The TamA Protein Fused to a DNA-Binding Domain Can Recruit AreA, the Major Nitrogen Regulatory Protein, to Activate Gene Expression in *Aspergillus nidulans*

Anna J. Small, Michael J. Hynes and Meryl A. Davis

Department of Genetics, University of Melbourne, Parkville, Victoria 3052, Australia

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ABSTRACT

The *areA* gene of *Aspergillus nidulans* encodes a GATA zinc finger transcription factor that activates the expression of a large number of genes subject to nitrogen metabolite repression. The amount and activity of the AreA protein under different nitrogen conditions is modulated by transcriptional, post-transcriptional, and post-translational controls. One of these controls of AreA activity has been proposed to involve the NmrA protein interacting with the DNA-binding domain and the extreme C terminus of AreA to inhibit DNA binding under nitrogen sufficient conditions. In contrast, mutational evidence suggests that the *tamA* gene has a positive role together with *areA* in regulating the expression of genes subject to nitrogen metabolite repression. This gene was identified by the selection of mutants resistant to toxic nitrogen source analogues, and a number of nitrogen metabolic activities have been shown to be reduced in these mutants. To investigate the role of this gene we have used constructs encoding the TamA protein fused to the DNA-binding domain of either the FacB or the AmdR regulatory proteins. These hybrid proteins have been shown to activate expression of the genes of acetate or GABA utilization, respectively, as well as the *amdS* gene. Strong activation was shown to require the AreA protein but was not dependent on AreA binding to DNA. The homologous *areA* gene of *A. oryzae* and *nit-2* gene of *Neurospora crassa* can substitute for *A. nidulans areA* in this interaction. We have shown that the same C-terminal region of AreA and NIT-2 that is involved in the interaction with NmrA is required for the TamA-AreA interaction. However, it is unlikely that TamA requires the same residues as NmrA within the GATA DNA-binding domain of AreA.

NITROGEN metabolite repression (NMR) is a global regulatory system that activates the expression of a large number of nitrogen catabolic genes when cells are nitrogen limited. A combination of classical and molecular genetic analyses have defined *areA* as the major nitrogen regulatory gene in *Aspergillus nidulans* (Arst and Cove 1973; Hynes 1975; see Marzluf 1997, for review). The *areA* gene encodes a positively acting DNA-binding protein that contains a single GATA zinc finger (Kudla *et al.* 1990). Similar positively acting nitrogen regulatory genes have been identified in a number of fungi including *Neurospora crassa* (Fu and Marzluf 1990), *Penicillium chrysogenum* (Haas *et al.* 1995), *A. oryzae* (Christensen *et al.* 1998), *Magnaporthe grisea* (Froelinger and Carpenter 1996), and *Saccharomyces cerevisiae* (Minehart and Magasanik 1991; Stanbrough *et al.* 1995; Coffman *et al.* 1997).

Detailed two-hybrid analyses and protein studies with NIT-2, the *N. crassa* homologue of AreA, suggest that the activator function of these proteins can be modified in response to changes in the nitrogen status of the cell. The product of the *nmr-1* gene has been shown to interact with the GATA finger and the extreme C terminus of NIT-2 to inhibit its DNA binding (Xiao *et al.* 1995). These *in vitro* results are supported by *in vivo* studies using *nit-2* mutations that result in specific alterations in these two regions of the protein (Pan *et al.* 1997). The GATA finger and C-terminal regions of NIT-2 are highly conserved with the AreA protein and mutational analysis indicates that both regions are also involved in the regulatory response in *A. nidulans* (Platt *et al.* 1996). The *A. nidulans* homologue of *nmr-1*, the *nmrA* gene, has recently been cloned and a gene inactivation strain shows partial derepression consistent with the phenotype of *nmr-1* mutants in *N. crassa* (Andrianopoulos *et al.* 1998). By analogy with the detailed studies in *N. crassa*, NmrA is proposed to act as a negative regulator of AreA function under nitrogen-sufficient conditions.

The *tamA* gene of *A. nidulans* encodes a protein required for full expression of genes under *areA* control (Kinghorn and Pateman 1975; Davis *et al.* 1996). *tamA* mutants were initially isolated by selection for simultaneous resistance to several toxic nitrogen source analogues resulting from reduced levels of nitrogen catabolic activ-

Corresponding author: Meryl A. Davis, Department of Genetics, University of Melbourne, Parkville, Victoria 3052, Australia.
E-mail: m.davis@genetics.unimelb.edu.au

ities (Kinghorn and Pateman 1975). These phenotypes are recessive to wild type, suggesting a positive role for *tamA*. The *tamA* gene encodes a predicted protein with homology to the UGA35/DAL81/DURL protein of *S. cerevisiae* (Davis *et al.* 1996). A possible Zn(II)2-Cys6 DNA-binding motif was identified in both the TamA and UGA35 predicted protein sequences; however, in both cases this putative DNA-binding motif was found to be dispensable for all functions tested (Bricmont *et al.* 1991; Davis *et al.* 1996).

To further investigate the role of TamA, we have tested its ability to function as an activator of gene expression when fused to known DNA-binding domains in *A. nidulans*. We have found that TamA is able to elevate expression of specific genes when targeted to the promoter by the DNA-binding domain. In addition, the inherent activity of these hybrid proteins is enhanced by AreA. This AreA-dependent activation requires two distinct regions of AreA and the most significant of these overlaps the C-terminal AreA residues required for interaction with NmrA.

MATERIALS AND METHODS

Strains, media, and growth conditions: Aspergillus media and growth conditions were as described by Cove (1966). Nitrogen sources were added at a final concentration of 10 mm and carbon sources were added at 1% (w/v) except for acetate medium, which contained 50 mm sodium acetate as the sole carbon source. Genetic manipulations were carried out using techniques described by Clutterbuck (1974). The Aspergillus strains used in this study are shown in Table 1. The *areA*Δ (*areA::riboB*) and *nmrA*Δ (*nmrA::Ble*^{R}) mutations have been described previously (Andrianopoulos *et al.* 1998; Christensen *et al.* 1998). The *tamA*Δ mutation was identified as a spontaneous mutant resistant to 100 mm methylammonium on medium containing 10 mm alanine as the sole nitrogen source (M. A. Davis, unpublished results). Gene symbols have been described previously (Clutterbuck 1974).

Aspergillus transformation and assays: *A. nidulans* strains were transformed according to the method of Andrianopoulos and Hynes (1988). Cotransformants were selected using the selectable marker plasmids pPL3 (RiboB$^+$; Oakley *et al.* 1987) on media lacking riboflavin or pI4 (PyroA$^+$; G. May, Baylor College of Medicine, Dallas, Texas) on media lacking pyridoxine. Cotransformants were identified by growth tests where appropriate and the presence of intact copies of the plasmid of interest was confirmed by Southern blot analysis. Genomic DNA was hybridized to a *Cla*I-*Xho*I fragment (−105 to +2041) of *tamA* and the number of intact copies was determined relative to the genomic band of *tamA*. Four independent transformants were characterized for each construct introduced and no copy number effects were detected for any of the constructs used. β-Galactosidase assays and soluble protein determinations of cell extracts from *A. nidulans* were performed as described in Davis *et al.* (1988).

***S. cerevisiae* strain, transformation, and assays:** The yeast strain YGH1 (**a** *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3* Can^r *gal4-542 gal80-538 LYS2::GAL1*$_{uas}$*-GAL1*$_{tata}$*-HIS3 URA3::GAL1*$_{uas}$*-GAL1*$_{tata}$*-lacZ*; Hannon *et al.* 1993) was grown in liquid and solid medium as described in Ausubel *et al.* (1987), with 2% sucrose used as a carbon source when testing for reporter gene activity. Transformation was essentially as described previously (Ito *et al.* 1983; Geitz *et al.* 1992). Transforming plasmids contained the *TRP1* (pGBT9) and *LEU2* (pGAD-GH) markers and transformants were selected on media lacking tryptophan and/or leucine as appropriate. β-Galactosidase activity was initially detected using the X-gal filter method of Hannon *et al.* (1993), and quantitative β-galactosidase assays were conducted as in Bonnefoy *et al.* (1995).

Construction of TamA and AreA fusion plasmids: pAS4148 (encoding AmdR-TamA) was constructed by subcloning a *Eco*RV-*Xba*I fragment from the *tamA* coding region (amino acids 153–658) into pALX129, which contains an *amdR Cla*I-*Hin*dIII fragment containing the promoter and sequences encoding amino acids 1–186 of AmdR. pAS4120 (encoding FacB-TamA) was constructed by cloning the *Eco*RV-*Xho*I fragment of the *tamA* coding region (amino acids 152–651) into pMH1055, which contains a *facB Sac*I-*Bam*HI fragment containing the promoter and sequences encoding amino acids 1–142 of FacB. The *Bam*HI site in this construct was then cut, endfilled, and religated to put *facB* and *tamA* in the same reading frame. GAL4DBD-TamA fusions were constructed in the vector pGBT9 (Bartel and Zhu 1993), which had been cut with *Eco*RI, endfilled, and religated to give the correct reading frame. Fragments encoding various regions of the TamA protein were cloned into *Eco*RI-endfilled pGBT9 cut with *Sma*I-*Sal*I, *Sal*I, or *Sma*I-*Sal*I to yield pAS4010 (*Eco*RV-*Sal*I, encoding amino acids 153–360), pAS3799 (*Sal*I-*Xho*I, encoding amino acids 359–650), and pAS4013 (*Eco*RV-*Xho*I, encoding amino acids 471–650). Fragments containing the *Pst*I-*Sal*I region of *tamA* encoding amino acids 29–360 and *Pst*I-*Xho*I (amino acids

TABLE 1

List of *A. nidulans* strains

Strain	Relevant genotype
MH1	*biA1*
MH6209	*biA1 amdR44 amdS::lacZ prn*Δ*309 riboB2 facB::Ble*^{R}
MH8767	*yA1 pabA1 amdR44 amdS::lacZ areA217 pyroA4 niiA4 riboB2*
MH8827	*yA1 amdR44 amdS::lacZ areA217 prn*Δ*309 pyroA4 riboB2 facB::Ble*^{R}
MH8845	*biA1 amdR44 amdS::lacZ areA*Δ*::riboB prn*Δ*309 pyroA4 facB::Ble*^{R}
MH9024	*biA1 amdR44 amdS::lacZ areA*Δ*::riboB pyroA4 facB::Ble*^{R} *[facB-tamA]*
MH9079	*biA1 amdR44 amdS::lacZ areA*Δ*844-876 pyroA4 facB::Ble*^{R} *[facB-tamA]*
MH9084	*biA1 amdR44 amdS::lacZ nmr::Ble*^{R} *pyroA4 facB::Ble*^{R} *[facB-tamA]*
MH9087	*biA1 amdR44 amdS::lacZ facB::Ble*^{R} *[facB-tamA]*
MH9147	*biA1 pabA1 amdR44 amdS::lacZ areA*Δ*743-864 facB::Ble*^{R} *[facB-tamA]*
MH9192	*biA1 amdR44 amdS::lacZ nmrA::Ble*^{R} *facB::Ble*^{R}
MH9211	*biA1 amdR44 amdS::lacZ areA*Δ*844-876 riboB2 facB::Ble*^{R} *[amdR-tamA]*
MH9212	*biA1 amdR44 amdS::lacZ areA*Δ*743-864 riboB2 facB::Ble*^{R} *[amdR-tamA]*
MH9219	*amdR44 amdS::lacZ pyroA4 tamA*Δ *riboB2 facB::Ble*^{R}
MH9220	*biA1 pabA1 amdR44 amdS::lacZ areA*Δ*743-864 pyroA4 facB::Ble*^{R}
MH9260	*biA1 amdR44 amdS::lacZ areA*Δ*844-876 facB::Ble*^{R}
MH9350	*biA1 amdR44 amdS::lacZ tamA*Δ *riboB2 facB::Ble*^{R} *[facB-tamA]*

29–650) were cloned into *Bam*HI-*Sal*I-cut pGBT9 with the *Eco*RI site endfilled, using a *Bam*HI plasmid polylinker site at the 5′ end of the *tamA* clone to yield pAS4009 and pAS4033, respectively. GAL4AD-AreA fusions were made using the vector pGAD-GH (Hannon *et al.* 1993). Fragments containing the *Pst*I-*Sal*I encoding amino acids 178–402, *Pst*I-*Xho*I encoding amino acids 178–755, and *Pst*I-*Eco*RI encoding amino acids 178–876 of *areA* were cloned, using a *Spe*I polylinker site at the 5′ end of the *areA* fragment to facilitate cloning into pGAD-GH cut with *Spe*I-*Sal*I or *Spe*I-*Eco*RI to yield pAS4004, pAS4003, and pAS4128, respectively. The integrity of the plasmid constructs was confirmed by sequencing.

RESULTS

TamA as an activator of gene expression: To determine whether TamA had activator function in *A. nidulans*, the TamA protein (residues 153–651 or 153–658) was fused to the Zn(II)2Cys6 DNA-binding domain of FacB (amino acids 1–142) or of AmdR (amino acids 1–186) to target TamA to specific promoters. The FacB protein is a transcriptional activator required for acetate induction of the *amdS* gene and the genes of acetate utilization in *A. nidulans* (Katz and Hynes 1989; Todd *et al.* 1997a). Loss-of-function *facB* mutants are unable to use acetate and compounds metabolized via acetate as sole carbon sources (Apirion 1965; Armitt *et al.* 1976; Hynes 1977). The AmdR protein mediates ω-amino acid induction of *amdS* and the genes of γ-amino butyric acid (GABA) and 2-pyrollidinone breakdown (Andrianopoulos and Hynes 1988, 1990). Loss-of-function *amdR* mutants are unable to use these compounds as sole nitrogen sources (Arst 1976; Arst *et al.* 1978). Domain-switch experiments have revealed that DNA-binding specificity resides in the respective N-terminal regions of FacB and AmdR and that these domains could function independently of the remainder of the protein (Parsons *et al.* 1992; Todd *et al.* 1997a). Neither the FacB nor the AmdR DNA-binding domain alone was able to restore growth on acetate or GABA, respectively (Parsons *et al.* 1992). The *facB-tamA* and *amdR-tamA* constructs were cotransformed into MH8694 (*tamA*Δ) and shown to complement the *tamA* mutation; indicating that residues 153–651 were sufficient for *tamA* function (data not shown).

To determine whether FacB-TamA and AmdR-TamA had activator function, these constructs were cotransformed into MH6209, which carries the *amdR44* and *facB::Ble*^{R} loss-of-function mutations and an *amdS::lacZ* reporter at the *amdS* locus. While the recipient strain was unable to use either acetate or GABA, cotransformants carrying *facB-tamA* constructs partially regained the ability to use acetate as a sole carbon source (Figure 1A). The phenotype of the cotransformants was not copy number dependent. Thus, with the DNA-binding domain of FacB tethering the TamA protein to the promoters of the acetate utilization genes, the FacB-TamA hybrid protein could substitute for the normal FacB function of activating gene expression. When TamA was fused to the AmdR DNA-binding domain, the fusion protein restored AmdR-dependent expression of the genes of GABA utilization while having no effect on acetate utilization (Figure 1B). Therefore, the activation of particular genes was determined by the DNA-binding domain used to target the TamA fusion protein to specific promoters (see Figure 1C).

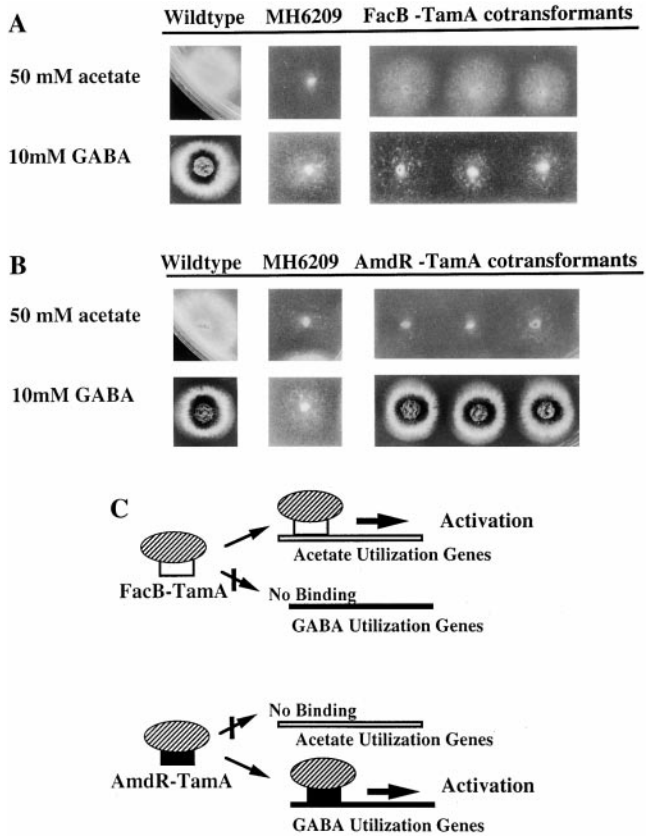

Figure 1.—Growth properties of transformants carrying FacB-TamA and AmdR-TamA. (A and B) The constructs pAS-4120 (encoding FacB-TamA) or pAS4148 (encoding AmdR-TamA) were cotransformed into MH6209 with pPL3 carrying the *riboB*$^+$ selectable marker. The growth of the wild-type (WT) strain MH1, the recipient strain MH6209, and cotransformants of MH6209 were tested for growth on 50 mm acetate as a sole carbon source and on 10 mm GABA as the sole nitrogen source. Growth was assessed after 2 days of incubation at 37°. (C) Schematic representation of the expression of the genes of acetate or GABA metabolism in cotransformants carrying either FacB-TamA or AmdR-TamA expressing constructs.

Both AmdR and FacB regulate *amdS* expression and β-galactosidase assays indicated that both AmdR-TamA and FacB-TamA were able to strongly activate expression of an *amdS::lacZ* reporter gene (Table 2). Expression of *amdS::lacZ* is regulated by nitrogen metabolite repression and relief of repression under nitrogen-free conditions results in a 10-fold increase in expression in an *areA*$^+$ background (Davis *et al.* 1988). Introduction of either *amdR-tamA* or *facB-tamA* led to a substantial increase in β-galactosidase levels under both repressed and derepressed conditions. Enzyme levels in the trans-

TABLE 2

β-Galactosidase levels of cotransformants expressing AmdR-TamA or FacB-TamA in different *areA* genetic backgrounds

	amdS::lacZ expression levels[a]					
	facB$^-$ *amdR*$^-$		+AmdR-TamA		+FacB-TamA	
areA background[b]	NH_4	N free	NH_4	N free	NH_4	N free
areA$^+$	0.7	11.3	12.1	114.2	33.7	209.1
	(0.1)	(1.2)	(2.2)	(21.5)	(9.9)	(37.3)
*areA*Δ	0.5	1.0	6.0	8.8	10.8	16.6
	(0.1)	(0.2)	(0.8)	(0.3)	(3.2)	(4.8)
areA217	0.6	1.1	62.1	114.8	67.0	139.4
	(0.1)	(0.1)	(5.7)	(13.8)	(6.2)	(17.0)

[a] Mycelia were grown overnight on 1% glucose medium containing 10 mm ammonium tartrate and transferred to 1% glucose medium containing either 10 mm ammonium (NH_4) or no added nitrogen source (N free) for 4 hr. Each of the relevant *areA* recipient strains and a representative cotransformant expressing either AmdR-TamA or FacB-TamA were assayed in triplicate. The mean β-galactosidase activities and standard errors (in parentheses) are shown. β-Galactosidase levels are expressed as units per minute per milligram soluble protein.

[b] Cotransformants of MH6209 (*areA*$^+$) were generated using pPL3 and pAS4148 (encoding AmdR-TamA) or pAS4120 (encoding FacB-TamA) and selection for riboflavin prototrophy. Cotransformants of MH8845 (*areA*Δ) were generated using pPI4 and pAS4148 (encoding AmdR-TamA) or pAS4120 (encoding FacB-TamA) and selection for pyridoxine prototrophy. Cotransformants of MH8767 (*areA217*) were generated using pPL3 and pAS4148 (encoding AmdR-TamA) or MH8827 using pPL3 and pAS4120 (encoding FacB-TamA) and selection for riboflavin prototrophy.

formants expressing FacB-TamA were higher than enzyme levels in those expressing AmdR-TamA. As both classes of transformants contained a similar range of copy numbers and there was no evidence of copy number dependency, this may reflect a greater affinity of the FacB DNA-binding domain for the *amdS* promoter. The partial restoration of growth on acetate by FacB-TamA is consistent with the suggestion that FacB has a structural role in acetate metabolism in addition to its regulatory function (Todd *et al.* 1997b).

TamA activation is AreA dependent: To investigate the contribution of AreA to the activation by FacB-TamA or AmdR-TamA, these constructs were cotransformed into *areA*Δ (MH8845) and *areA217* (MH8767 or MH8827) strains. Both *areA*Δ and *areA217* mutations result in an extreme loss-of-function phenotype. The *areA*Δ mutation deletes the entire *areA* gene and *areA217* mutation is a loss-of-function allele due to a missense mutation that alters glycine 698, adjacent to the last cysteine of the GATA finger, to an aspartate residue (Hynes 1975; Kudla *et al.* 1990). AmdR-TamA and FacB-TamA activation was strikingly different in these two mutant *areA* backgrounds.

The expression of AmdR-TamA restored growth on GABA in an *areA217* background, but not in an *areA*Δ background (Figure 2A). Similarly, AmdR-TamA and FacB-TamA strongly activated *amdS::lacZ* expression in *areA217* strains but not in an *areA*Δ background (Table 2). In both the *areA*Δ and *areA217* strains, *amdS::lacZ* expression did not respond to the relief of nitrogen metabolite repression due to loss of AreA function. In

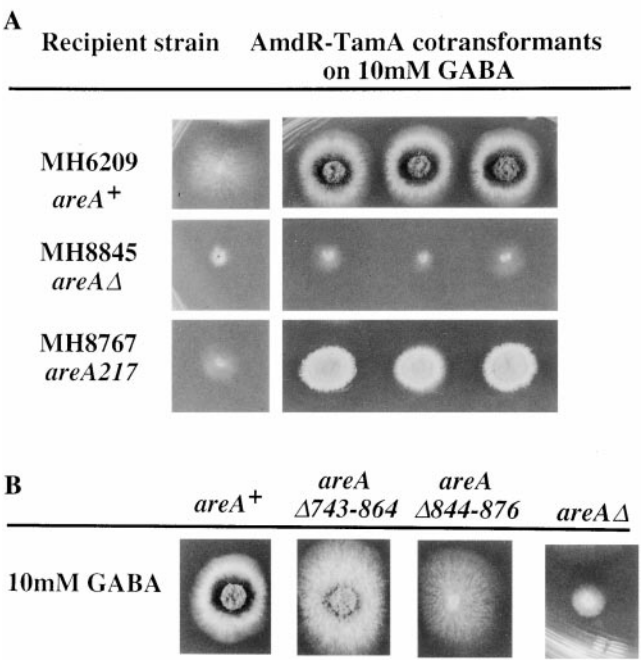

Figure 2.—Effect of AmdR-TamA expression in different *areA* genetic backgrounds. (A) The growth on GABA of recipient strains and the respective cotransformants expressing AmdR-TamA. Cotransformants of MH6209 and MH8827 were isolated using pPL3 and pAS4148 and selection for riboflavin prototrophy; cotransformants of MH8845 were isolated using pI4 and pAS4148 and selection for pyridoxine prototrophy. (B) The growth on GABA of wild-type and *areA* mutant strains expressing AmdR-TamA. Growth was assessed after 2 days of incubation at 37° on glucose-minimal medium containing 10 mm GABA as the sole nitrogen source.

an *areA*Δ background, neither AmdR-TamA nor FacB-TamA was able to activate expression to *areA*$^+$ levels. The residual activation in the absence of AreA suggested that the hybrid proteins also had *areA*-independent activator function.

In contrast to an *areA*Δ background, in an *areA217* mutant background FacB-TamA and AmdR-TamA were able to strongly activate *amdS::lacZ* expression. β-Galactosidase levels were as high or higher than the levels detected in an *areA*$^+$ background under both repressed and derepressed conditions. Therefore, the AreA protein is required for high levels of activation by the TamA hybrid proteins but AreA DNA binding is not a prerequisite. This suggests that TamA bound to the *amdS* promoter by either the FacB or AmdR DNA-binding domains could recruit AreA as a transcriptional activator.

Interaction requires the C terminus of AreA: The loss-of-function phenotype of an *areA*Δ mutant can be complemented by the *A. nidulans areA* gene or the *A. oryzae areA* (*oareA*) or *N. crassa nit-2* homologues (Davis and Hynes 1987; Fu and Marzluf 1990; Christensen *et al.* 1998). When the *A. nidulans areA* gene was transformed into an *areA*Δ strain expressing FacB-TamA (MH9024), activation of *amdS::lacZ* expression was restored to levels equivalent to *areA*$^+$ strains cotransformed with FacB-TamA. The *A. oryzae areA* gene also activated expression of *amdS::lacZ* in combination with the *A. nidulans* FacB-TamA, consistent with its structural and functional similarity to *A. nidulans areA* (Figure 3).

A series of *A. oryzae areA* deletion plasmids, which encode AreA products able to complement an *A. nidulans areA* deletion mutant for growth on nitrate (Christensen *et al.* 1998), was tested for their ability to promote activation of *amdS::lacZ* by FacB-TamA (Figure 3). The oAreA proteins lacking N-terminal regions were all able to function as well as wild-type oAreA in replacing native AreA function in the absence of FacB-TamA. However, deletions that removed N-terminal amino acids 44–218 or greater resulted in lower levels of *amdS:lacZ* expression in combination with FacB-TamA, suggesting that amino acids within this region of oAreA, while not essential, may contribute to the interaction with FacB-TamA.

In contrast, the C terminus of oAreA was critical for interaction with FacB-TamA. Deletions removing the C-terminal residues affected oAreA function only slightly when tested in the absence of FacB-TamA yet virtually abolished the AreA-dependent component of FacB-TamA activation. Therefore, the C-terminal 58 amino acids of *A. oryzae* AreA are essential for interaction with TamA. An internal deletion that removes amino acids 326–648 of oAreA resulted in a protein with weak activator function in the presence or absence of FacB-TamA. This deletion is thought to remove AreA activation domains and to allow complementation of an *areA* loss-of-function mutant only on those nitrogen sources such as nitrate or proline that have strong induction signals to compensate for poor AreA activation function (Christensen *et al.* 1998). It is most likely that this loss of activator capacity prevented significant activation via FacB-TamA, although it cannot be excluded that this mutation may also interfere with TamA interaction.

Several *A. nidulans areA* mutants that specifically affect the C-terminal region of the encoded protein have been created (Platt *et al.* 1996). These *areA* mutations were introduced by genetic crosses into strains carrying *amdS::lacZ* and expressing AmdR-TamA or FacB-TamA (Table 3). The *areA*Δ*743–864*-mutant encodes an AreA protein that lacks C-terminal residues 743–864 but retains the extreme C-terminal residues from 865 to 876 while the *areA*Δ*844–876*-encoded protein lacks the last 32 amino acids of the AreA protein. Neither mutant version of the AreA protein activated *amdS::lacZ* expression as well as full-length AreA in the absence of FacB-TamA. However, the *areA*Δ*743–864*-encoded protein was able to increase *amdS::lacZ* expression via FacB-TamA, indicating that the residues 743–864 of AreA are not required for the interaction. In contrast, activation of *amdS::lacZ* via FacB-TamA was greatly impaired in the *areA*Δ*844–876* background. The levels of expression were comparable to those seen with the C-terminal deletion of oAreA. Comparison of the predicted protein products of the two *areA* mutants indicated that amino acids 865–876 were required for FacB-TamA interaction.

Activation by FacB-TamA is independent of NmrA and TamA: Interestingly the amino acids 865–876 of AreA required for interaction with TamA are the same C-terminal residues predicted to interact with the negatively acting NmrA protein of *A. nidulans* (Platt *et al.* 1996; Andrianopoulos *et al.* 1998). Therefore, the possibility that TamA interacts indirectly with AreA via NmrA was tested using the strain MH9084, which carries an *nmrA* deletion mutation and expresses FacB-TamA. Activation of *amdS:lacZ* expression via FacB-TamA was still able to occur in the absence of a functional NmrA product (Table 3). In fact, the level of FacB-TamA-dependent expression was elevated in the *nmrA*Δ background particularly on ammonium compared to levels seen in a wild-type background. Therefore, NmrA was not required for FacB-TamA activation. In addition, the wild-type TamA protein was not required. In the absence of FacB-TamA, the *tamA*Δ mutant MH9219 had reduced levels of *amdS::lacZ* expression compared to wild type due to the loss of *tamA* function. The FacB-TamA-dependent expression of *amdS::lacZ* was significantly elevated in a *tamA*Δ background compared to a wild-type background (Table 3). Rather than being required for FacB-TamA activation, these results suggested that the wild-type TamA protein may partially interfere with FacB-TamA activation.

TamA and NmrA interact with common but not identical regions of AreA/NIT-2: It was of considerable interest to determine whether the regions of AreA involved in TamA and NmrA interaction completely overlap. The

Transformed plasmid	- FacB-TamA		+ FacB-TamA	
	NH_4	N-Free	NH_4	N-Free
None	0.5	1.0	8.0	12.1
	(0.1)	(0.2)	(1.1)	(1.4)
pAR4-1 (1–876)	nd	nd	33.4	141.8
			(5.3)	(6.5)
pTOC895 (1–866)	0.7	8.5	32.0	110.6
	(0.1)	(0.5)	(6.7)	(3.3)
pTOC181 (1–43, 219–866)	0.6	8.6	16.8	60.6
	(0.1)	(0.9)	(1.7)	(7.6)
pTOC206 (1–218, 326–866)	0.9	10.1	42.2	134.9
	(0.2)	(0.7)	(7.8)	(17.4)
pTOC257 (271–866)	1.0	8.2	13.3	31.7
	(0.3)	(2.9)	(2.0)	(4.2)
pTOC180 (1–325, 649–866)	1.0	1.5	15.0	17.3
	(0.2)	(0.3)	(1.1)	(1.5)
pTOC183 (1–749)	0.8	2.4	12.3	24.5
	(0.1)	(0.5)	(1.2)	(2.6)
pTOC184 (1–818)	1.0	6.0	10.9	19.5
	(0.2)	(0.5)	(0.7)	(1.6)

Figure 3.—β-Galactosidase levels of transformants carrying different *A. oryzae areA* constructs in the presence of FacB-TamA. Constructs encoding *A. nidulans areA* (pAR4-1; M. A. Davis and M. J. Hynes, unpublished results) and full-length or truncated versions of the *A. oryzae areA* gene (Christensen *et al.* 1998) were introduced into the *areA*Δ strain MH8845 and the *areA*Δ strain MH9024, which also expresses FacB-TamA. The GATA zinc finger region is indicated by dark shading and the acidic region potentially involved in activation is indicated by light shading. The amino acid coordinates of the predicted *A. oryzae* AreA products are shown. Transformants were selected for growth on nitrate as a sole nitrogen source and the mean β-galactosidase activities and standard errors (in parentheses) of four independent transformants were determined. Mycelia were grown overnight on 1% glucose medium containing 10 mm ammonium tartrate and transferred to 1% glucose medium containing either 10 mm ammonium (NH_4) or no added nitrogen source (N free) for 4 hr. The β-galactosidase activities are expressed as units of activity per minute per milligram of soluble protein.

N. crassa nit-2 gene transformed into *A. nidulans* strain MH9024 (*areA*Δ; FacB-TamA) was able to substitute for AreA consistent with previous studies showing that *nit-2* complemented the *areA217* loss-of-function mutation (Davis and Hynes 1987). The levels of activation of *amdS::lacZ* by FacB-TamA in strains expressing NIT-2 were similar to those with *A. nidulans* AreA (Figure 4). Therefore, the key amino acids or protein structures are conserved in NIT-2, allowing this heterologous interaction. Two *nit-2* mutant alleles, both of which encode proteins unable to bind *N. crassa* NMR-1, were tested in *A. nidulans* (Figure 4). Mutation *nit2-P1* results in a leucine-proline substitution (L1032P) in the C terminus of NIT-2 and mutation *nit2-2* leads to two substitutions (L770F and H773Q) in the GATA zinc finger region (Pan *et al.* 1997). Although transformants able to grow on nitrate were obtained using both mutant versions of *nit-2*, neither functioned as well as the wild-type *nit-2* gene. In both cases, the transformants grew more poorly on nitrate and, in the absence of FacB-TamA, the levels of *amdS::lacZ* expression were 40 and 10%, respectively, of levels in wild-type *nit-2* transformants. However, the *nit-2* mutant alleles differed greatly in their ability to allow activation via FacB-TamA. The *nit2-P1* mutant allele resulted in greatly reduced FacB-TamA-dependent activation of *amdS::lacZ*. This mutation also prevented

TABLE 3

Effect of mutations in *areA, tamA, or nmrA* genes on FacB-TamA activation on *amdS::LacZ* expression

	amdS::lacZ expression levels[a]			
	−FacB-TamA		+FacB-TamA	
Relevant genotype	NH_4	N free	NH_4	N free
areA$^+$	0.7	11.4	20.7	105.1
	(0.1)	(1.2)	(0.6)	(15.0)
areAΔ743-864	0.4	2.5	31.9	110.1
	(0.1)	(0.6)	(3.0)	(12.1)
areAΔ844-876	0.6	3.3	10.1	20.5
	(0.1)	(0.4)	(0.9)	(2.4)
nmrΔ	1.5	8.7	45.5	146.6
	(0.1)	(1.2)	(4.8)	(30.5)
tamAΔ	0.5	3.1	39.1	218.1
	(0.1)	(0.6)	(3.9)	(11.7)

[a] β-Galactosidase levels were determined in strains lacking *facB-tamA* (−FacB-TamA) and in strains carrying *facB-tamA* (+FacB-TamA) in an *areA*$^+$ (MH6209, MH9087), *areAΔ743-864* (MH9220, MH9147), *areAΔ844-876* (MH9260, MH9079), *nmrΔ* (MH9192, MH9084), and *tamAΔ* (MH9219, MH9350) background. Mycelia were grown overnight on 1% glucose medium containing 10 mm ammonium tartrate and transferred to 1% glucose medium containing either 10 mm ammonium (NH_4) or no added nitrogen source (N free) for 4 hr. each strain was assayed in triplicate and the mean β-galactosidase activities and standard errors (in parentheses) are given. β-Galactosidase activities are expressed as units of activity per minute per milligram of soluble protein.

NMR-1 interaction (Pan *et al.* 1997), indicating that L1032 in NIT-2 (equivalent to L872 in AreA) is required for both NMR-1 and FacB-TamA interactions. In contrast, the *nit2-2* mutant allele did not affect FacB-TamA-dependent activation as transformants expressing this altered NIT-2 protein showed similar *amdS::lacZ* levels to wild-type NIT-2. The finding that alteration within the DNA-binding domain did not affect FacB-TamA activation was consistent with the evidence that the *areA217*-encoded protein is also functional in this assay (see Figure 2A). Together with the suggestion that FacB-TamA has a partial requirement for the N-terminal region of AreA (see Figure 3), these data indicate that NmrA and TamA recognize a common C-terminal region of AreA but require additional nonoverlapping regions of AreA to exert their effects.

TamA and AreA interact in *S. cerevisiae*: A series of constructs in which TamA (amino acids 30–651, 30–359, 153–159, 360–651, or 470–651) was fused to the GAL4 DNA-binding domain (GAL4DBD) were made. Each of these GAL4DBD-TamA fusions was transformed into *S. cerevisiae.* None were able to activate expression of the *HIS3* and *lacZ* reporter genes sufficiently to allow transformants to grow on media lacking histidine or produce detectable levels of β-galactosidase (data not shown). Therefore, the TamA protein lacks sequences able to function as activation domains in *S. cerevisiae.*

The yeast two-hybrid system was used to test for interaction between the TamA and the AreA proteins. A variety of constructs in which the GAL4 activation domain (GAL4AD) was fused to the AreA protein (amino acids 178–876, 178–755, or 178–402) were made. These constructs were transformed into *S. cerevisiae* in combination with GAL4DBD and none allowed growth on minimal media (data not shown). Each of the GAL4DBD-TamA fusions was tested in combination with the GAL4AD construct and each of the GAL4AD-AreA fusions. Most combinations gave no evidence of interaction (data not shown) although transformants carrying GAL4DBD-TamA(30–651) in combination with GAL4AD alone were able to grow on minimal media.

Transformed *nit2* construct		- FacB-TamA		+ FacB-TamA	
		NH_4	N-Free	NH_4	N-Free
nit2	1 — 1036	2.1	11.0	56.7	99.9
		(0.3)	(0.8)	(6.3)	(6.4)
nit2-P1	1 — 1036, L1032P	1.1	4.3	12.8	20.9
		(0.2)	(0.7)	(1.2)	(1.3)
nit2-2	1 — 1036, L770F H773Q	0.5	1.1	69.6	78.9
		(0.1)	(0.2)	(9.4)	(12.3)

Figure 4.—Effect of mutations in the *nit-2* gene on FacB-TamA activation of *amdS::lacZ.* The effects of mutations in the *N. crassa nit-2* gene were assessed by introducing constructs encoding either wild-type or mutant versions of NIT2 as indicated into the *areAΔ* strain MH8845 and the *areAΔ* strain MH9024, which also expresses FacB-TamA. Transformants were selected for growth on nitrate as a sole nitrogen source and the mean β-galactosidase activities and standard errors (in parentheses) of four independent transformants were determined. Mycelia were grown overnight on 1% glucose medium containing 10 mm ammonium tartrate and transferred to 1% glucose medium containing either 10 mm ammonium (NH_4) or no added nitrogen source (N free) for 4 hr. β-Galactosidase activities are expressed as units of activity per minute per milligram of soluble protein.

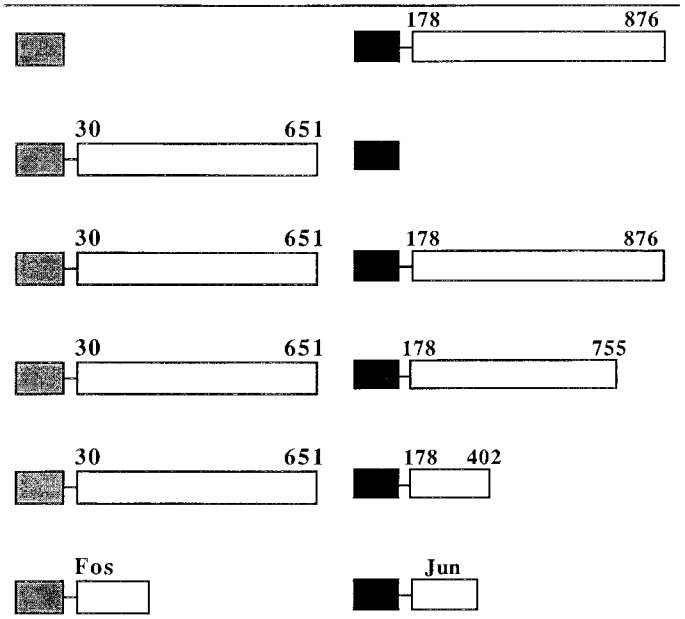

Figure 5.—Interaction between TamA and AreA in *S. cerevisiae*. The GAL4 DNA binding domain (GAL4DBD) amino acids 1–147, shown as a lightly shaded box, or the GAL4 activation domain (GAL4AD) amino acids 768–881, shown as a dark box, were fused in frame to various portions of TamA or AreA, respectively. The amino acid coordinates of the TamA or AreA proteins are shown. *S. cerevisiae* transformants with the various GAL4DBD-TamA and GAL4AD-AreA constructs indicated were tested for growth on minimal media lacking histidine (−his media) where +++ represents strong growth, ++ represents reduced growth; and − represents no growth. Colonies were tested for *lacZ* expression by the plate X-gal filter test where +++ represents a rapid and intense color change within 10 min of exposure to X-gal, + represents a detectable color change within 2 hr, ± represents a very low level of expression detectable after approximately 24 hr, and − represents no detectable color change. β-Galactosidase activities were determined in cells grown in liquid culture and are expressed in Miller units.

These transformants were positive for the X-gal filter test although levels were extremely low. However, transformants carrying both GAL4DBD-TamA(30–651) and GAL4AD-AreA(178–876) were able to grow strongly on media lacking histidine and were positive on X-gal filter tests (Figure 5). These results provide evidence for a weak interaction between TamA and AreA in *S. cerevisiae* as determined by quantitative β-galactosidase assays relative to the strong interaction of the Jun and Fos control (Figure 5). The level of interaction between TamA and AreA detected in *S. cerevisiae* is similar to the weak, but significant, interaction of the mammalian Rb and E2F1 proteins (Bartek *et al.* 1996).

The interaction between the AreA and TamA hybrid proteins was abolished when the N-terminal (30–359 or 153–359) or C-terminal (360–651 or 470–651) residues of TamA were expressed in the presence of GAL4AD-AreA(178–876) suggesting that an intact TamA protein was required. Significantly, C-terminal truncation of AreA (178–755 or 178–402) also prevented a positive interaction. Therefore, the C-terminal region of AreA required for interaction with FacB-TamA or AmdR-TamA in *A. nidulans* was also required for interaction to form a functional two-hybrid GAL4 activator in *S. cerevisiae*.

DISCUSSION

An understanding of the role of the *tamA* gene in nitrogen metabolite repression in *A. nidulans* has been elusive. The gene was initially defined by the isolation of mutants that produced lowered levels of a variety of nitrogen metabolic enzymes sufficient to give resistance to toxic nitrogen source analogues (Kinghorn and Pateman 1975). Although a regulatory role for *tamA* has been disputed (Arst *et al.* 1982), the cloning and sequencing of the *tamA* gene has provided new approaches for studying its function (Davis *et al.* 1996). The predicted TamA protein contains a number of regions with similarity to a yeast regulatory gene *UGA35/DAL81/DURL*. Curiously, the putative N-terminal Zn (II)2Cys6 DNA-binding domains of both TamA and UGA35 are dispensable for function despite the conserved six cysteine residues and the basic region between cysteines 2 and 3 (Bricmont *et al.* 1991; Davis *et al.* 1996). Thus a significant role for TamA as a DNA-binding protein in the context of known phenotypes seems unlikely.

We have addressed the possibility that TamA functions as a transcriptional activator. As TamA may not bind DNA directly, it may be brought to a promoter by interaction with a DNA-binding protein. We have mimicked this situation by fusing TamA directly to a functional DNA-binding domain. These TamA hybrid proteins are strong activators of gene expression in *A. nidulans* and AreA is a major source of their activation potential. We propose that the TamA fusions are recruiting AreA to the relevant promoters. This is supported by the finding that the mutant AreA217 protein that

lacks DNA-binding activity was able to activate expression when recruited by interaction with TamA. This result also indicates that AreA was not required indirectly to activate transcription of an unknown protein that then interacts with the TamA fusion protein. If that were so, then the *areA217* strain would lack the activation of expression of this protein.

Two regions of *A. oryzae* AreA are required for TamA interaction; the most significant is in the C terminus and a less critical region is in its N-terminal segment. Using *areA* mutants of *A. nidulans* (Platt *et al.* 1996) we have further localized the C-terminal interacting region to the last 12 amino acids of AreA. A specific prediction is that the phenotype of the *areAΔ844–876* mutant should resemble that of a *tamA* mutant. This is supported by the observations of Platt *et al.* (1996) that *areAΔ844–876* strains have a partial loss-of-function phenotype and increased resistance to certain toxic nitrogen source analogues. The C-terminal 9 amino acids are absolutely conserved in *A. nidulans*, *A. oryzae*, and *N. crassa* as is a similar sequence (residues 116–131 of AreA) within the region of a possible second TamA interaction site. Interestingly, deletion of the N-terminal AreA residues 2–389 results in resistance to chlorate and β-aspartate hydroxymate consistent with slightly reduced *areA* function (Caddick and Arst 1998). It is possible that this phenotype results, at least in part, from a reduced interaction with TamA.

The C terminus of AreA is involved in interaction with the negatively acting NmrA protein (Platt *et al.* 1996; Andrianopoulos *et al.* 1998). Significantly, the *N. crassa nit2-P1* mutation that prevents NMR-1 interaction (Pan *et al.* 1997) also prevents TamA interaction. It is unlikely that TamA interacts with AreA indirectly by binding to NmrA as FacB-TamA was able to promote activation in an *nmrAΔ* strain. Therefore, TamA and NmrA may compete for the same C-terminal region and the role of TamA may be to displace NmrA under nitrogen-limiting conditions. Consistent with this proposal, *amdS::lacZ* expression was higher in an *nmrAΔ* background where AreA may be more accessible to the TamA fusion proteins in the absence of NmrA competition. However, the AreA-TamA interaction was also detectable under nitrogen-sufficient conditions, indicating that NmrA is unable to outcompete TamA for access to AreA.

In the absence of AreA, the TamA fusion protein retained detectable activation activity. This could be due to activation domains within TamA itself, although when linked to the GAL4 DNA-binding domain, TamA had no apparent activation capacity in *S. cerevisiae*. This highlights possible species-specific differences between *A. nidulans* and *S. cerevisiae*. TamA, bound to DNA by the FacB or AmdR DNA-binding domains, was able to bring about strong AreA-dependent activation in *A. nidulans*. However, TamA bound to DNA by the GAL4 DNA-binding domain was not able to bring the homologous nitrogen regulators, GLN3 and/or NIL1/GAT1, into the proximity of the relevant promoters in *S. cerevisiae*. The C-terminal amino acid sequences of GLN3 and NIL1/GAT1 are not highly conserved with AreA or NIT-2. Therefore, divergence in these sequences may prevent *A. nidulans* TamA from interacting with the *S. cerevisiae* nitrogen regulators. There is no evidence that additional factors were preventing this interaction as we were able to detect an interaction between TamA and AreA in *S. cerevisiae*. This interaction was weak, but the two-hybrid assay may underestimate the strength of the TamA-AreA interaction as the GAL4AD-AreA component contains the AreA GATA finger. This hybrid protein could bind to GATA sites within the *S. cerevisiae* genome limiting the amount of product available to interact with GAL4DBD-TamA. A similar phenomenon has been observed with constructs expressing the FacB DNA-binding domain in *S. cerevisiae* (Todd 1995).

Our experiments indicate that TamA, once bound to DNA, can recruit the transcriptional capacity of AreA to specific promoters. However, in a wild-type situation, it is more likely that the GATA finger of AreA allows recognition of the target promoters of nitrogen-regulated genes and TamA is recruited through interaction with AreA (Figure 6). The positive role of TamA in this interaction remains unclear. The previously described interactions of AreA, NIT-2, and other mammalian or Drosophila GATA transcription factors that modify the activity of the GATA factor all involve interactions with the GATA finger (see Mackay and Crossley 1998). The ability of the AreA217 and NIT2-2 proteins to function in this assay argues against involvement of the GATA finger in the TamA-AreA interaction, making it unlikely that the positive role of TamA is to enhance AreA DNA binding. Another possibility is that AreA, once bound to DNA, can use the TamA interaction to recruit additional AreA molecules to a promoter. Alternatively, TamA may modify the transcriptional potency of AreA, either by inducing conformational

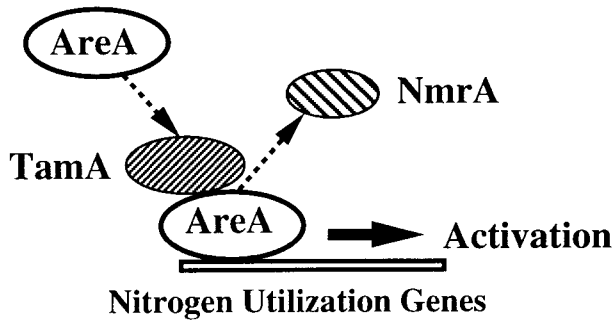

Figure 6.—Schematic representation of the TamA and AreA interaction *in vivo*. In a wild-type cell, AreA binds to the promoters of genes involved in nitrogen utilization. The interaction between AreA and TamA may lead to increased expression by recruitment of additional AreA molecules, modification of the transcriptional capacity of AreA, and/or displacement of the negatively acting NMRA (see text for discussion).

changes that increase the accessibility of the AreA activation domains or by forming additional activation domains within the TamA-AreA complex. TamA itself may provide additional activation domains to the complex. The AreA-TamA interaction may also have a role in the displacement of NmrA from the AreA-NmrA complex under nitrogen-limiting conditions. It will be of interest to further define the nature of the AreA-TamA interaction, particularly the regions of the TamA protein required for this interaction.

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