Nitrate and the GATA factor AreA are necessary for in vivo binding of NirA, the pathway-specific transcriptional activator of Aspergillus nidulans

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Summary
In Aspergillus nidulans, the genes coding for nitrate reductase (niaD) and nitrite reductase (niiA), are transcribed divergently from a common promoter region of 1200 basepairs. We have previously characterized the relevant cis-acting elements for the two synergistically acting transcriptional activators NirA and AreA. We have further shown that AreA is constitutively bound to a central cluster of four GATA sites, and is involved in opening the chromatin structure over the promoter region thus making additional cis-acting binding sites accessible. Here we show that the asymmetric mode of NirA–DNA interaction determined in vitro is also found in vivo. Binding of the NirA transactivator is not constitutive as in other binuclear C6-Zn₂⁺-cluster proteins but depends on nitrate induction and, additionally, on the presence of a wild-type areA allele. Dissecting the role of AreA further, we found that it is required for intracellular nitrate accumulation and therefore could indirectly exert its effect on NirA via inducer exclusion. We have tested this possibility in a strain accumulating nitrate in the absence of areA. We found that in such a strain the intracellular presence of inducer is not sufficient to promote either chromatin rearrangement or NirA binding, implying that both processes are directly dependent on AreA.

Introduction
In Aspergillus nidulans, most genes coding for activities involved in the utilization of nitrogen sources are subject to two forms of regulation, pathway-specific induction and nitrogen metabolite repression. Nitrate and nitrite induction require the pathway specific activator NirA, whereas nitrogen metabolite repression is mediated by the positive-acting wide-domain regulatory protein AreA. Genetic evidence shows that the two regulatory proteins NirA and AreA act synergistically but with independently defined roles to assure a co-ordinated regulation of nitrate utilization in A. nidulans (for a review, see Scanzocchio and Arst, 1989).

Nitrate assimilation involves nitrate uptake, and subsequent conversion via nitrite to ammonia. The genes which code for these activities are clustered on chromosome VIII: crnA, coding for a nitrate permease, niaD, encoding nitrate reductase (NR) and niiA, encoding nitrite reductase (NiR). The two latter genes encoding these enzymes are divergently transcribed and are separated by 1200 basepairs (bp). The expression of crnA, niiA and niaD structural genes is strictly dependent on both functional NirA and AreA (Brownlee and Arst, 1983; Scanzocchio and Arst, 1989; Unkles et al, 1991). Genetic and biochemical evidence suggests that at least one other nitrate transporter exists in A. nidulans as crnA+ mutants are still able to utilize nitrate as a sole nitrogen source. In plants, in which nitrate serves as a prevalent nitrogen source, the transport process is a key step in nitrate assimilation. On the basis of chlorate resistance mutants and polymerase chain reaction (PCR) approaches using crnA-based degenerate primers, low- and high-affinity uptake systems have been cloned (reviewed by Daniel-Vedele et al., 1998). It appears that the high-affinity plant uptake systems are regulated by available nitrate and reduced nitrogen in a similar way to that described for fungi (Aslam et al., 1992). So far, no regulatory genes have been identified in plants either for the transporters or for the structural genes encoding nitrate and nitrite reductase.

The corresponding regulatory genes of A. nidulans have been cloned and characterized as DNA-binding transcription factors (Kudla et al., 1990; Burger et al., 1991a; b). NirA contains a binuclear Zn₂⁺-cluster domain of the GAL4p type but specific recognition does not involve the typical inverted, everted or direct repeat sequences as shown for many Zn₂⁺-cluster proteins (Schjerling and Holmberg, 1996). Instead, NirA binds as a dimer to a non-repeated, asymmetrical 5’-CTCCGHHGG-3’ sequence. The NirA protein was the first to be described in which a dimer binds such a sequence, presumably
each monomer making different contacts on each strand (Strauss et al., 1998). AreA contains a C$_2$C$_2$Zn$^{2+}$-finger and belongs to the so-called GATA factor family of transcription factors (recognizing a DNA motif with a 5'–WGATAR–3' core sequence (Omichinski et al., 1993a; b; Starich et al., 1998). This class of eukaryotic transcriptional activators is known to be involved in the regulation of a variety of metabolic and developmental processes (Wilson and Arst, 1998; Scagazzochio, 2000 and references therein). It is proposed that promoter specific recognition and discrimination between the different set of genes regulated by GATA factors is determined by subtle differences in the hydrophobic protein-DNA interactions among GATA factors (Charron et al., 1999) or by interaction with pathway-specific transcription factors (Feng and Marzluf, 1998). In Saccharomyces cerevisiae, regulated nitrogen catabolic gene transcription is also mediated by GATA factors (reviewed by Coffman and Cooper, 1997).

The contribution of the four NirA and 10 AreA binding sites in the niaD–niiA intergenic region (IGR) to nitrate-responsive expression of NR and NiR was analysed by deletion studies in vivo (Punt et al., 1995). The central NirA site 2 acts bidirectionally and contributes to more than 80% of niiA and niaD transcription in an areA$^-$$^-$ background. It is located in the proximity of a cluster of four AreA sites. These GATA sites are responsible for about 80% of the transcriptional activity in a nirA$^-$$^-$ background (Muro-Pastor et al., 1999). These authors showed in vivo that a GATA factor is directly involved in the remodelling of chromatin. NirA site 2 is located at the border of positioned nucleosome-1 and the clustered GATA sites 5–8 are situated in a nucleosome-free region. AreA-dependent remodelling occurs upon nitrate induction in the absence of ammonia. Mutation of all four GATA sites could not prevent this nucleosomal rearrangement, which suggests either a mechanism acting at distance from the remaining six AreA binding sites in the IGR, or a mechanism which is independent of DNA binding. The repositioning of four nucleosomes out of five monitored in this region is independent from the NirA-specific transcription factor and hence from the onset of transcription. In vivo footprinting, which was adapted to filamentous fungi by Wolschek and colleagues (Wolschek et al., 1998) showed that AreA binds to site 5 under derepressed conditions (regardless of whether non-induced or induced conditions). Upon repression, occupancy of AreA site 5 decreases but protection of this site is still clearly visible even under conditions of full repression. Only in areA loss-of-function mutants, such as areA600 or areA18, is protection completely lost.

Here we report the in vivo binding characteristics of the pathway-specific NirA transcription factor. NirA recognizes in vivo the same asymmetrical, non-repeated sequence as it does in vitro. We show that in contrast to the yeast GAL4p activator which is bound under non-induced conditions (Kodadek, 1993), binding of NirA is dependent on intracellular nitrate and on a functional AreA protein.

Results

The kinetics of nitrate induction

We have previously shown that the genes coding for nitrate reductase (niaD) and nitrite reductase (niiA) are co-regulated in response to the availability of nitrate (Punt et al., 1995). To monitor the appearance and disappearance of the niaD transcript in detail, we have set up an experimental procedure in which mycelium was precultured under non-inducing conditions (on urea as sole nitrogen source, NI). These cultures were aliquoted and transferred to medium containing only nitrate (induced, I), simultaneously nitrate plus ammonia (induced/repressed, I/R) or ammonia following 20 min of nitrate induction (I20 + R). Aliquots were incubated for different times (5–90 min) and the cultures were harvested for Northern analysis, reporter enzyme assays, in vivo footprinting and chromatin analysis.

The results of the Northern experiments are shown in Fig. 1 for the wild-type (Fig. 1A) and for nirA and areA mutant strains (Fig. 1B). In the wild type, under non-induced conditions, niaD transcript can hardly be detected. Nitrate induction results in a rapid accumulation of niaD transcripts already after 10 min. The simultaneous

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**Fig. 1.** Northern blot analysis of niaD transcription in Aspergillus nidulans wild-type strain (A) after incubation on different nitrogen sources and in A. nidulans wild-type, nirA and areA loss-of-function mutants (B) after 20 min of nitrate induction. The strains were pre-grown and processed as detailed in Experimental procedures. Different nitrogen sources (NI, non-induced; I, induction by nitrate; I/R, simultaneous induction by nitrate and repression by ammonia; I20 + R, induction by nitrate for 20 min and subsequent addition of ammonia) were then added and the cultures were incubated further for the time indicated (10, 20, 45 and 90 min). Finally, the samples were harvested by filtration and total RNA was isolated. Per sample, 20 μg of total RNA was loaded onto the gels. Rehybridization with a probe of the A. nidulans actin gene was used as a loading control. Numbers below the panel indicate the normalized relative niaD signal intensity for each condition in relation to the loading control.

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addition of nitrate plus ammonia prevents the induction process (I/R10 to I/R90). Induction for 20 min and subsequent addition of ammonia to the culture (I20 + R10 to I20 + R90) medium results in a rapid decrease in niaD mRNA abundance, which is in accordance with an areA-mediated repression mechanism (Kudla et al., 1990). As expected, the nirA 637 and areA600 loss-of-function mutant strains, which are unable to utilize nitrate as a sole nitrogen source, did not accumulate niaD transcript over the investigated time-period (Fig. 1B).

Simultaneously, we investigated niiA and niaD expression using the GUS and lacZ double reporter vector pTRAN3–1 A (Punt et al., 1995), which monitors expression of niaD-GUS and niiA-lacZ from the intergenic region. We found that the expression profiles detected in the Northern experiments were mirrored by the enzyme assays of the reporter (data not shown).

NirA binding in vivo to its cognate sequence is nitrate-dependent

We have previously shown by in vitro binding and a number of protection and interference in vitro footprinting studies that NirA, a member of the Zn$_6$-cluster family of transcription factors, binds as a dimer to a non-repeated, asymmetrical sequence with the consensus 5'-CTGGCGGG-3' (Strauss et al., 1998). We have further established the physiological role of the four NirA binding sites in the niaD-niiA intergenic region by deletion and mutagenesis studies (Punt et al., 1995). These studies revealed a major bi-directional contribution of the central NirA site 2 of about 80% of the total induction potential. A cluster of four AreA-binding GATA sites is neighbouring this central NirA site 2 and, in a recent mutagenesis study (Muro-Pastor et al., 1999), we have allocated the main physiological role to these four sites acting synergistically with NirA site 2 to achieve maximal induction levels.

We were now interested to see whether the unusual recognition mode of NirA in vitro binding is also mirrored in vivo and whether binding of the activator is inducer-dependent or not. Numbering of the bases within the NirA site was carried out as in the work by Strauss and colleagues (Strauss et al., 1998), i.e. 1–8 on the strand showing the 5′'-consensus sequence (5′-CTGGCGGG-3′) and 1′ to 8′ in the 5′'-orientation of the complementary strand (5′-CAGAATCGCGGC-3′). The in vivo methylation protection pattern shown in Fig. 2 reveals three conserved guanines within the complementary strand of the consensus sequence C1′GCGGGAG8′ as strongly protected, i.e. G3, G5, and G6. Footprints carried out on the opposite strand of NirA site 2 (Fig. 3) showed a protection pattern consistent with the in vitro NirA-DNA contacts, i.e. guanines G6, G7, and G8 are protected from methylation under inducing conditions. Additionally, adenine A10, which is hypersensitive in in vitro methylation assays (Strauss et al., 1998), also becomes hypersensitive in the in vivo assay (Fig. 3; A10 is marked with arrows). From these results, it becomes apparent that the unusual DNA–protein interaction profile obtained in in vitro studies with a truncated NirA protein (representing the bi-nuclear Zn-cluster and the dimerization domain) is also seen with the native, full-length transcription factor in vivo.

Our data show that binding of NirA is strictly dependent on the presence of inducer (Figs 2 and 3, lanes 110 to 190). Binding of NirA to its DNA target is very fast and can be monitored even 5 min after induction (Fig. 2, I5). The nirA637 mutant, which contains an in-frame deletion of the DNA-binding domain (Muro-Pastor et al., 1999), as...
expected, does not show protected bases in the binding site.

NirA dissociates under conditions of induction plus repression despite sufficient intracellular nitrate concentrations

When we assayed binding of NirA under conditions of induction and repression (Fig. 3, I/R10 to I/R90 and I20 + R10 to I20 + R90), we found that NirA is bound to its cognate site for 20 min even in the presence of ammonia. At 45 and 90 min of repression, occupancy of NirA site 2 is lost. As described above, there are neither niaD transcript nor reporter enzyme activities accumulating under conditions of induction plus repression, and the lack of transcriptional activation can be attributed to the inactivation of AreA by ammonium. As binding of NirA is dependent on inducer, a possible explanation for NirA dissociation from its target after 20 min would be low internal nitrate concentrations after this prolonged repression time. We therefore analysed intracellular nitrate concentrations of cells incubated with the relevant nitrogen sources.

Figure 4 shows intracellular nitrate concentrations of cells subjected to non-inducing, inducing and inducing-repressing conditions. The nitrate uptake systems are rapidly induced and this results in an intracellular nitrate level of more than 200 ng mg⁻¹ dry weight (DW) after 10 min (Fig. 4A). Under conditions of prolonged induction (90 min) this level drops to roughly 30 ng mg⁻¹ DW. This level is sufficient for NirA to remain bound to site 2 when nitrate alone serves as nitrogen source, and is also sufficient to maintain transcription. Under conditions of induction and repression (Fig. 4B), nitrate accumulates to a level of 250 ng mg⁻¹ DW, which is to be expected as nitrate is taken up without being metabolized. Despite the fact that almost no NR activity is present, the internal nitrate level drops to roughly 100 ng mg⁻¹ DW after 20–45 min (Fig. 4B and C). This finding suggests that a nitrate secretion mechanism is operating in A. nidulans. However, this result indicates that low internal nitrate concentrations do not account for the loss of NirA binding after 45 min under I/R and I20 + R conditions. The intracellular nitrate concentrations measured under these conditions (70–150 ng mg⁻¹ DW) are well above the concentrations of I90 (30 ng mg⁻¹ DW) in which NirA remains bound.

Repression does not lead to NirA degradation

We also considered the possibility that NirA might be subjected to ammonia-triggered protein degradation. Owing to the extremely low NirA level in cells that do not allow detection of the protein by Western blotting (M. Muro-Pastor and C. Scazzocchio, personal communication), we were not able to monitor a putative nitrogen source-dependent degradation process. We therefore tested this process indirectly. We pre-grew the wild-type mycelium under repressing conditions, and transferred aliquots to induction in the presence and absence of the protein synthesis inhibitor cyclohexamide (Strauss et al., 1999). We reasoned that cyclohexamide should prevent
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de novo synthesis of NirA in ammonia-grown cultures, which are then transferred to nitrate. In in vivo footprinting assays we did not see any difference between the control mycelium and the cyclohexamide-treated mycelium. In both cases, NirA binding site 2 was occupied already after 5 min of induction (data not shown). Thus, it seems very unlikely that NirA is subject to ammonia-mediated degradation and the results are consistent with the view that loss of NirA site 2 occupancy under inducing/repressing conditions is not due to increased turnover of the activator.

Binding of NirA to its DNA target is dependent on a functional areA allele

In Figs 2 and 3, we also show that NirA binding does not respond to nitrate in the absence of a functional AreA protein (Figs 2 and 3, lanes I areA600). A possible explanation is that in an areA mutant background, complete inducer exclusion prevents nitrate-dependent association of NirA with binding site 2.

In fact, measuring intracellular nitrate levels under inducing conditions (Fig. 5), we found that areA600 does not follow wild-type kinetics but only accumulates up to 15 ng mg⁻¹ DW intracellular nitrate over the whole incubation time of 90 min. It is interesting to note that in the non-induced control experiments (NI), carried out with nitrate and nitrite-free urea as sole nitrogen source, a considerable intracellular nitrate level accumulates in the mutant strains. We have repeatedly observed this effect also in other mutants lacking nitrate reductase activity, such as niaD26 (unpublished). For the moment, it is not clear how nitrate can be generated in the fungal cell as the known pathway via nitric oxide synthase activity (Stuehr, 1997) has not been described in ascomycetes. However, strongly reduced intracellular nitrate levels in the induced areA mutant strain suggest that inducer exclusion could be at least in part responsible for our observation that NirA does not bind in an areA loss-of-function strain. To circumvent the inducer exclusion effect, we constructed a strain expressing the crnA nitrate transporter gene (Unkles et al., 1991) under the AreA-independent gpdA promoter (Punt et al., 1990) in an areA600 background. The gpd-crnA construct was tested for functionality and was found to complement the crnA1 mutation. We transformed this construct into an areA600/argB2 strain as a single copy integrated at the argB locus. Figure 6A compares the expression pattern of crnA and niaD of the recipient strain areA600/argB2 with

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observed during a 90 min incubation time a slowly in-
incorporation under inducing conditions (Fig. 5), we
as sole nitrogen source.

These data confirm our earlier observation that
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reductase gene (crnA/areA

Fig. 6. Northern blot (A) and chromatin structure analysis (B) of wild-type, are4600 mutant strain (defective in nitrate uptake) and of gpd-
crnA/are4600, which takes up nitrate independently of AreA.
A. The cultures were grown under standard conditions and transferred to non-inducing conditions (NI), inducing conditions for 90 min (I90) and
simultaneous inducing and repressing conditions (I/R 90) for 90 min. The membranes were probed to monitor the expression of the nitrate
reductase gene (niaD), the nitrate transporter (crnA) and reprobed again with the actin gene as a loading control.
B. Chromatin organization of the niiA-niAD intergenic region in a wild-type, are4600 and a gpd-crnA/are4600 strain. Growth conditions are as described in Experimental procedures. Crude nuclei were treated for 5 min with 125 or 250 U MNase per gram of mycelia (wet weight) at
30°C. Increasing quantities of enzyme are symbolized on top of the lanes. DNA was digested with HindIII and subjected to indirect end-
labelling using a HindIII–EcoRV fragment of the niiA gene as a probe. The positions of nucleosomes are pictured at the right as ellipses and
are numbered divergently from the niiA free region (nfr) as referred to by Muro-Pastor and colleagues (Muro-Pastor et al., 1999).

Under induced conditions in the wild type (I90 wild type), in which the open chromatin structure prevails, nucleosomes are not positioned,
whereas under the same conditions in gpd-crnA/are4600 (I90 gpd-crnA/are4600) and are4600 (I90 are4600) strains positioned nucleosomes
indicate a closed chromatin structure.

these of the gpd-crnA/are4600 transformant together with
a wild-type control. crnA is not expressed to a detectable
level in either condition in an are4600 background but is
clearly expressed under all conditions in the gpd-crnA/
are4600 transformant. On the other hand, the constitutive
expression of the transporter does not lead to induction
of niaD in the are4600 strain. Thus, reversal of inducer
exclusion does not bypass an areA loss-of-function muta-
tion. These data confirm our earlier observation that gpd-
crnA/are4600 transformants are not able to utilize nitrate
as sole nitrogen source.

When we tested the gpd-crnA/are4600 strain for nitrate
incorporation under inducing conditions (Fig. 5), we
observed during a 90 min incubation time a slowly in-
creasing intracellular nitrate level of up to 35 ng mg⁻¹
DW. Interestingly, this strain did not reconstitute wild-type
uptake kinetics. This effect might be due to areA depend-
ent function of the second nitrate transporter ntrB
(Unkles et al., 2001) and/or another factor necessary for
efficient nitrate uptake. However, this intracellular nitrate
level is sufficient to promote NirA DNA binding in the
wild type. We therefore used these conditions to test in vivo if
NirA binding is directly dependent on AreA. In the gpd-
crnA/are4600 strain, site 2 is not occupied by NirA under
these conditions (data not shown) which provides evidence
that, independently of sufficient intracellular nitrate,
functional AreA is necessary for in vivo NirA–DNA inter-
action at least for site 2.

It was previously shown also that the rearrangement of
chromatin structures in the niiA-niD IGR is inducer- and
AreA-dependent. Our nitrate accumulation studies in an
areA⁻ background prompted us to question whether AreA
dependence could be indirect because of inducer exclu-
sion. We therefore analysed the gpd-crnA/are4600 strain
under inducing conditions for chromatin remodelling in the
nitrate-responsive niiA-niAD IGR (Fig. 6B). We observed
that in this strain the nucleosomal structure in the
bidirectional promoter is not disrupted despite sufficient
intracellular nitrate levels. This result extends our earlier
observation in respect to AreA-dependent chromatin
remodelling: AreA seems to be directly involved in this
process and not only indirectly via inducer exclusion.

Discussion

Almost all members of the fungal binuclear Zn-cluster
proteins have been shown to recognize DNA sequences
of diad symmetry and as homodimers (reviewed in
Schjerling and Holmberg, 1996). Exceptions to this rule
are the A. nidulans NirA and AlcR proteins and the
Saccharomyces cerevisiae CYP1p (HAP1p) activator. In
A. nidulans, AlcR binds as a monomer to multiple
repeated sites in the ethanol regulon (Cerdan et al., 1997;
Panozzo et al., 1997). CYP1p was shown to recognize
tandemly repeated half sites in a highly asymmetric
manner as a homodimer (King et al., 1999a; b). In CYP1p,
asymmetric dimerization and a head-to-tail orientation of
the two monomers align the protein to the half sites of
different polarities. It is not known if the mode of AlcR or
CYP1p binding determined in vitro is also mirrored in vivo.
NirA is the only known protein of this family of transcription factors which recognizes a non-repeated, fully asymmetric sequence as a dimer (Strauss et al., 1998). In vivo footprinting of both strands of the crucial NirA site 2 in the nirA-niaD IGR has provided evidence that the base contacts determined in vitro are also protected from methylation in vivo. Additional features such as hypersensitive adenosines outside the consensus sequence (A10) are also revealed in the in vitro footprint. From the in vitro work, it was proposed that NirA contacts bases within the recognition sequence in such a way that amino acid side chains in the binding domain of each monomer are able to make specific base and phosphate contacts to the entire separate half sites. To our knowledge, NirA is the only case showing such a binding mode, not only among Zn-cluster proteins but also among all DNA binding proteins.

In our in vitro binding assays, we could not detect any effect of nitrate on the affinity of the truncated NirA protein carrying about one third of the native form (J. Strauss and C. Scaccocchio, unpublished). In contrast to this finding, we now observe inducer-dependent DNA binding in vivo. NirA is the first member of the binuclear Zn-cluster family to be shown to bind in vivo only in response to the induction signal. So far, all regulators of this family are known to interact without inducer with their cognate sites. In S. cerevisiae GAL4p is bound to UASGAL in the presence of non-inducing glycerol as well as inducing galactose (Ginger et al., 1985; Lohr and Hopper, 1985; Selleck and Majors, 1987). PUT3p, the transcriptional activator of the proline utilization pathway (Axelrod et al., 1991) and LEU3p, a transcriptional activator that regulates leucine biosynthesis (Kirkpatrick and Schimmel, 1995) are both bound constitutively to their targets in vivo. To perform their activation function, however, these factors need the presence of an inducing compound, which can be either an extracellular inducer or intracellular metabolites. Recent data indicate that at variance with PUT3 also the PmA activator, regulating proline utilization in A. nidulans, needs the presence of proline to bind to its cognate sites in vivo (Gomez et al., 2002). Inducer-dependent DNA binding mechanisms are only known for other DNA binding protein families such as the hormone receptor family of transcription factors (reviewed in Dittmar and Pratt, 1997).

Regulatory processes, traditionally assayed after several hours of induction and/or repression in A. nidulans and other fungal experimental systems, were shown here to result in transcriptional responses within a few minutes. This suggests that the specific molecular machinery for the nitrate induction process must pre-exist and only needs to be activated. areA transcripts exist under all physiological conditions (Platt et al., 1996a; b) and AreA is bound permanently but with different affinity to GATA site 5 in the nirA-niaD IGR under the different physiological conditions. The nirA gene has been shown to be constitutively transcribed at a low level (Burger et al., 1991a; b) and, in this work, we provided evidence that occupancy of NirA site 2 in the IGR is independent of de novo NirA synthesis, but is strictly dependent on a functional AreA. NirA is never bound in an areA' strain.

This situation conflicts with our observation that under induced/repressed conditions, in which AreA is non-functional, NirA is able to bind for a certain time (at least 20 min). Repression-mediated nuclear export of AreA and/or NirA would be a possibility to explain this time-delayed response. In the yeast S. cerevisiae Gln3p, a GATA factor similar to AreA involved in the activation of nitrogen catabolite repressible genes is retained in the cytoplasm under repressing conditions (Beck and Hall, 1999). In Aspergillus, this mechanism is not very likely to play an important role in AreA regulation, as we have shown in a previous report (Muro-Pastor et al., 1999) that the physiologically essential GATA site 5 in the nirA-niaD IGR remains at least partially protected even under fully repressed conditions. However, future experiments using tagged versions of these two transcription factors will show if the nitrogen source in fact stimulates nuclear translocation of AreA and NirA. Additionally, physical interaction between NirA and AreA might be another prerequisite for NirA binding. As a matter of fact, direct protein–protein interactions have been shown for the NirA and AreA homologues of Neurospora crassa, Nit4 and Nit2 respectively. A Nit2 mutant, which possesses wild-type DNA binding activity in vitro but is greatly impaired in its interaction with Nit4, showed significantly reduced expression of the target gene, nitrate reductase (Feng and Marzluf, 1998). Other members of the GATA-factor family such as hGATA1 and hGATA2 also require cooperative interactions with specific transcription factors for transcriptional activation (Kawana et al., 1995; Gregory et al., 1996).

However, on the transcriptional level the repression mechanism inactivating AreA seems to be effective immediately after the addition of ammonia as under induced/repressed conditions no nirA or niaD transcripts are accumulating. The inactivation of AreA or its homologue Nit2 of N. crassa involves the interaction with a negatively acting protein, NMR (Xiao et al., 1995; Andrianopoulos et al., 1998) and hence it is likely that NMR prevents the transcriptional activation function of AreA but not its function necessary for NirA binding.

**Experimental procedures**

**Strains, plasmids and genetic techniques**

Aspergillus nidulans strains used throughout this work are listed in Table 1. Escherichia coli strain JM109 [F’ traD36
lac\(^{\Delta(lacZ)M15}\) pro\(^{A'B'/e14^-}\) (McrA\(^{-}\)) & \(\Delta(lac-proAB)\) thigyrA96 (Nal\(^{R}\)) endA1 hsdR217 (K\(^{m+}\)) relA1 supE44 recA1

This was used for routine plasmid propagation.

Plasmid pNTC3 expressing the *A. nidulans* crnA nitrate transporter gene under the *A. nidulans* gpd promoter was constructed as follows: the argB gene of pMS12 (obtained from Fungal Genetics Stock Center) was isolated as BamHI fragment, the restriction site was filled in and cloned into the unique XbaI site (filled in) of pAN52-1 (Punt et al., 1987) resulting in plasmid pNTC1. The crnA gene was amplified with polymerase chain reaction (PCR) using primer crnA-for (5'-GGTGGAAGAGATAGATCTCGCCAAGCTGCTGG-3') and crnA-rev (5'-GGAAAATAAGATCTAACTCAGATGCCGCG-3'), which overlap the translational start codon and the stop codon of crnA (Unkles et al., 1991). Both primers contain a BglII site not present in the original crnA sequence. The resulting PCR fragment was cloned as BglII fragment into the BamHI site of pNTC1 resulting in plasmid pNTC3.

The gpd-crnA construct was tested for functionality by transformation into a cmr1/argB2 strain and found to complement the cmr1 mutation for hypersensitivity to 75 mM caesium chloride in the presence of nitrate. The transformants of the areA600/argB2 recipient strain were analysed using Southern hybridization, and the strain used in this study showed integration of the plasmid into the argB locus in a single copy. Sexual crosses to obtain the recipient strain for transformation were carried out according to standard procedures. Transformation protocols followed the procedures published by Tilburn and colleagues (Tilburn et al., 1983).

**Culture conditions**

Strains were grown for 12 h at 30°C in minimal medium (Pontecorvo et al., 1953) with appropriate supplements plus 5 mM urea and 1.25 mM ammonium D-(+)-tartrate as nitrogen source. This medium allows the growth of all strains, including areA600. The mycelia were then harvested by filtration, washed with sterile water, and transferred to the same medium without any nitrogen source. Incubation was continued for 20 min in this medium. To this end, the cultures were divided into aliquots of 20 ml when the following nitrogen sources were added: 5 mM urea (non-induced conditions, NI); 10 mM NaNO\(_3\) (induced conditions, I); 10 mM NaNO\(_3\) and 5 mM ammonium D-(+)-tartrate (simultaneous induced-repressed conditions, I/R); or 10 mM NaNO\(_3\) as sole nitrogen source for 20 min following by the addition of 5 mM ammonium D-(+)-tartrate for the remaining incubation time (sequential induced-repressed conditions, I20 + R). The aliquots were incubated for different times (10, 20, 45 and 90 min); the mycelium was harvested by filtration and frozen in liquid nitrogen for further processing.

**Northern blots**

Strains were grown under conditions described above. Total RNA isolation and Northern hybridization was carried out as described in Strauss et al., 1999). The probe for detecting the niaD transcript was obtained by PCR amplification with primers niaD-F and niaD-R (Muro-Pastor et al., 1999) from chromosomal DNA. The cmrA transcript was detected with a PCR-generated probe using primers cmrA-F (5'-GGTGGAA GAGATAGATCGCCAAGCTGCTGG-3') and cmrA-R (5'- GGAAAATAAGATCTAACAGATGCCGCCG-3'). The actin probe was derived from plasmid pSF5 (Fidel et al., 1988). Probes were labelled by random priming. Relative intensities of signals were calculated with the IMAGEQUANT software (Molecular Dynamics) from densitometric analysis of autoradiographs. Normalized niaD signal intensity was obtained by dividing the intensity of the niaD signal by the intensity of the actin signal.

**Nitrate measurement and reporter enzyme assay**

For reporter enzyme assays and nitrate measurement, mycelia were harvested and extensively washed with deionized water after incubation with the different nitrogen sources for exactly the same time as indicated for Northern and in vivo footprinting analysis. Filtered mycelia were immediately frozen in liquid nitrogen. The GUS and β-GAL assays were carried out as described by Punt and colleagues (Punt et al., 1995).

For nitrate measurement, mycelia were ground under liquid nitrogen and resuspended in 1 ml of 70% ethanol. Cell debris was removed by centrifugation at 13 000 g for 15 min. The supernatant was transferred into a new tube and ethanol was removed by drying the samples in a speed-vac desiccator. The remaining material was dissolved in 1 ml of distilled water and 100 μl was used for nitrate assay. Nitrate concentrations in samples were measured with the colorimetric nitrate/nitrite assay from Roche according to the manufacturer’s instructions, except that the assay was carried out in microtitre plates and the volume of reagents was downscaled to one fifth of the original amounts. All measurements including the determination of mycelial dry weight were carried out in three independent repetitions.

**In vivo footprint studies**

For *in vivo* footprint studies, all strains were grown under

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**Table 1. Aspergillus nidulans strains.**

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type:</td>
<td>pabaA</td>
<td>Pontecorvo et al. (1953)</td>
</tr>
<tr>
<td>nirA 637</td>
<td>pabaA, nirA637</td>
<td>Muro-Pastor et al. (1999)</td>
</tr>
<tr>
<td>nirA 87</td>
<td>biA, FvA, nirA87</td>
<td>Tollervey and Arst (1981)</td>
</tr>
<tr>
<td>areA600</td>
<td>pantoBl100, biA, areA600</td>
<td>Al Taho et al. (1984)</td>
</tr>
<tr>
<td>areA600/argB</td>
<td>pabaA1, argB2, areA600</td>
<td>This work</td>
</tr>
<tr>
<td>gpd-crnA/areA600</td>
<td>pabaA1, [pNTC3], areA600</td>
<td>This work</td>
</tr>
<tr>
<td>cmrA1/argB2</td>
<td>biA, cmrA1, argB2</td>
<td>This work</td>
</tr>
</tbody>
</table>

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exactly the same conditions as for the Northern Blot analysis. Methylation conditions, DNA extraction and detection of in vivo protein–DNA interactions were carried out as described by Wolschek and colleagues (Wolschek et al., 1998). Briefly, 2 min before harvesting the mycelia, dimethyl-sulphate was added to the cultures and further incubated. Mycelia were harvested by filtration and methylated DNA was isolated. Following cleavage of methylated guanines by piperidine, DNA–protein interactions were visualized by radioactive ligation-mediated PCR (LM-PCR).

The primers detecting NirA–site 2 interaction on the niaD-coding strand were: NIR II/1, 5’-tgctagcgcggcgtcagataagcatgatgttggc-3’, NIR II/2, 5’-gagccggcgtcagataagcatgatgttggc-3’; and NIR II/3, 5’-ccggcgataagcatgatgttggcgcgtc-3’.

The primers detecting NirA–site 2 interaction on the niiA-coding strand were: NII R II/1, 5’-ttgctagcgcggcgtcagataagcatgatgttggc-3’; NII R II/2, 5’-gagccggcgtcagataagcatgatgttggc-3’; and NIR II/3, 5’-ccggcgataagcatgatgttggcgcgtc-3’.

Relative intensities of signals were calculated with the IMAGEQUANT software (Molecular Dynamics) from densitometric analysis of autoradiographs. The percentage of signal intensity within the binding site was obtained by dividing the mean intensity of bands within the binding site by the mean intensity of four bands located outside the binding site. The ratio obtained for the bands inside and outside the binding sequence in the ‘vitro’ lane was set to 100% and the ratios obtained for the other lanes are relative to the ‘vitro’ lane.

**Chromatin structure analysis**

Strains were grown under exactly the same conditions as described for Northern Blot analysis and in vivo footprinting. For the analysis of the chromatin structure in the niiA-niaD intergenic region, DNase I and MNase sensitivity was tested using the method described by Gonzalez and Scanzocchio (1997). Culture conditions, transfer of mycelium to different media and further incubation under different nitrogen regimes were as described for Northern and in vivo footprinting analysis. Details on incubation times are given in the legends to the figures. The HindIII–EcoRV fragment of the niiA-niaD intergenic region (IGR) was used for indirect end labelling and was isolated from plasmid pAN302 (Strauss, 1993).

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**References**


