–6656 Vol. 179, No. 21
0021-9193/97/$04.00+0

Deletion of the N-Terminal Region of the AREA Protein Is Correlated with a Derepressed Phenotype with Respect to Nitrogen Metabolite Repression

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Received 20 March 1997/Accepted 2 September 1997

The entire *areA* gene and a truncated version lacking the sequence encoding the N-terminal 389 amino acids were expressed from the *qutE* promoter and terminator in an *Aspergillus nidulans* strain with the endogenous *areA* gene deleted. This expression system was used to decouple the effects of transcription regulation and mRNA stability mediated by the native promoter and terminator from any posttranslational modulation of AREA activity. Both the full-length AREA protein and the truncated form were able to function in the deletion strain, conferring the ability to use alternate nitrogen sources. Transformants containing the entire *areA* gene had a repressible phenotype with respect to nitrogen metabolite repression, whereas those containing the truncated form of the *areA* gene had a derepressed phenotype. The truncated *areA* gene was expressed in an *A. nidulans* strain containing a normally regulated wild-type *areA* gene, and transformants displayed a quinate-inducible nitrogen metabolite derepressed phenotype. Northern blot analysis of transformed strains showed that *areA*-specific mRNAs of the expected sizes were being produced. The truncated AREA protein was overproduced in *Escherichia coli* as a fusion protein and purified to homogeneity by a single-step immobilized metal affinity chromatography, and the purified protein was shown to bind specifically to the *niaD* promoter. Revised sequences of the 5′ region of the *areA* gene and the entire *meaB* gene are reported.

The *areA* gene of *Aspergillus nidulans* encodes a positively acting transcription regulator, AREA, which controls the expression of many of the genes encoding enzymes and permeases necessary for the use of alternative nitrogen sources (7, 25, 33). Many of the alternative nitrogen utilization pathways are regulated by pathway-specific proteins, but when the preferred nitrogen sources ammonium and glutamine are provided, these pathways remain inactive—a state called nitrogen metabolite repression (33). The equivalent function in *Neurospora crassa* is carried out by the NIT2 protein (encoded by the *nit-2* gene), and mutational analysis, deduced amino acid sequence alignments, and heterologous expression studies of the cloned *nit-2* and *areA* genes have confirmed that the two proteins are homologous (8, 14, 19, 23). An equivalent gene, *nre*, has been isolated from *Penicillium chrysogenum*, and its nucleotide sequence has been determined (16). AREA, NIT2, and NRE proteins contain a single zinc finger motif which is proposed to facilitate DNA binding to specific target sequences.

Several different models have been proposed for the mechanism by which the transcription activating status of the NIT2 and AREA proteins is modulated in response to ammonium or glutamine (9, 11, 15, 17). In *N. crassa*, mutation analysis has identified a second gene, *nmr-1*, which is proposed to be involved in mediating nitrogen metabolite repression (12, 35). A molecular analysis (34) has shown that the NMR1 protein is directly involved in mediating nitrogen metabolite repression by binding to two α-helical regions of NIT2, one region that is located within the DNA-binding domain and the other that encompasses the C-terminal 30 amino acids. No firm conclusions could be drawn on any possible interactions between the NMR1 protein and the N terminus of NIT2, as the plasmid constructs used produced NIT2 proteins with extensions of heterologous amino acids added to the wild-type N terminus (34). Platt et al. (28) state that "It is conceivable that the C-terminal domain in AREA interacts with, and possibly binds to, a second protein such as an NMR1 equivalent." They further report experiments in which expression of *areA* sequences in *A. nidulans* encoding the carboxy-terminal 159 residues of the AREA protein (which might therefore interact with and titrate out a second regulatory molecule like NMR1) apparently have no phenotypic consequences. These latter data have been discussed in detail elsewhere (27), concluding with the statement "The interpretation of the data produced by the overexpression of the C-terminus is far from conclusive." On the basis of mutant phenotypes, the most likely genetically identified gene in *A. nidulans* that corresponds to *nmr-1* has been suggested to be the *meaB* gene (1, 17). The *meaB* gene has recently been cloned, its nucleotide sequence has been determined, and the deduced amino acid sequence has been reported as being unrelated to that of the NMR1 protein (29).

In *A. nidulans*, the extreme C terminus of AREA and sequences within the last 218 nucleotides of the *areA* transcript have been identified as being important in mediating nitrogen metabolite repression in response to the presence of glutamine (4, 19, 28). It has been reported that, under repressing conditions, the half-life of the wild-type *areA* mRNA is shorter than that under derepressing conditions and a sequence in the 3′ untranslated region of the *areA* mRNA has been implicated in this differential mRNA stability (28).

A three-domain model for the structure of the AREA and NIT2 proteins has been proposed (17); this model predicts that the N-terminal domain acts, at least in part, as a molecular

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TABLE 1. *A. nidulans* strains used in this study

Strain	Genotype	*areA* copy number(s)
NKRB3-2	Δ*areA ya2 pyroA4 riboB2*::*ribo*$^+$	0
G191	*fwA1 pyrG189 pabaA1 uaY9*	1 (wild type)
R153	*wA3 pyroA4*	1 (wild type)
QWA2	Δ*areA*::*areA*$^+$ *ya2 pyroA4 riboB2*::*ribo*$^+$	3 (*areA1-876*)
QWA3	Δ*areA*::*areA*$^+$ *ya2 pyroA4 riboB2*::*ribo*$^+$	2 (*areA1-876*)
QCF1	Δ*areA*::*areA390-876 ya2 pyroA4 riboB2*::*ribo*$^+$	2 (*areA390-876*)
QCF2	Δ*areA*::*areA390-876 ya2 pyroA4 riboB2*::*ribo*$^+$	3 (*areA390-876*)
CF77/38	*fwA1 pyrG189*::*pyr-4 pabaA1 uaY9*	1 (wild-type host), 5 (*areA390-876*)
CF77/42	*fwA1 pyrG189*::*pyr-4 pabaA1 uaY9*	1 (wild-type host), 1 (*areA390-876*)

sensor to detect the presence of glutamine. This model predicts that the two carboxy-terminal domains are transcriptionally active in the absence of the amino-terminal domain and are also insensitive to glutamine- or ammonium-mediated nitrogen metabolite repression (17). Here, we report the results of experiments that test these predictions.

MATERIALS AND METHODS

Materials. Chemicals and solvents were purchased from local suppliers and were of analytic grade or greater purity. Enzyme substrates were purchased from Sigma, and molecular biology reagents (which were used in accordance with the manufacturer's recommendations) were purchased from GIBCO/BRL, Pharmacia, Boehringer Mannheim, or the University of Newcastle upon Tyne Facility for Molecular Biology. The genotypes of the strains used in this study are given in Table 1.

Methods. Purification of total RNA was done by the method of Cathala et al. (5), and poly(A)$^+$ mRNA was prepared from total RNA by using Dynabeads Oligo(dT) and following the manufacturer's protocol. Images from Southern and Northern blot experiments that involved the *areA* and *actA* genes of *A. nidulans* were recorded by quantitative phosphorimaging, using a Fuji phosphorimager, and analyzed with Tina software. Exposures of the probed filters varied from 1 h (for transformants containing multiple copies of the integrated plasmids) to 96 h (for strains with low levels of wild-type regulatory gene mRNA). An [α-^{32}P]dCTP-labelled 0.83-kb *Nco*I-*Kpn*I fragment from the *A. nidulans actA* gene (13) was used as a probe to identify the *actA* mRNA as an internal standard for Northern blots. The relative copy number of the *areA* gene in Southern blots of transformant DNA from strain NKRB3-2 was quantified by comparison to the *actA* gene of *A. nidulans* (13) as an internal reference. In the case of the G191 transformants (which have a resident copy of the wild-type *areA* gene), the relative copy number of the *areA* gene was established by using the resident *areA* gene as an internal marker. Transformation of *A. nidulans* (using KCl as the osmotic stabilizer) was as previously described (21). Cell extracts of *A. nidulans* were assayed for nitrate reductase as previously described (3) with a Shimadzu UV 1601 visible spectrophotometer. All other routine molecular biology protocols followed individual manufacturer's recommendations or were as previously described (24).

Construction of recombinant plasmids for expression in *A. nidulans*. The *areA* DNA sequences corresponding to the entire coding sequence or a truncated sequence lacking the N-terminal 389 amino acids were amplified by PCR with 5′ sense oligonucleotides (containing an *Nco*I recognition site) of sequence 5′ TCCAGCCCACGGCCATGGCTGGGTTGACTC 3′ (entire *areA*) or of sequence 5′ TTGCCTCTTCACCCATGGCAACCGACTTCT 3′ (truncated *areA*) and a 3′ antisense oligonucleotide (containing a *Hin*dIII site) of sequence CTT TAGAAGCTTACAAACTCA. After digestion with the appropriate restriction endonuclease, the DNA sequence encoding the entire *areA* gene was subcloned into the *A. nidulans* expression vector pNUFC102 (20), placing it under the control of the *qutE* promoter and the *qutE* terminator. Similarly, the DNA sequence encoding the truncated *areA* gene was subcloned into the *A. nidulans* expression vector pNUFC77 (20). The vectors pNUFC77 and pNUFC102 differ only in that the latter has a naturally occurring *Nde*I site altered to *Xba*I by site-directed mutagenesis (20). The recombinant plasmid containing the entire *areA* gene was designated pNUFC266, and the recombinant plasmid containing the truncated *areA* gene was designated pNUFC205. The truncated sequence was also subcloned into the *Escherichia coli* expression vector pRSETB to produce plasmid pRF14. Oligonucleotides specific to the 5′ end of the *qutE* promoter (5′ ACGAATTAATTCAGATCTACTGGGGCAATC 3′) and the *qutE* terminator (5′ CGGCCAGTGCCAAGATCTTTCATGCAGAAT 3′) and containing restriction sites for *Bgl*II were subsequently used to PCR amplify the *areA* DNA sequences in plasmid pNUFC205 as a cassette with the promoter and terminator attached. Following digestion with the appropriate restriction endonucleases, the PCR-amplified DNA sequences were subcloned in the transformation vector pCAP2 (31) at a unique *Bam*HI site to produce plasmid pACF1. Use of restriction endonucleases followed manufacturers' recommendations and amplification of target DNA sequences by PCR was done as previously described (26, 32). All other routine molecular biology protocols followed the individual manufacturer's recommendations or were as previously described (24). The relevant features of the plasmids constructed as part of this work are given in Table 2.

Growth tests. Derepression of nitrogen circuit enzymes in transformants and wild-type strains were detected by combinations of the following four diagnostic plate tests: (i) sensitivity to 100 mM chlorate toxicity with 10 mM ammonium or glutamine as the nitrogen source, indicating derepression of nitrate reductase (2); (ii) clearing the turbidity to produce a halo on medium containing 1.5% (wt/vol) powdered skim milk with 10 mM ammonium or glutamine, indicating derepression of extracellular protease (6); (iii) sensitivity to 0.32 mM DL-β-aspartyl hydroxamate (10), indicating derepression of asparaginase; and (iv) reduction of nitrite in the presence of 10 mM ammonium such that proton depletion allows 0.25% (wt/vol) bromothymol blue in the growth medium to develop a blue color as opposed to the yellow colon of the wild-type strain which is caused by a drop in pH (19), indicating derepression of nitrite reductase. Plate tests were also used to determine the sensitivity of some transformants and wild type to 15 mM nitrite in the presence of 10 mM ammonium or glutamine. In all cases, 0.1% (wt/vol) quinate was used to induce transcription from the *qutE* promoter, and 0.5% (vol/vol) glycerol or 0.5% (wt/vol) glucose was used as the carbon source.

Overproduction and purification of a truncated AREA protein. Cultures (400 ml) of *E. coli* BL21 carrying plasmids pLysE and pRF14 were grown to mid-exponential phase in Luria broth at 37°C in the presence of 50 μg of ampicillin ml^{-1} and 20 μg of chloramphenicol ml^{-1}; 0.2 mg of isopropyl-β-D-thiogalactopyranoside (IPTG) ml^{-1} was then added, and growth continued for a further 3 h. After the cells were harvested by centrifugation, the cell pellets were resuspended in 40-ml portions of a solution containing 50 mM potassium phosphate, 0.5 M NaCl (pH 7.2), 1 mM dithiothreitol (DTT), and 1 mM benzamidine (buffer 1), and following sonication, a cell extract was prepared by centrifugation (26). Proteins in the sonicate capable of zinc binding were adsorbed onto a 27.15-ml chelating Sepharose column that had been charged to one-third capacity with zinc and equilibrated in buffer 1. Loosely bound proteins were eluted from the column with a 100-ml step wash of buffer 1 containing 2.0 M glycine. Tightly bound proteins were then eluted with a single 50 mM potassium phosphate linear pH gradient (pH 7.2 to 4.0). The AREA protein-containing fractions were pooled and dialyzed overnight at 4°C against 5 liters of buffer (pH 7.0) containing 50 mM potassium phosphate, 0.5 M KCl, 1 mM DTT, and 10 μM $ZnSO_4$ and subsequently concentrated 10-fold by pressure concentration, using a ChemLab concentrator and a YM10 DIAFLO ultrafiltration membrane.

EMSA. Gel mobility shift experiments were performed by the method of Fu and Marzluf (14) and carried out in a final volume of 10 μl containing 10 mM HEPES (pH 7.9), 2.0 mM DTT, 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 4.0 mM spermidine, 10% (vol/vol) glycerol, 20 μg of bovine serum albumin ml^{-1}, 1.0 mg of poly(dI-dC) ml^{-1}, 6×10^4 cpm of [α-^{32}P]dCTP-labelled probe, and approximately 30 to 250 ng of protein. A section of the *niaD* gene promoter region (EMBL accession no. XM58291) was amplified by PCR with a 5′ oligonucleotide of sequence 5′ AGATGGAGCCCTGAAGT 3′ and a 3′ oligonucleotide of sequence 5′ GAATTCGCAGCGACCAC 3′. The target sequence used in the electrophoretic mobility shift assay (EMSA) was generated by a subsequent *Bam*HI digestion of the PCR-amplified DNA and corresponded to coordinates 1 to 296 as designated in the previously published EMBL accession no. XM58291.

RESULTS AND DISCUSSION

Sequence analysis of the *areA* and *meaB* genes. When these experiments were undertaken, a revision of the analysis of the 5′ region of the *areA* gene using unpublished sequence data

TABLE 2. Relevant features of the plasmids used in this study

Plasmid	Vector	Promoter or terminator	Organism for expression	*areA* nucleotide coordinates	AREA amino acid coordinates
pNUFC266	pNUFC102	*qutE*	*A. nidulans*	1–2631	1–876
pNUFC205	pNUFC77	*qutE*	*A. nidulans*	1168–2631	390–876
pACF1	pCAP2	*qutE*	*A. nidulans*	1168–2631	390–876
pRF14	pRSETB	T7	*E. coli*	1168–2631	390–876

TABLE 3. Results of diagnostic growth tests for nitrogen metabolite derepression in transformants of *A. nidulans* NKRB3-2[a]

Transformant	Extracellular protease (milk clearing)		Nitrite reductase (bromothymol blue test)		Nitrate reductase (chlorate sensitivity)	
	Phenotype	Effect observed under repressing conditions	Phenotype	Effect observed under repressing conditions	Phenotype	Effect observed under repressing conditions
NKRB3-2	Repressed	No halo	Repressed	Yellow color	Repressed	Growth insensitive to chlorate
QWA2	Repressible	No halo	Repressible	Yellow color	Repressible	Growth insensitive to chlorate
QWA3	Repressible	No halo	Repressible	Yellow color	Repressible	Growth insensitive to chlorate
QCF1	Derepressed	Halo of milk clearing	Derepressed	Blue color	Derepressed	Growth sensitive to chlorate
QCF2	Derepressed	Halo of milk clearing	Derepressed	Blue color	Derepressed	Growth sensitive to chlorate
R153	Repressible	No halo	Repressible	Yellow color	Repressible	Growth insensitive to chlorate

[a] The phenotypes reported are those observed after 24 h of growth in the presence of 0.1% quinate.

had been reported in two reviews (4, 7). The presence (but not the position) of a previously unreported intron in the extreme 5′ coding region of the *areA* gene was reported. To facilitate the cloning of the entire *areA* gene into *A. nidulans* expression vectors and place it under the control of the *qutE* promoter, the extreme 5′ end of the *areA* gene was resequenced and the revised sequence was placed in the public domain (EMBL database accession no. X90649). This sequence (which was independently confirmed by Langdon et al. [23]; EMBL database accession number X52491), demonstrated the presence of an intron and additionally identified a missing sequence of 82 nucleotides compared with the sequence originally published by Kudla et al. (19).

In order to overproduce the *A. nidulans* MEAB protein in *E. coli* and thereby facilitate in vitro studies on potential protein-protein interactions between AREA and MEAB, we independently cloned the *meaB* gene. We found that an experimentally determined restriction map did not agree with that predicted from the published sequence (29), so we independently determined the complete nucleotide sequence of this gene. This sequence analysis revealed a number of changes compared with that previously published (29), including changes consistent with the new restriction map. A sequence of 53 nucleotides was missing immediately downstream of the previously reported AUG translational start codon. When this missing sequence is incorporated, four in-frame potential AUG start codons are predicted in the N-terminal 17 amino acids. This observation has implications for strategies for overproducing the native MEAB protein in *E. coli*, as it is no longer clear which AUG codon represents the true in vivo translational start site. Other revisions to the *meaB* nucleotide sequence include (i) a frameshift in the coding region such that a MEAB protein 37 residues shorter at the extreme C terminus than that previously reported is predicted and (ii) changes that remove predicted *Hin*dIII and *Bsp*HI sites and create an *Nco*I site (the absence or presence of these sites was confirmed by the insensitivity or sensitivity of PCR products derived from the *meaB* gene from three strains of *A. nidulans* to *Hin*dIII, *Bsp*HI, and *Nco*I). The complete revised sequence is available in the EMBL database under accession no. Y13700.

All sequencing was achieved by PCR amplification from chromosomal DNA (*areA* and *meaB*) and cDNA (*areA*), subsequent cloning into M13 sequencing vectors, and automated nucleotide sequence determination. Sequences for each gene were determined on both strands from clones derived from two independent PCRs to ensure that any differences from the previously published sequence were not due to artifacts caused by PCR included in the subcloning strategy.

Expression of *areA* and truncated forms of *areA* in *A. nidulans* NKRB3-2. Amino acids 1 to 389 correspond to the coordinates of the N-terminal domain in the three-domain model for the structure of the AREA and NIT2 proteins proposed by Hawkins et al. (17). The entire *areA* gene and a truncated form lacking the codons encoding the N-terminal 389 amino acids were subcloned into *A. nidulans* expression vectors to generate plasmids pNUFC266 and pNUFC205, respectively (see Materials and Methods). This cloning strategy places these sequences under the control of a quinic acid-responsive promoter and teminator derived from the *qutE* gene of *A. nidulans*. The promoter and terminator were changed to un-

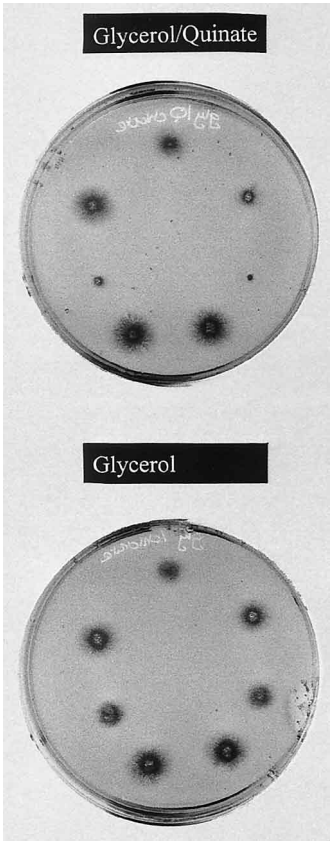

FIG. 1. Typical responses of wild-type control, host transformation strain and selected transformants in the plate test for derepression of nitrate reductase (sensitivity to the presence of chlorate). The growth tests were carried out as described in Materials and Methods, with the various strains arranged as follows: clockwise starting at the top, NKRB3-2, QWA2, QCF1, R153 (wild-type control), R153 (wild-type control), QCF2, and QWA3. The carbon sources included are shown above the plates.

TABLE 4. Nitrate reductase levels in control and transformed strains of *A. nidulans* NKRB3-2[a]

Strain	Induction conditions[b]			Nitrate reductase activity[c]			
				Expt 1		Expt 2	
	Nitrate	Quinate	Ammonium	Specific	Relative	Specific	Relative
R153	+	+	−	10.29	1.00	29.9	1.00
	+	+	+	0.00	0.00	6.43	0.22
	+	−	+	0.00	0.00	0.00	0.00
QWA2	+	+	−	3.38	1.00	12.70	1.00
	+	+	+	0.00	0.00	2.57	0.20
	+	−	+	0.00	0.00	0.00	0.00
QCF1	+	+	−	6.59	1.00	3.53	1.00
	+	+	+	5.95	0.90	3.22	0.91
	+	−	+	0.00	0.00	0.00	0.00

[a] Spores from desired strains were grown for 18 h at 37°C in minimal medium with glucose (0.4% [wt/vol]) and ammonium tartrate (5 mM) as the carbon and nitrogen sources, respectively. For the QWA and QCF transformants and the associated R153 control, the harvested (by filtration) washed mycelium was then divided into three 1-g portions and reincubated for 8 h in minimal medium containing the following supplements: (i) nitrate (10 mM), quinate (0.1% [wt/vol]), and glucose (0.05% [wt/vol]); (ii) nitrate (10 mM), ammonium (5 mM), quinate (0.1% [wt/vol]), and glucose (0.05% [wt/vol]); (iii) nitrate (10 mM), ammonium (5 mM), and glucose (0.05% [wt/vol]). Following incubation, harvested washed mycelium was blotted between paper towels and snap frozen in liquid nitrogen, and 0.5-g portions of frozen mycelium were immediately processed to produce cell extracts by grinding to a powder in liquid nitrogen and subsequent shaking in extraction buffer (100 mM potassium phosphate [pH 7.2], 150 mM NaCl, 1 mM DTT) for 1 h at 4°C. After incubation in extraction buffer, cell extracts were prepared by centrifugation at 4°C and assayed as previously described (3) within 3 h of preparation with a Shimadzu UV 1601 visible spectrophotometer.

[b] The symbols + and − denote the presence or absence, respectively, of the particular addition to the medium.

[c] Experiments 1 and 2 are repeat experiments carried out on different days. Specific nitrate reductase activity is reported in units per milligram of protein, where 1 U is the amount of enzyme necessary to oxidise 1 nmol of NADPH per min at 32°C. Relative nitrate reductase activity is the specific activity of individual strains expressed relative to the value obtained for the individual strain in the absence of ammonium (given an arbitrary value of 1).

couple the effects of transcription regulation and mRNA stability (28) imparted by the native promoter and terminator and allow the study of any posttranslational modulation of AREA activity in isolation from these effects.

The quinic acid-responsive *qutE* promoter has another useful advantage for the study of any posttranslational modulation of AREA protein activity, as it is subject to carbon catabolite repression. Therefore, when analyzing the properties of transformants, the levels of AREA protein produced can be modulated by growing the strains on glucose (strongly catabolite repressing for quinate promoters) or glycerol (weakly catabolite repressing for quinate promoters). We also chose to use nontargeted integration of the plasmid-borne sequences to maximize the chances of obtaining transformants with a range of copy numbers. In this way, the phenotypic consequences with respect to nitrogen metabolite repression of producing a range of concentrations of the full-length and truncated AREA proteins can be investigated in vivo. We have successfully used this strategy for varying the amount of recombinant protein production in *A. nidulans* previously (21).

(i) Strain NKRB3-2 transformed with plasmids pNUFC266 and pNUFC205 can utilize nitrate. The *A. nidulans* mutant strain NKRB3-2 has the entire chromosomal *areA* gene deleted and is unable to make efficient use of nitrogen sources other than ammonium. We have previously observed that transformation frequencies are sharply reduced when carried out in the presence of quinate (22), and this phenomenon was repeated with strain NKRB3-2 (3 to 12 transformants μg^{-1}). Transformants of *A. nidulans* NKRB3-2 were recovered on the basis of the ability to use nitrate in the presence of quinate after exposure to the plasmids pNUFC266 and pNUFC205. Both plasmid-borne *areA* sequences therefore specify functional AREA proteins that allow an alternate nitrogen source to be used. Transformants containing the truncated *areA* gene were unable to utilize nitrate as the sole nitrogen source unless quinate was present in the growth medium. In contrast, transformants containing the entire *areA* gene were able to utilize nitrate in the absence of quinate, but their growth was enhanced by its addition. This effect is consistent with two alternate explanations. The first explanation proposes that sufficient complete AREA protein is being produced from basal-level read-through of the *qutE* promoter to allow nitrate utilization, whereas the second explanation proposes that basal-level mRNA production is facilitated by sequences within the 5′ 389 codons of the *areA* gene which may act as cryptic promoters (16).

(ii) Diagnostic growth tests for nitrogen metabolite repression in transformants of strain NKRB3-2. Diagnostic growth tests for nitrogen metabolite repression were carried out on a range of transformants selected in the presence of quinate: the QWA transformants (13 tested), which contain the entire *areA*

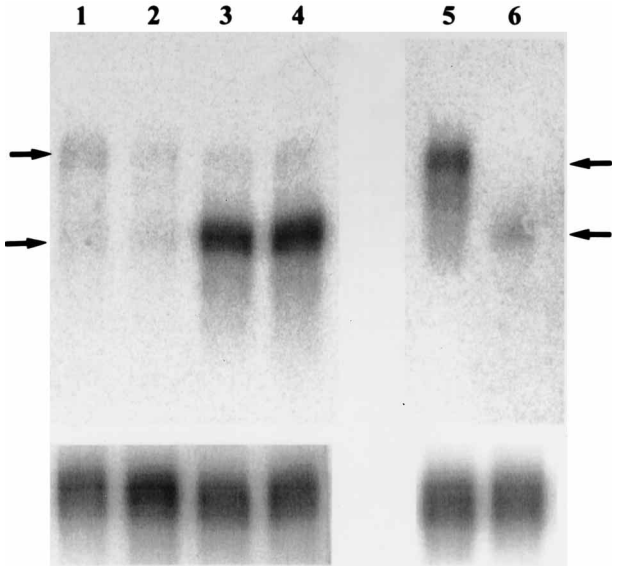

FIG. 2. Northern blot analysis of transformants CF77/38, QWA2, and QCF2. In two separate blots, the arrows mark the positions of the wild-type (approximately 2.9-kb) and truncated *areA* (approximately 1.6-kb) mRNAs in transformant CF77/38 (lanes 1 to 4) and the positions of the *qutE*-driven full-length *areA* gene (approximately 2.8-kb) and the truncated *areA* gene (approximately 1.6-kb) mRNAs in transformants QWA2 and QCF2 (lanes 5 and 6). The reference *actA* mRNA is shown in the blots at the bottom of the figure. Transformant CF77/38 was grown at 37°C for 18 h in liquid minimal medium containing proline (10 mM) and glucose (0.4% [wt/vol]), and the harvested mycelium was divided equally into minimal medium supplemented with 0.5% glycerol and 10 mM nitrate (lane 1), 0.5% glycerol (wt/vol), 10 mM nitrate, and 10 mM glutamine (lane 2), 0.5% glycerol (wt/vol), 10 mM nitrate, 10 mM glutamine, and 0.1% (wt/vol) quinate (lane 3), or 0.5% (wt/vol) glycerol, 10 mM nitrate, and 0.1% (wt/vol) quinic acid (lane 4) and incubated for a further 5 h. Transformants QWA2 (lane 5) and QCF2 (lane 6) were grown with ammonium tartrate (5 mM) and glucose (0.4% [wt/vol]), and the harvested mycelium was incubated in 0.5% glycerol (wt/vol), 10 mM ammonium tartrate, 10 mM nitrate, and 0.1% (wt/vol) quinate for a further 5 h. Approximately 1.5 μg of poly(A)$^+$ RNA (2, 12) from each sample was screened in the Northern blot using [α-^{32}P]dCTP-labelled *areA* DNA as a probe and a commercial (GIBCO BRL) RNA ladder to provide molecular size markers (not shown).

TABLE 5. Nitrate reductase levels in control and transformed strains of *A. nidulans* G191[a]

Strain[b]	Induction conditions[c]						Nitrate reductase activity[d]			
							Expt 1		Expt 2	
	Nitrate	Quinate	Glutamine	Proline	Glycerol	Glucose	Specific	Relative	Specific	Relative
2043	+	−	−	−	−	+	98.4	1.00	70.5	1.00
	+	−	+	−	−	+	0.0	0.00	0.0	0.00
	−	−	−	+	−	+	0.0	0.00	0.0	0.00
	+	+	−	−	+	−	71.3	0.72	34.8	0.49
	+	+	+		+	−	0.0	0.00	0.0	0.00
	−	+	−	+	+	−	0.0	0.00	0.0	0.00
CF77/38	+	−	−	−	−	+	88.0	0.89	28.2	0.40
	+	−	+	−	−	+	0.0	0.00	0.0	0.00
	−	−	−	+	−	+	0.0	0.00	0.0	0.00
	+	+	−	−	+	−	35.0	0.36	17.3	0.25
	+	+	+	−	+	−	29.1	0.30	17.6	0.25
	−	+	−	+	+	−	0.00	0.00	0.0	0.00
CF77/42	+	−	−	−	−	+	113.0	1.10	57.1	0.81
	+	−	+	−	−	+	0.0	0.00	0.0	0.00
	−	−	−	+	−	+	0.0	0.00	0.0	0.00
	+	+	−	−	+	−	28.1	0.29	11.1	0.16
	+	+	+	−	+	−	15.0	0.15	6.2	0.09
	−	+	−	+	+	−	0.0	0.00	0.0	0.00

[a] Spores from desired strains were grown for 18 h at 37°C in minimal medium with glucose (0.4% [wt/vol]) and proline (10 mM) as the carbon and nitrogen source, respectively. Proline was used as the nitrogen source, as it is neutral with respect to nitrogen metabolite repression, and preliminary experiments had shown that its use led to the greatest induction of nitrate reductase in wild-type strains. The harvested (by filtration) washed mycelium from each strain was then divided into six equal portions and reincubated for 5 h in minimal medium containing the following supplements: (i) nitrate (10 mM) and glucose (0.4% [wt/vol]); (ii) nitrate (10 mM), glucose (0.4% [wt/vol]), and glutamine (5 mM); (iii) proline (10 mM) and glucose (0.4% [wt/vol]); (iv) nitrate (10 mM), quinate (0.1% [wt/vol]), and glycerol (0.1% [vol/vol]); (v) nitrate (10 mM), glutamine (5 mM), quinate (0.1% [wt/vol]), and glycerol (0.1% [vol/vol]); (vi) proline (10 mM), quinate (0.1% [wt/vol]), and glycerol (0.1% [vol/vol]). Following incubation, the harvested washed mycelium was blotted between paper towels and snap frozen in liquid nitrogen, and 0.5-g portions of frozen mycelium were immediately processed to produce cell extracts for assay of nitrate reductase as described in footnote *a* of Table 4.

[b] Strain 2043 is strain G191 transformed with unmodified plasmid pCAP2 (20).

[c] The symbols + and − denote the presence or absence, respectively, of the particular addition to the medium.

[d] Experiments 1 and 2 are repeat experiments carried out on different days. Specific activity is defined in Table 4, footnote *c*. Relative activity is the specific activity expressed relative to the activity of strain 2043 induced in the absence of glutamine.

gene, and the QCF transformants (15 tested), which contain the truncated *areA* gene. Table 3 summarizes the responses of wild-type and *areA* deletion mutant strains along with four transformants, QWA2, QWA3, QCF1, and QCF2. QWA2 and QCF1 were further assayed for the levels of nitrate reductase (see Table 4 below). The growth responses shown in Table 3 refer to the phenotype observed in the presence of quinate after 24 h of growth at 37°C with glycerol (weakly catabolite repressing) as a carbon source. As can be seen from Table 3, the transformants containing the entire *areA* gene were sensitive to ammonium-mediated nitrogen metabolite repression, whereas the transformants containing the truncated *areA* gene were insensitive. When the tests were repeated in the absence of ammonium, the QWA transformants had a derepressed phenotype, confirming that the repressed phenotype seen in the presence of ammonium was not due to the absence of the AREA protein. The contrasting phenotypes of the QWA and QCF transformants are independently confirmed by the nitrate reductase assays shown in Table 4, and typical growth responses are shown in Fig. 1.

All QCF transformants, which express the truncated *areA* gene, had a quinate-inducible nitrogen metabolite derepressed phenotype that was strongly expressed after 24 h of growth. These transformants were unable to use nitrate as the sole nitrogen source on minimal medium unless quinate was present, showing that quinate induction was necessary to produce sufficient quantities of the truncated AREA protein for growth. Similarly, the QCF transformants were unable to produce halos in the milk test unless quinate was present. In all transformants (QWA or QCF), any derepressed phenotype associated with quinate induction was always less pronounced with glucose (0.4% [wt/vol]) as the carbon source (strongly catabolite repressing the *qutE* promoter) compared with equivalent growth tests with glycerol as the carbon source (weakly catabolite repressing the *qutE* promoter).

The QWA transformants were able to utilize nitrate as a nitrogen source and grow on minimal medium in 24 h, demonstrating, that in the plate tests for derepression, there is sufficient AREA protein for growth. In the bromothymol blue and milk clearing plate tests, the phenotypes of 2 QWA transformants (of a total of 13) changed gradually over a 56-h period from a repressed to a derepressed phenotype in the presence of ammonium; however, the remaining 11 transformants remained sensitive to nitrogen metabolite repression. The atypical temporally sensitive phenotype seen in 2 of the 13 QWA transformants under repressing conditions is consistent with the explanation that the AREA concentration in these strains increases slowly with time. This effect may be a direct consequence of utilizing the *qutE* terminator in place of the native *areA* terminator, as the native mRNA in wild-type strains has a shorter half-life under repressing conditions (28).

(iii) In vitro nitrate reductase assays. In vitro nitrate reductase assays were carried out on two selected transformants as an independent way of assessing their phenotype with regard to nitrogen metabolite repression. The nitrate reductase levels determined in transformants QWA2 and QCF1 (chosen as they show the repressible and derepressed phenotypes, respectively, in the plate tests) are summarized in Table 4. Inspection

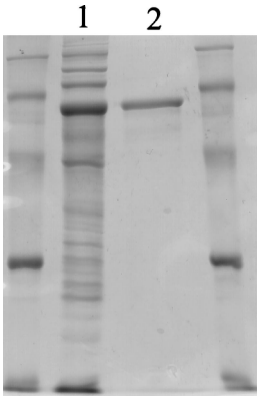

FIG. 3. Overproduction and purification of the truncated AREA protein. The truncated AREA protein was purified from a sonicated extract of *E. coli* BL21(DE3) by IMAC as described in Materials and Methods. The sodium dodecyl sulfate-polyacrylamide gel shown had a 5% acrylamide stacking gel and a 10% acrylamide separating gel. Lane 1 contains approximately 5 μg of protein of the total soluble protein following sonication and clarification by centrifugation. Lane 2 contains approximately 1 μg of the truncated AREA protein following purification by IMAC. The two unlabelled lanes contain the M_r markers phosphorylase (97,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), and β-lactoglobulin (18,000). Proteins were made visible by staining with Coomassie blue.

of Table 4 shows that the production of nitrate reductase in transformant QWA2 is mostly sensitive to repression with the level of repression indistinguishable from that of the wild type (on average 90% repressed). In contrast, the production of nitrate reductase in transformant QCF1 is mostly insensitive to repression (on average 10% repressed). Taken together, the in vitro nitrate reductase assays on the QCF and QWA transformants confirm the conclusion drawn from the growth tests that an AREA protein lacking the 389 N-terminal amino acids is insensitive to nitrogen metabolite repression.

Expression of the truncated form of the *areA* gene in *A. nidulans* G191. The transformants used in the preceding section were isolated on the basis of function using regimens that selected for a particular range of activity. It is possible that, in the case of the QCF transformants, the derepressed phenotype observed was due to plasmid integration events that inactivate hitherto unidentified genes essential for wild-type regulation. If the derepressed phenotype were due solely to the effect of the truncated AREA protein, then this effect should be reproducible in a wild-type strain by overproducing the truncated AREA protein from a plasmid in which there was no selection for the function of the truncated *areA* gene. For this experiment to be valid, any derepressed phenotype must be quinate inducible, and the transformants must have a wild-type phenotype with respect to nitrogen metabolite repression in the absence of quinate. As a consequence, the truncated *areA* gene must be expressed in a strain which is expressing a normally regulated wild-type *areA* gene.

A. nidulans G191 contains the *pyrG* marker, and transformants were isolated by selection for growth in the absence of uracil (hence, there was no selection for function of the truncated *areA* sequence) after exposure of protoplasts to the plasmid pACF1 (which contains the *N. crassa pyr-4* gene, allowing complementation of the *pyrG* mutation, and the truncated *areA* sequence). All transformants tested produced extracellular protease in the presence of glutamine when quinate was supplied in the medium, but not when quinate was omitted (data not shown). These results imply that the truncated AREA protein is responsible for the quinate-inducible, derepressed phenotype and that nitrogen metabolite repression is mediated as in the wild type in the absence of quinate. DNAs from six randomly selected transformants were probed by Southern blot analysis, and transformants with *areA* copy numbers ranging from 1 to 7 were identified: in each case, the transforming plasmid had integrated at a site away from the resident *areA* gene (data not shown). The six transformants were tested for their sensitivity to the presence of chlorate (indicating the presence of nitrate reductase), β-aspartyl hydroxamate (indicating the presence of asparaginase), and nitrite (data not shown). In these tests, the transformants had a quinate-inducible derepressed phenotype, the extent of which was correlated with increased copy number. We note that the sensitivity of the transformants to the presence of nitrite is a phenotype associated with mutations in the *meaB* gene (1, 29).

The levels of nitrate reductase were assayed in cell extracts of the mycelia of two transformants derived from strain G191 (transformant CF/7742, one copy of *areA390-876*; and transformant CF77/38, five copies of *areA390-876*), grown under a variety of conditions. These enzyme assay data are summarized in Table 5, inspection of which leads to the following conclusions. (i) In the absence of quinate, the transformed strains mediated nitrogen metabolite repression in a manner indistinguishable from that of the wild type. This observation rules out the possibility that the derepressed phenotype associated with transformants transcribing the truncated *areA* gene can be caused only by plasmid integration into hitherto undetected genes essential for the mediation of wild-type nitrogen metabolite repression. (ii) In the presence of quinate, both transformants had a nitrogen metabolite derepressed phenotype that was, at least in part, insensitive to the presence of glutamine. The high-copy-number transformant CF77/38 retained 83 to 100% of the quinate-inducible nitrate reductase activity in the presence of glutamine; the low-copy-number transformant CF77/42 retained approximately 50% activity. We attribute the repressibility of a portion of the nitrate reductase activity to the sensitivity of the endogenous wild-type AREA protein to nitrogen metabolite repression. The combined data from the growth tests and the in vitro enzyme assays in the G191 transformants are consistent with a simple mass action effect in which increasing concentrations of the truncated AREA protein are competing with the wild-type AREA protein for common binding sites in promoters sensitive to nitrogen metabolite

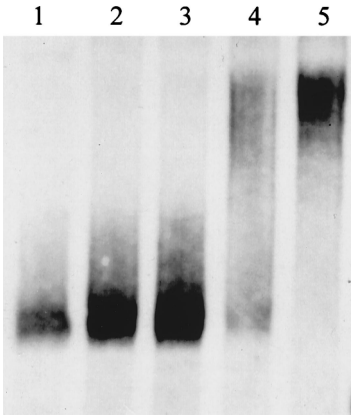

FIG. 4. In vitro binding of the truncated AREA protein to the *niaD* promoter. EMSA, utilizing the *niaD* promoter, was carried out according to the protocol in Materials and Methods and used to probe the specificity of binding of the truncated AREA protein. Lane 1, no protein (control); lanes 2 to 6, approximately 30, 60, 130, and 250 ng of truncated AREA protein, respectively.

repression. We note that the derepressed *areA* allele *xprD1* has a semidominant phenotype (6).

Northern blot analysis. An analysis of mRNA production in transformants CF77/38, QCF2, and QWA2 is shown in Fig. 2. Inspection of Fig. 2 shows that under repressing conditions, (i) transformant CF77/38 produced a quinate-inducible, *areA*-specific mRNA of approximately 1.6 kb in addition to the wild-type mRNA of approximately 2.9 kb; (ii) transformants QCF2 produced an *areA*-specific mRNA of approximately 1.6 kb; and (iii) transformant QWA2 produced an *areA*-specific mRNA of approximately 2.8 kb. The sizes of the *areA*-specific mRNAs detected are those predicted from a knowledge of the sizes of the *areA* sequences expressed from the *qutE* promoter in plasmids pNUFC266 and pNUFC205.

Dot blot analysis of mRNA levels showed that approximately 2.5-fold-more *areA* mRNA was produced in transformant QWA2 than in QCF2 (data not shown), implying corresponding levels of AREA protein production. Therefore, the derepressed phenotype seen in the QCF2 transformant cannot be explained by proposing that this is due to elevated levels of the truncated AREA protein being produced relative to the levels in the transformant QWA2.

The truncated AREA protein is stable in vitro and binds to the *niaD* promoter in EMSA. If the nitrogen metabolite derepression observed in the transformed strains is mediated by the truncated AREA, then this protein must be both stable in vivo and able to specifically recognize the required promoters. To confirm that the truncated AREA protein could bind specifically to its cognate promoters in vitro, it was overproduced in *E. coli*, purified, and used in EMSA with the nitrogen-metabolite sensitive *niaD* promoter (which drives transcription of the nitrate reductase-encoding *niaD* gene [30]). The purified, truncated AREA protein (Fig. 3) was shown to bind specifically to the *niaD* promoter in the presence of 1.0 mg of poly(dI/dC) ml^{-1} (Fig. 4). This specific binding was insensitive to the presence of 10 mM ammonium or glutamine (data not shown). The truncated AREA protein used in these EMSAs has a sequence of 41 heterologous amino acids attached to its N terminus which are encoded by the plasmid pRSETB. Contained within this sequence are 6 His residues which can coordinate with Zn^{2+}, allowing purification by immobilized metal affinity chromatography (IMAC). Control experiments using the *A. nidulans* quinate dehydrogenase enzyme fused to the same 41 heterologous amino acids (18) failed to show a shift of the *niaD* promoter (data not shown), confirming that the specific shifting was due to the *areA*-encoded sequence.

In conclusion, we have experimentally tested predictions that could have refuted the three-domain model for the structure of the AREA protein (17). This model predicts that the N-terminal domain (amino acid coordinates 1 to 389) acts, at least in part, as a glutamine sensor and that expression of a truncated *areA* gene lacking the sequence encoding this domain leads to a derepressed phenotype. The data presented here verify experimentally the latter prediction, implying that the N-terminal region of AREA is involved in mediating nitrogen metabolite repression. Previously, other data have implicated the C-terminal region of the AREA protein and the 3′ untranslated region of the *areA* mRNA in mediating the wild-type strain's response to nitrogen metabolite repression (4, 19, 28).

ACKNOWLEDGMENTS

This research was supported by UK Research Council funding.

We are indebted to Meryl Davis for generously providing us with *A. nidulans* NRKB3-2. We thank Ian Fillingham for constructing plasmid pNUFC205 as part of his final-year undergraduate project and Giles Newton for critically reading the manuscript.

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