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Deletion of the 389 N-Terminal Residues of the Transcriptional Activator AREA Does Not Result in Nitrogen Metabolite Derepression in *Aspergillus nidulans*

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Utilizing a homologous gene replacement in order to retain the native promoter and 5′ and 3′ untranslated messenger regions (and thereby ensure physiological validity), we have shown that deletion of the N-terminal 389 amino acids of the transcriptional activator AREA does not result in nitrogen metabolite derepression in *Aspergillus nidulans*. Our results provide no evidence for a modulating interaction involving the N terminus of AREA and contrast with those of H. K. Lamb, A. L. Dodds, D. R. Swatman, E. Cairns, and A. R. Hawkins (J. Bacteriol. 179:6649–6656, 1997), who used nontargeted ectopic copies of a construct containing a heterologous promoter and untranslated regions. Results obtained with this deletion mutant, nevertheless, provide further evidence for the dispensability of large portions of AREA.

The *areA* gene of the ascomycete *Aspergillus nidulans* encodes a GATA family transcriptional activator (AREA) mediating nitrogen metabolite repression, the regulatory mechanism whereby the preferred nitrogen sources ammonium and L-glutamine prevent expression of genes required for utilization of less favorable nitrogen sources (2, 7, 9, 15, 17). The precise mechanism by which ammonium (probably via its conversion to glutamine [see reference 19]) and glutamine exert nitrogen metabolite repression is not known. However, two important and additive components of this mechanism have been identified (1, 13). One is a sequence in the 3′ untranslated region of the *areA* mRNA which confers instability under repressing conditions (13). The other is a protein-protein interaction of AREA with the negatively acting NMRA protein. The regions of AREA which apparently interact with NMRA are part of the DNA binding domain and the 12 C-terminal residues (13). Evidence for corresponding modulation by a negatively acting protein was first reported for the isofunctional AREA homolog NIT2 of *Neurospora crassa* (12, 20).

Recently Lamb et al. (8) reported that deletion of the 389 N-terminal residues of AREA results in nitrogen metabolite derepression and proposed that this supports their hypothesis for a modulating protein-protein interaction involving this region. We (3) and others (10, 11) have previously shown that their hypothesis lacks a sound scientific basis. Here we show, using directed gene replacement, that deletion of the N-terminal 389 residues of AREA does not lead to nitrogen metabolite derepression. This suggests that the findings of Lamb et al. (8) are artifactual, probably resulting from the use of nontargeted ectopic transformants.

We were initially doubtful about whether deletion of the 389 N-terminal residues of AREA would result in nitrogen metabolite derepression, because we had never observed derepression in mutants having smaller deletions or compensating frameshifts (in which a portion of the normal protein sequence is replaced by a peptide translated in another reading frame) affecting this region. For example, neither *areAΔE1* strains, which lack the N-terminal 157 residues (9), nor *areA396* strains, which lack the N-terminal 289 residues (4), are derepressed. Furthermore, in *N. crassa*, deletion of NIT2 residues 199 to 334 (corresponding in alignment [9] to AREA residues 135 to 265) or 352 to 458 (corresponding in alignment [9] to AREA residues 286 to 400) does not lead to nitrogen metabolite derepression (12). In contrast, deletion of the sequence conferring instability in the 3′ untranslated region of the mRNA, mutations affecting the 12 C-terminal residues, and certain missense and deletion mutations within the region (codons 671 to 722) encoding the DNA binding domain clearly result in derepression (7, 13, 14, 16).

We therefore constructed by homologous gene replacement the same deletion mutation that Lamb et al. (8) had investigated by using nontargeted ectopic transgenes. This allele encodes an AREA protein in which a single alanine residue substitutes for residues 2 to 389, giving a protein composition of MA + 390 → 876 (designated *areAΔ2-389*). Briefly, the deletion was constructed by recombinant PCR as described previously (9, 13, 14), except for use of the Expand High Fidelity PCR system (Boehringer Mannheim), employing oligonucleotide combinations 5′-GTTGTTATCCGGCCGTAT plus 5′-G GTTGCCATGACCGTGGGCT (EMBL accession no. X52491, coordinates 1083 to 1100 and 2727-2703C1479-1466, respectively) and 5′-CACGGTCATGGCAACCGACTTCTTCTCT plus 5′-ATGTCTCCCACGCCATGTTG (coordinates 1470-1479G2703-2719 and 3185 to 3166, respectively) and subsequently amplifying the combined products with the first and last of the above-mentioned oligonucleotides. The recombinant PCR fragment was then transformed into a strain with a *pabaA1 yA2 areA49* genotype (*p*-aminobenzoate requiring, having yellow conidial color, lacking *areA* function), and transformants able to utilize 10 mM nitrate as a nitrogen source were selected as described previously (9). *areA49* is an 8-bp deletion beginning in codon 75 (9) which would therefore be replaced by homologous integration of the transforming PCR fragment. Numerous transformants were obtained. A total of 24 transformants subjected to preliminary growth tests were

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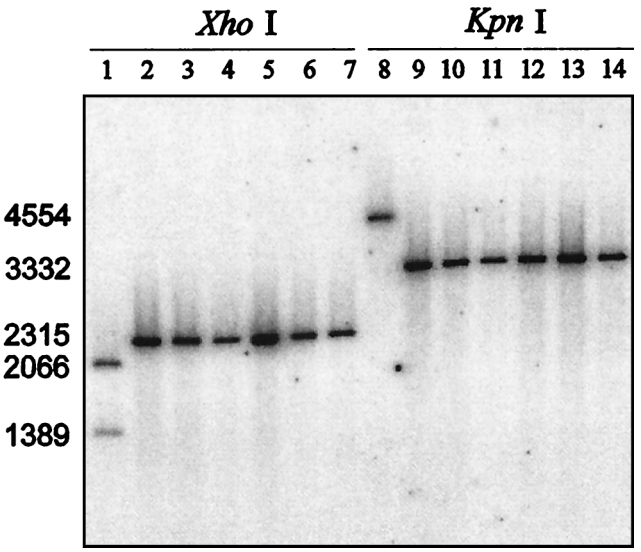

FIG. 1. Southern hybridization analysis of *areAΔ2-389* transformants. Genomic DNA from the wild type (lanes 1 and 8) and six transformants (lanes 2 to 7 and 9 to 14) was digested with either *Xho*I (lanes 1 to 7) or *Kpn*I (lanes 8 to 14), electrophoresed on a 0.8% (wt/vol) agarose gel, blotted, and probed with the transforming DNA. The expected sizes of the restriction fragments are given (in base pairs) to the left of the gel. An 82-bp *Xho*I fragment, present in the wild type but deleted from the transformants, has presumably migrated off the gel and is not visible. The banding pattern is fully consistent with all six transformants having a single copy of the transforming DNA integrated homologously at *areA*.

phenotypically indistinguishable. Of these, 6 were analyzed by Southern blotting and found to be single-copy gene replacement integrants, consistent with the presence of the 1,222-bp deletion (Fig. 1). Of these 6, 1 (analyzed in lanes 3 and 10 of Fig. 1) was shown by sequencing (using primers outside the region covered by the transforming fragment) to contain a faithful copy of the transforming sequence with no extraneous mutations. Compared to the wild type, the deletion mutation reduces growth to various degrees on all nitrogen sources tested, including adenine, L-alanine, L-arginine, L-aspartate, δ-amino-*n*-valerate, nitrate, L-ornithine, and 2-pyrrolidone, facilitating genetic analysis. Utilizing the sequenced transformant and recognizing the *areAΔ2-389* deletion mutation by its partial impairment of nitrogen source utilization, *areAΔ2-389* was shown to segregate as a single mutation in a cross with a wild-type (*areA*$^+$) strain and to fail to give (fully functional) *areA*$^+$ recombinants in a cross with an *areA49* strain.

Using plate tests (reference 7 and references therein) with sensitivities equal to or greater than those performed by Lamb et al. (8), four gene replacement (as verified by Southern analysis) transformants, including that sequenced and analyzed genetically along with 50 outcrossed *areAΔ2-389* strains, were tested for derepression. None were derepressed for synthesis of nitrate reductase (i.e., they did not result in hypersensitivity to 500 mM chlorate in the presence of 3.3 mM ammonium), nitrite reductase (i.e., they did not result in a green or blue color [alkalinization] in the colony's vicinity in the presence of 10 mM nitrite, 10 mM ammonium chloride, and 0.025% [wt/vol] bromothymol blue), asparaginase (i.e., they did not result in hypersensitivity to 5 mM DL-β-aspartylhydroxamate in the presence of 3.3 mM ammonium), or extracellular protease (i.e., they did not result in a halo of clearing of the turbidity of 0.6% [wt/vol] powdered skim milk in the presence of 10 mM ammonium). In fact, *areAΔ2-389* strains were actually more resistant to chlorate and β-aspartylhydroxamate in these tests than were wild-type control strains, consistent with a diminution of *areA* function. Quantitative data in Table 1 confirm that introduction of the *areAΔ2-389* mutation does not result in derepression. By way of comparison, an *areA* mutant with a deletion of the nine C-terminal amino acids (*areAΔ868-876*), used as a control, was significantly derepressed.

This raises the question of why the transformants studied by Lamb et al. (8) behaved differently. At the outset it is important that their transforming constructs were integrated ectopically and without targeting. Levels of functional protein encoded by nontargeted ectopic gene copies can vary considerably (5, 6, 18), presumably due to genome position and the site(s) of crossover(s). Moreover, they did not measure AREA protein levels. Even in strains in which the mRNA levels are similar there is no guarantee that changes in mRNA sequence will not affect the translatability of the mRNA. As AREA interacts with the negatively acting NMRA protein through its DNA binding domain and C terminus (1, 12, 13, 20), overproduction of any AREA protein containing the DNA binding domain and C terminus could, in principle, titrate the NMRA protein, whose depletion would result in derepression. When Lamb et al. (8) selected transformants in an *areA* null recipient on nitrate (plus quinate) medium, they were imposing a selective pressure for overproduction of the AREAΔ2-389 protein stronger than that for the wild-type AREA protein, because *areAΔ2-389* strains, which lack >44% of the coding region, utilize nitrate more poorly than *areA*$^+$ strains. It is thus likely that transformants receiving the *areAΔ2-389* construct contain higher levels of the AREA DNA binding domain and C terminus than transformants receiving the *areA*$^+$ construct. Such AREA overproduction could also explain the behavior of their transformants selected for pyrimidine prototrophy in an *areA*$^+$ recipient.

Irrespective of the basis for the transformant phenotypes reported by Lamb et al. (8), the fact that the *areAΔ2-389* allele of the endogenous resident *areA* gene does not lead to derepression (whereas mutations affecting a portion of the DNA binding domain or C terminus do [see reference 13; Table 1]) shows that there is no evidence for a physiologically relevant modulating interaction involving the N terminus of AREA.

Although there is no evidence for a modulating interaction involving the 389 N-terminal amino acids of AREA, our present study does provide further evidence for the dispensability of large portions of AREA, augmenting the data presented in references 4, 7, 9, and 14.

TABLE 1. Repression of nitrate reductase in an *areAΔ2-389* strain

Relevant genotype[a]	Nitrate reductase sp act for strain grown in presence of[b]:		Derepression (%)[c]
	20 mM NO_3^-	20 mM NO_3^- + 40 mM NH_4^+	
Wild type (*areA*$^+$)	144 ± 9.0	9.7 ± 2.6	6.7
areAΔ2-389	122 ± 6.6	3.9 ± 1.1	3.2
areAΔ868-876	101 ± 9.0	56.2 ± 1.2	55.6

[a] The full genotypes of the *areA*$^+$, *areAΔ2-389*, and *areAΔ868-876* strains are *pabaA1* (*p*-aminobenzoate requiring), *yA2* (yellow conidial color) *pabaA1 areAΔ2-389*, and *areAΔ868-876 pantoB100* (D-pantothenate requiring) *fwA1* (fawn conidial color), respectively. *areAΔ868-876* has been described previously (13).

[b] Growth and assay conditions were as described in reference 13. Specific activities are presented as nanomoles of NO_2^- produced per minute per milligram of protein (means ± standard deviations).

[c] Calculated by dividing the specific activity on NO_3^- + NH_4^+ by that on NO_3^- and expressing it as a percentage.

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ADDENDUM IN PROOF

Additional evidence against a modulating interaction involving the N terminus of AREA comes from a very recent paper by Christensen et al. (T. Christensen, M. J. Hynes, and M. A. Davis, Appl. Environ. Microbiol. **64:**3232–3237, 1998), who complemented an *A. nidulans areA* null mutation with full-length and deletion derivatives of its *Aspergillus oryzae* isofunctional homologue. The full-length *A. oryzae* gene complements fully and results in normal nitrogen metabolite-repressible regulation. Deletion of *A. oryzae* AREA residues 1 to 270, 44 to 218, 219 to 325, or 326 to 648 (corresponding in alignment to *A. nidulans* AREA residues 1 to 276, 53 to 223, 225 to 332, or 333 to 659, respectively) does not lead to nitrogen metabolite derepression, whereas deletions removing the C terminus do.

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