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Genetic Regulation of Nitrogen Metabolism in the Fungi

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INTRODUCTION

Nitrogen is a major component of nearly all of the complex macromolecules central to the structure and function of all living organisms. Accordingly, most prokaryotic and eukaryotic organisms have elaborate control mechanisms to provide a constant supply of nitrogen. The fungi can use a surprisingly diverse array of compounds as nitrogen sources and are capable of expressing upon demand the catabolic enzymes of many different pathways. Extensive studies of nitrogen metabolism and its regulation have been conducted with *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *Neurospora crassa*. This review will focus primarily on the regulatory mechanisms which govern nitrogen metabolism in these model experimental organisms. Recent, impressive progress that has been achieved by studying nitrogen metabolism in other fungal species, including both plant and animal pathogens, will be described. A number of authoritative reviews of earlier aspects of nitrogen metabolism and closely related topics are available (4, 20, 23, 60, 74–76); thus, this review will emphasize the recent breakthroughs in our understanding which have emerged from the powerful combination of genetic, biochemical, and molecular biological studies.

Certain nitrogenous compounds—ammonia, glutamine, and glutamate—are preferentially used by these fungi, and in yeast, asparagine is also a preferred nitrogen source. However, when these primary nitrogen sources are not available or are present in concentrations low enough to limit growth, many different nitrogen sources can be used, e.g., nitrate, nitrite, purines, amides, most amino acids, and proteins. Utilization of any of the various secondary nitrogen sources is highly regulated and nearly always requires the synthesis of a set of pathway-specific catabolic enzymes and permeases which are otherwise subject to nitrogen catabolite repression. Nitrogen control actually involves activation of the structural genes, which is prevented in the presence of preferred nitrogen sources. The de novo synthesis of the permeases and catabolic enzymes of a particular catabolic pathway is controlled at the level of transcription and often requires two distinct positive signals: first, a global signal indicating nitrogen derepression, and second, a pathway-specific signal which indicates the presence of a substrate or an intermediate of that pathway. This two-step requirement permits the selective expression of just the enzymes of a specific catabolic pathway from many potential candidates within the nitrogen regulatory circuit. However, some systems are controlled only by nitrogen metabolite repression and do not involve induction. Positive-acting global regulatory genes, i.e., *areA* in *Aspergillus* (11, 64), *nit-2* in *Neurospora* (46, 108), *gln-3* in *Saccharomyces* (80), and *nre* in *Penicillium* (54), specify GATA-type zinc finger transcription factors which activate nitrogen structural genes when preferred nitrogen sources are lacking; i.e., these factors mediate nitrogen catabolite derepression. Regulatory proteins encoded by the pathway-specific control genes each mediate induction only of the enzymes for the specific pathway.

NITRATE ASSIMILATION

Inorganic nitrate serves as an excellent nitrogen source for *Aspergillus*, *Neurospora*, and many other fungal species, but in nearly all cases it will not be utilized unless the cells lack a favored nitrogen source, such as ammonia, glutamine, or glutamate (22, 23, 47, 61, 91). Utilization of nitrate requires the de novo synthesis of nitrate reductase and nitrite reductase, which requires both nitrogen derepression and specific induction by nitrate. Nitrate reductase, a large homodimeric multiredox protein, catalyzes the conversion of nitrate to nitrite. Nitrate

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TABLE 1. *A. nidulans* and *N. crassa* genes which function in nitrate assimilation

Function	Genetic locus		Growth of mutant on[a]:		
	Neurospora	*Aspergillus*	Nitrate	Nitrite	Xanthine
Encodes nitrate reductase	*nit-3*	*niaD*	−	+	+
Encodes nitrite reductase	*nit-6*	*niiA*	−	−	+
Genes which specify a molybdenum cofactor (or its assembly)	*nit-1*	*cnxABC*	−	+	−
	nit-7	*cnxE*	−	+	−
	nit-8	*cnxF*	−	+	−
	nit-9	*cnxG*	−	+	−
		cnxH	−	+	−
Pathway-specific control gene (mediates induction)	*nit-4*	*nirA*	−	−	+
Globally acting nitrogen regulatory gene (mediates nitrogen repression)	*nit-2*	*areA*	−	−	−

[a] Wild-type strains and the *N. crassa nmr* mutant grow on all of these nitrogen sources. Wild-type strains and all mutants grow on medium containing ammonium salts or glutamine.

reductase contains three separate domains which are separated by short hinge regions (12). Electrons derived from NADPH are transferred stepwise to a carboxy-terminal flavin domain which contains FAD, then to a central heme-containing domain, and finally to an amino-terminal molybdopterin-containing domain, where the actual reduction of nitrate to nitrite takes place (12). Site-directed mutagenesis of the cloned *N. crassa nit-3* gene, specifying nitrate reductase, has been used to identify amino acid residues which are critical for catalytic function or stability. Substitution in the central domain of alanine for histidine residues believed important in chelating the heme cofactor resulted in a stable but catalytically inactive protein (83). Similarly, residues in the flavin domain which appear to function in binding the flavin adenine dinucleotide or pyridine nucleotide cofactors have been examined (52).

Mutants which lack nitrate reductase can be readily isolated in many organisms because, unlike the wild type, they are resistant to chlorate. Thus, a simple two-way selection system permits the isolation of mutants which lack nitrate reductase (chlorate resistant) and of revertants and suppressor mutants which restore nitrate reductase (use of nitrate). This feature can be exploited to obtain mutants of fungi which possess the nitrate assimilatory pathway, and it is particularly valuable in developing transformation systems for various filamentous fungi (118).

Mutations at many loci can result in chlorate resistance and loss of nitrate reductase (23). These loci include the structural gene which encodes the nitrate reductase polypeptide (*A. nidulans niaD* and *N. crassa nit-3*) and multiple genes which are required for synthesis and assembly of Mo-Co, the molybdopterin cofactor, which is also a component of xanthine dehydrogenase (Table 1). Mutants with mutations in the cofactor genes, i.e., the *cnx* mutants of *Aspergillus* and the *nit-1*, *nit-7*, *nit-8*, and *nit-9* mutants of *Neurospora* can be readily distinguished because they lack both nitrate reductase and xanthine dehydrogenase activity and thus fail to grow with either nitrate or xanthine as nitrogen sources. In addition, mutants with mutations in the globally acting and pathway-specific regulatory genes, *areA* and *nirA* of *Aspergillus* and *nit-2* and *nit-4* of *Neurospora*, respectively, lack both nitrate and nitrite reductase and can readily be distinguished by testing for growth with several nitrogen sources (Table 1).

In *A. nidulans*, *niaD* and *niiA*, the structural genes which encode nitrate reductase and nitrite reductase, respectively, are closely linked but transcribed divergently from a common intergenic control region (61). A third gene, *crnA*, which encodes a nitrate transporter, is also located in this cluster and is coregulated with *niaD* and *niiA* (119). In *N. crassa*, the structural genes for the two reductases are unlinked, although they are also regulated in a parallel fashion (38, 47). Accumulation of mRNA for these enzymes in both *Aspergillus* and *Neurospora* requires both nitrogen limitation and nitrate induction, suggesting control at the transcriptional level (38, 47, 61). In *N. crassa*, upon induction and derepression, the synthesis of nitrate reductase mRNA occurs very rapidly and reaches a steady-state level within 15 min; this mRNA also turns over rapidly with a half-life of approximately 5 min, and the nitrate reductase enzyme itself is subject to turnover (74, 82, 103). These features allow for a quick response to changing environmental nitrogen sources. A fascinating aspect of nitrate reductase is the well-documented feature that in addition to its catalytic function, it appears to play a regulatory role in the process of nitrate induction, controlling at the transcriptional level its own expression and that of nitrite reductase (22, 23, 48, 76).

PROMOTER ANALYSIS OF NITRATE ASSIMILATORY GENES

The upstream promoter region of the *N. crassa nit-3* gene, which encodes nitrate reductase, is organized in an intriguing fashion (Fig. 1). A strong binding site for the globally acting NIT2 protein occurs at −180, and two additional NIT2 binding sites are located more than 1 kb upstream. Two binding sites for NIT4, the pathway-specific transcription factor, also occur approximately 1 kb upstream, immediately downstream of the distal NIT2 sites (45). In addition, long AT-rich segments occur at two potentially significant locations. One distal site is located just downstream (3′) of the distal cluster of NIT2 and NIT4 sites; the other, more proximal AT-rich segment is situated immediately 5′ of the major transcription start site. The role, if any, of the distal AT-rich segment remains to be demonstrated. When transformed into a *nit-3* mutant, constructs with a precise deletion of the distal AT-rich segment were properly regulated and expressed at the same level as a completely wild-type gene (113). In contrast, deletion of the proximal AT-rich region led to an 80% decline in *nit-3* transcription. This AT-rich region, just upstream of the sites for initiation of transcription, may allow RNA polymerase II to more readily melt open the duplex DNA.

The functional role, if any, of the various NIT2 and NIT4 sites upstream of the *nit-3* gene has been examined by deletion analysis and by single-base substitutions which eliminated the binding of the respective factors but otherwise maintained the normal sequence and spacing throughout the entire promoter (16). A 5′ deletion which removed the upstream cluster of

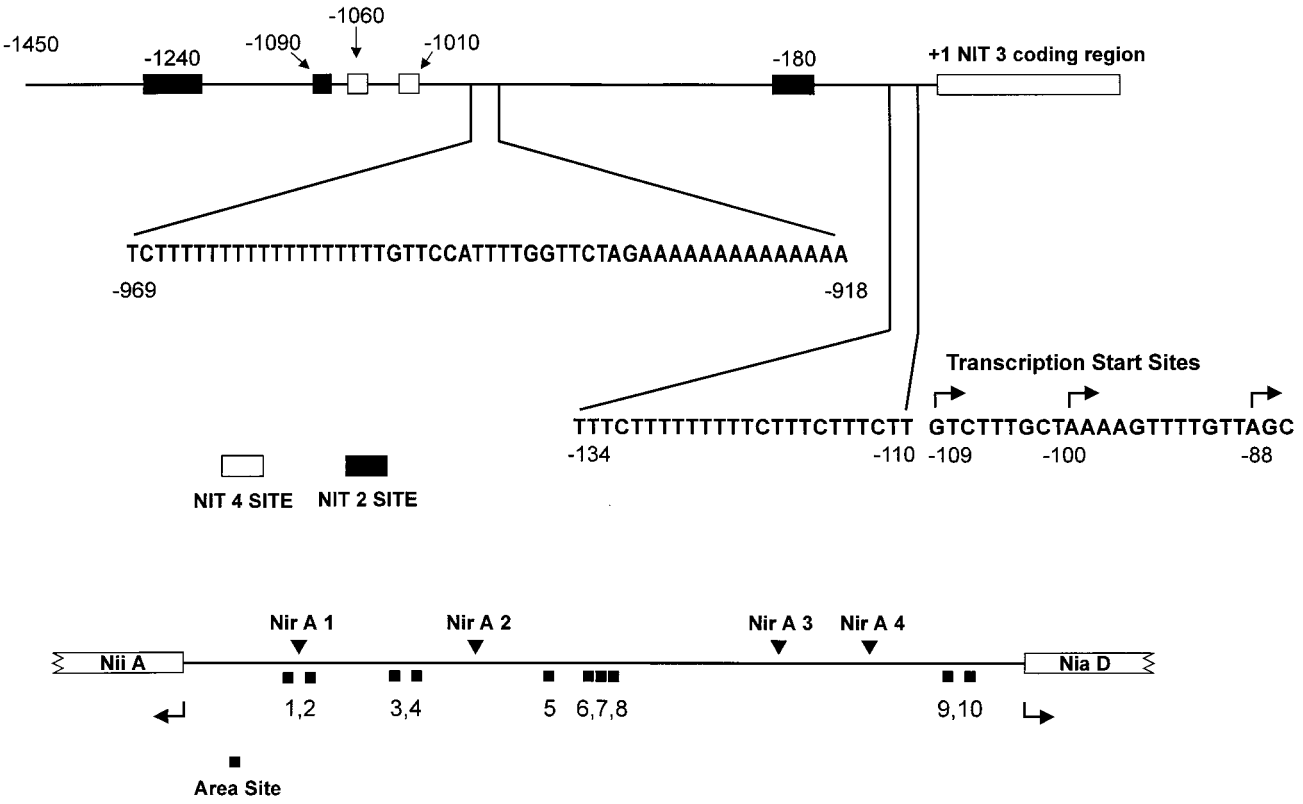

FIG. 1. Promoter of nitrate assimilatory genes of *N. crassa* and *A. nidulans*. The promoter of the *N. crassa nit-3* gene (encodes nitrate reductase) showing NIT2 and NIT4 binding sites, AT-rich regions, and transcription start sites is shown at the top. Locations are given relative to the initiation codon (+1) for protein synthesis. The 1,200-bp intergenic control region between the divergently transcribed *niiA* (encodes nitrite reductase) and *niaD* (encodes nitrate reductase) genes of *A. nidulans* is shown at the bottom. The four binding sites for NIRA, the pathway-specific factor, and 10 GATA elements, potential sites for the globally acting AREA protein, are shown.

NIT2 and NIT4 sites resulted in a completely inactive *nit-3* gene, clearly demonstrating that the distal cluster of sites was essential for any *nit-3* gene expression. An internal deletion from −969 to −200, which thus removed the entire upstream region between the distal sites and the proximal NIT2 site, resulted in a *nit-3* construct which was regulated and expressed at the wild-type level, implying that no significant positive or negative elements were located in this stretch of nearly 800 bp. Mutation of any of the three NIT2 binding sites reduced the expression of the *nit-3* gene, showing that they all played a regulatory role; however, site II, which contains a single GATA element, was most important, and its loss alone led to a completely inactive gene (16). Similarly, both NIT4 binding sites function in *nit-3* gene expression, since mutational loss of either resulted in significantly reduced enzyme levels. The clustering of sites suggested that a close proximity of NIT2 and NIT4 elements might be important for positive regulation of *nit-3*. This possibility was tested and eliminated by increasing the distance between the NIT2 and NIT4 sites by introducing neutral sequences between them. When the spacing between the NIT2 and NIT4 sites was increased by various distances up to 200 bp, the *nit-3* gene was expressed at least as well as a wild-type control (114). Thus, although the expression of *nit-3* absolutely requires the positive action of both NIT2 and NIT4 proteins, their respective binding sites need not be tightly clustered.

A sophisticated study of the regulation of the nitrate assimilatory genes in *A. nidulans* employed deletions and mutations of potential control sites in the intergenic control region with β-galactosidase and β-glucuronidase serving as reporters for *niaD* (nitrate reductase) and *niiA* (nitrite reductase), respectively (93). The *niiA-niaD* intergenic control region contains four binding sites for NIRA, the pathway-specific factor that mediates nitrate induction, and 10 GATA elements which were identified by in vitro studies as binding sites for AREA, the globally acting nitrogen control factor (Fig. 1). A NIRA-GST fusion protein binds in vitro to each of the four NIRA sites, whose consensus was demonstrated to be the nonpalindromic sequence CTCCGHGG (H = A, C, or T) (93). All four NIRA binding sites serve a regulatory function for nitrate induction, with sites A2, A3, and to a lesser extent A4 acting bidirectionally, i.e., for both *niiA* and *niaD* induction, whereas site A1 controls only *niiA*. The simultaneous loss of all four NIRA sites results in complete noninducibility of the bidirectional promoter. Somewhat surprisingly, of the 10 AREA binding sites identified by in vitro binding studies, only 4 centrally located AREA sites (sites 5, 6, 7, and 8) appear to be physiologically important for nitrogen repression/derepression in vivo; e.g., loss of AREA sites 1 and 2, near the transcriptional start site of *niiA*, and of sites 9 and 10, near the *niaD* gene, did not show a demonstrable effect on either gene. These results underscore the fact that a binding site recognized by its sequence or even identified by in vitro DNA binding studies may not play a discernible role in controlling gene expression.

As with *A. nidulans*, a number of other fungal species can utilize nitrate and possess linked structural genes for nitrate reductase and nitrite reductase which are divergently transcribed and regulated by nitrate induction and nitrogen repres-

sion. These include *Aspergillus niger*, *Aspergillus oryzae*, and *Penicillium chrysogenum*. The nitrate reductase genes of *Fusarium oxysporum* and *Leptosphaeria maculans* have also been isolated and sequenced (93). In each case, at least one, and usually three or four, putative NIRA-type binding sites are evident in the promoter region. This result, coupled with the finding that the *A. nidulans niaD* gene is normally controlled when introduced into several of these other species, reinforces the concept that these fungi share a very similar system for the regulation of nitrate assimilation (93).

CONTROL OF PURINE METABOLISM

Scazzocchio and his colleagues have used elegant genetic and molecular approaches to examine the genetic control of purine metabolism in *A. nidulans* (35, 110, 111), which is regulated in a similar fashion in *N. crassa* (67, 68, 81, 95). The use of purines requires the de novo synthesis of a set of enzymes that is produced only upon nitrogen derepression, mediated by the globally acting AREA protein, and upon simultaneous induction with uric acid, mediated by *uaY*, a pathway-specific regulatory gene. The *uaY* gene encodes a positive-acting regulatory protein that possesses a *S. cerevisiae* GAL4-like Zn_2/Cys_6 DNA binding domain (see Fig. 3). The UAY protein is required for the expression of at least nine unlinked genes which specify permeases and enzymes that function in the transport and metabolism of purines. The UAY polypeptide has an estimated mass of 118,394 Da and binds as a homodimer at promoter elements with a TCGG-N_6-CCGA sequence (35, 109, 110). This element has been demonstrated to be required for the in vivo expression of *uap*, which encodes a specific urate-xanthine permease (109, 110).

The *uaY* regulatory gene is expressed constitutively and is not itself subject to nitrogen derepression or uric acid induction; moreover, *uaY* expression does not require a functional AREA protein, nor is it autogenously controlled (35). Therefore, a model of sequential gene action, in which AREA controls *uaY*, seems unlikely. Rather, it appears certain that the active forms of both the UAY and AREA proteins must each bind to specific elements in the promoters of the purine-catabolic genes to cause transcriptional activation.

ALLANTOIN CATABOLISM IN YEAST

A complex pattern of genetic regulation governing allantoin catabolism occurs in *S. cerevisiae*; Cooper, who has pioneered much of the work in this area, has provided an authoritative review of this topic (20). Yeast can use exogenous allantoin as a nitrogen source and also accumulates significant levels of allantoin in vacuoles as a reserve which is utilized during "hard times," i.e., when extracellular nitrogen sources are absent. Eight structural genes encoding permeases and catabolic enzymes all have been isolated and sequenced, and their pattern of expression has been characterized. The structural and regulatory genes involved in the allantoin catabolic pathway are located on seven different yeast chromosomes, although a cluster of five structural genes lies on chromosome IX (20). Some of the structural genes of the pathway require induction, whereas others do not; however, all are sensitive to nitrogen catabolite repression. Three types of *cis*-acting elements are found in the 5′ promoter region of DAL7 and other inducible genes: UAS_{NTR} (upstream activating nitrogen control sequence), URS (upstream repressing sequence), and UIS (upstream induction sequence). The UIS element is a dodecanucleotide, 5′-GAAAATTGCGTT-3′ (121). An UIS element alone does not appear to act as a UAS but instead seems to increase the activation potential of an adjacent UAS_{NTR}.

Five genes, *GLN3*, *DAL81*, *DAL82*, *DAL80*, and *URE2* encode regulatory proteins that participate in control of the allantoin catabolic genes; some are pathway specific, whereas others are more global in action. *GLN3* encodes a global, positive-acting GATA binding protein that is required for the expression of all of the allantoin pathway genes and many other genes subject to nitrogen catabolite repression/derepression (80). Gln3p is presumed to recognize the UAS_{NTR} elements which contain a GATA core sequence. The N-terminal 140 amino acids of Gln3p possesses a net charge of −20, which could function as an acidic activation domain, although this has not yet been explored experimentally. The *DAL80* protein is a global, negative-acting factor which is also a member of the GATA-binding family of proteins; it binds at sites designated URS_{GATA}, which contain two GATA (URS) elements 15 to 20 bp apart and oriented either head to tail or tail to tail (27, 28). The *GLN3* protein is expressed constitutively; in contrast, *DAL80* expression is controlled by *GLN3* and nitrogen repression and is also autogenously negatively regulated (26).

The *DAL81* (also known as *UGA35*) and *DAL82* regulatory proteins are both required for induction of the structural genes served by an UIS element(s). The *DAL81*/*UGA35* protein has a helix-turn-helix motif and a GAL4-like Zn_2/Cys_6 DNA binding domain; surprisingly, however, deletion of the zinc finger does not interfere with Dal81p function in vivo. In *Aspergillus*, the *tamA* gene, which appears to be involved in some aspect of global nitrogen metabolite repression, encodes a protein which has two regions that show significant similarity to segments of the *S. cerevisiae DAL81*/*UGA35* protein (31). Moreover, the TAMA protein contains a sequence that would be predicted to form a Zn_2/Cys_6 binuclear DNA binding domain but which is not required for $tamA^+$ function (31). The yeast *DAL81*/*UGA35* protein does not appear to bind at the UIS elements but instead plays a more general role, since its action is not limited to allantoin-specific genes. The *DAL82* protein has fewer recognizable motifs but contains a number of predicted α-helices and a putative nuclear localization signal. Recently, it has been demonstrated that the *DAL82* protein expressed in *Escherichia coli* binds specifically in vitro to DNA fragments that contain a UIS (36), although the exact nature of the motif responsible for sequence-specific DNA binding by Dal82p remains to be established. These interesting results should serve as a caution not to rely too greatly upon sequence comparisons to indicate functions until concepts have been experimentally tested. A number of allantoin catabolic genes, e.g., *DAL7*, are controlled in a dual fashion by nitrogen repression and pathway-specific induction, and their promoters contain three types of *cis*-acting elements. A useful and intriguing model for the complex interactions which occur in these promoters has been proposed by Cooper (20). The *DAL7* promoter contains UAS_{NTR} sites, recognized by the positive-acting *GLN3* protein, and URS sites for the negative-acting DAL80 protein. These sites completely or partially overlap, so that Gln3p and Dal80p compete for binding; thus, during nitrogen derepression, the promoter is poised for action but still quiescent. When inducer is present, UIS, the third element, is occupied, presumably by Dal81p and Dal82p, and the balance between Gln3p (positive) and Dal80p (negative) is tipped in favor of Gln3p, leading to enhanced expression of *DAL7* and similarly controlled genes, e.g., *DAL1*, *DAL2*, and *DUR1.*

Mutation or deletion of the *URE2* gene results in a loss of nitrogen catabolite repression for some nitrogen catabolic genes, whereas other loci are still subject to nitrogen repression. This suggests that *URE2* functions in only one of two

branches of the nitrogen regulatory network. The *URE2* gene encodes a protein of 354 amino acids, but its molecular mechanism of action is still a mystery, although it has been suggested that it modifies Gln3p by attaching a glutathione residue to it (21). It appears that *URE2* is involved in regulation of the same subset of nitrogen catabolic genes which are controlled by *GLN3* and that Ure2p acts via some interaction with Gln3p (8). Further investigation of this predicted interaction remains an important goal. One particularly fascinating aspect of *URE2* is that a mutant form appears to be inherited in a non-Mendelian fashion and has the properties of a prion, an infectious protein that can convert the normal protein into the mutant form (77).

Some findings concerning nitrogen metabolism in yeast led to the inescapable conclusion that *GLN3* does not uniquely act as the global mediator of nitrogen derepression. Many, but clearly not all, structural genes controlled by nitrogen repression require Gln3p for expression. For example, *UGA1* (which encodes GABA transaminase) and *PUT2* (which encodes pyrroline-5-carboxylate dehydrogenase) both are sensitive to nitrogen catabolite repression, but their expression does not require *GLN3*. Moreover, at least some structural genes remain sensitive to nitrogen catabolite repression in a *gln3 ure2 dal80* mutant strain, deleted for all three genes previously identified to function in nitrogen repression/derepression (18). This result also demonstrates that Ure2p does not exclusively process the environmental signal, indicating the presence of a sufficient level of a good nitrogen source. It thus appears that the nitrogen repression circuit is partially redundant or, alternatively, contains at least two parallel control networks. This paradox has been at least partially resolved by the identification of a new gene, *GAT1* (also called *NIL1*), which encodes an additional GATA binding protein with significant homology to Gln3p, including the presence of an acidic region in the N terminus (18, 106). Stanbrough and Magasanik (105) have demonstrated that both Nil1p/Gat1p and Gln3p recognize the same GATAAG sites to activate the expression of the *GAP1* gene. Expression of *GAT1*/*NIL1* is sensitive to nitrogen repression, partially dependent upon *GLN3*, and controlled by *DAL80*. The *GAT1*/*NIL1* protein appears to be a positive activator that is required for full expression of numerous nitrogen-related genes; e.g., *DAL5*, *PUT1*, and *UGA4* all require both Gln3p and Gat1p/Nil1p for strong expression. The positive effects of *GLN3* or *GAT1*/*NIL1* appear to differ depending upon the presence of different nitrogen sources (106). Significantly, the expression of *UGA1* appeared to require Gat1p/Nil1p but not Gln3p (18). A major objective for future work is to gain insight into how the two GATA-binding activators, Gln3p and Gat1p/Nil1p (which appear to bind to similar elements), act synergistically in some cases but also can act individually to turn on certain genes. Interestingly, at present there is no definitive evidence for a similar dual system governing nitrogen-regulatory activities in *Aspergillus* or *Neurospora.*

UTILIZATION OF PROLINE AS A NITROGEN OR CARBON SOURCE

Proline can be utilized by *A. nidulans* as both a nitrogen and a carbon source; a cluster of five genes are involved in its metabolism (59, 100). A cluster of five genes, *prnA*, *prnX*, *prnD*, *prnB*, and *prnC*, are responsible for proline metabolism; *prnA* encodes a positive-acting regulatory protein that mediates proline induction of the structural genes (Fig. 2). The function of *prnX* is unknown. Although the genes are tightly linked in this *prn* cluster, each is expressed as a monocistronic mRNA (100). Expression of this set of proline-specific genes requires induction by proline and is also controlled by both nitrogen and carbon catabolite repression. When proline is present, both glucose and ammonia are required to repress the expression of these genes. A central control region lies between *prnD* and *prnB*; it is thought to contain an enhancer element that is active for the entire cluster as well as control elements for the PRNA protein and for CREA and AREA, the globally acting factors for carbon and nitrogen control, respectively. There is also a binding site for one additional positive-acting factor, whose identity is still unknown (101). The primary point of control appears to converge on *prnB*, which encodes the proline permease, and inducer exclusion is at least in part responsible for regulation of the other *prn* genes of the cluster.

The pathway-specific protein encoded by *prnA* is a DNA binding protein of 818 amino acid residues with a GAL4-like Cys_6/Zn_2 domain and a glutamine-rich putative activation domain in its C terminus. The PRNA protein is activated in an unknown manner by the inducer proline and binds at elements with the sequence CCGG-N_{16}-CCGG (direct repeats of CCGG separated by 16 bp whose sequence is unimportant).

Nitrogen catabolite derepression requires a functional AREA protein, and two AREA binding sites are present in the *prnD-prnB* intergenic control region. Carbon repression is exerted by the negative-acting CREA protein, which possesses two Cys_2/His_2-type zinc fingers which mediate sequence-specific DNA binding. An *areA* mutant cannot utilize proline if a specific region in the central control region is deleted. This region is believed to represent a binding site for an unknown positive-acting factor which turns on the expression of the *prn* cluster in the absence of carbon repression, the condition when CREA is inactive (96).

One special feature of *prnB*, which encodes the proline-specific permease, is that it is not only turned on by high proline levels under conditions where proline will be metabolized as a C or N source, but it is also turned on by proline starvation, which may involve a separate control mechanism. Control of the *prnB* gene and the entire *prn* cluster represents a well-defined example of complex regulation in which multiple signals converge to control expression. Derepression/repression is signaled by global positive (AREA)- and negative (CREA)-acting factors and the unknown positive factor mentioned above; activation of the *prn* genes additionally requires induction, mediated by the pathway-specific PRNA protein.

Proline serves as a nitrogen source but not a carbon source for *S. cerevisiae*, and its catabolism involves the same pathway and set of enzymes as found in *A. nidulans*. Expression of the yeast proline utilization genes, *PUT1* and *PUT2*, which encode proline oxidase and pyrroline 5-carboxylate dehydrogenase, respectively, requires proline induction. *PUT1* and *PUT2* expression also requires nitrogen derepression, which, however, does not involve the *GLN3* protein (32). These two *PUT* genes are negatively regulated by the Ure2p protein, which suggests that *GAT1*/*NIL1* might be affected by *URE2*. The *PUT3* gene encodes a positive regulator which has clear homology in its amino-terminal Cys_6/Zn_2 GAL4-like DNA binding domain to that of PRNA of *A. nidulans* (97). Put3p, which is composed of 979 amino acids exists as a homodimer and possesses two acidic segments located in positions analogous to those in Gal4p, suggesting that they activate transcription (73). In vivo footprinting studies demonstrate clearly that the *PUT3* protein binds to its DNA sites in both the presence and absence of the inducer, proline (6). Thus, Put3p binds to its target promoters and is poised to function even in the absence of the inducer, which implies that the presence of proline somehow converts Put3p into a form that activates *PUT* structural gene expression. *GAL4*/*PUT3* fusions were used to study the *trans*-activa-

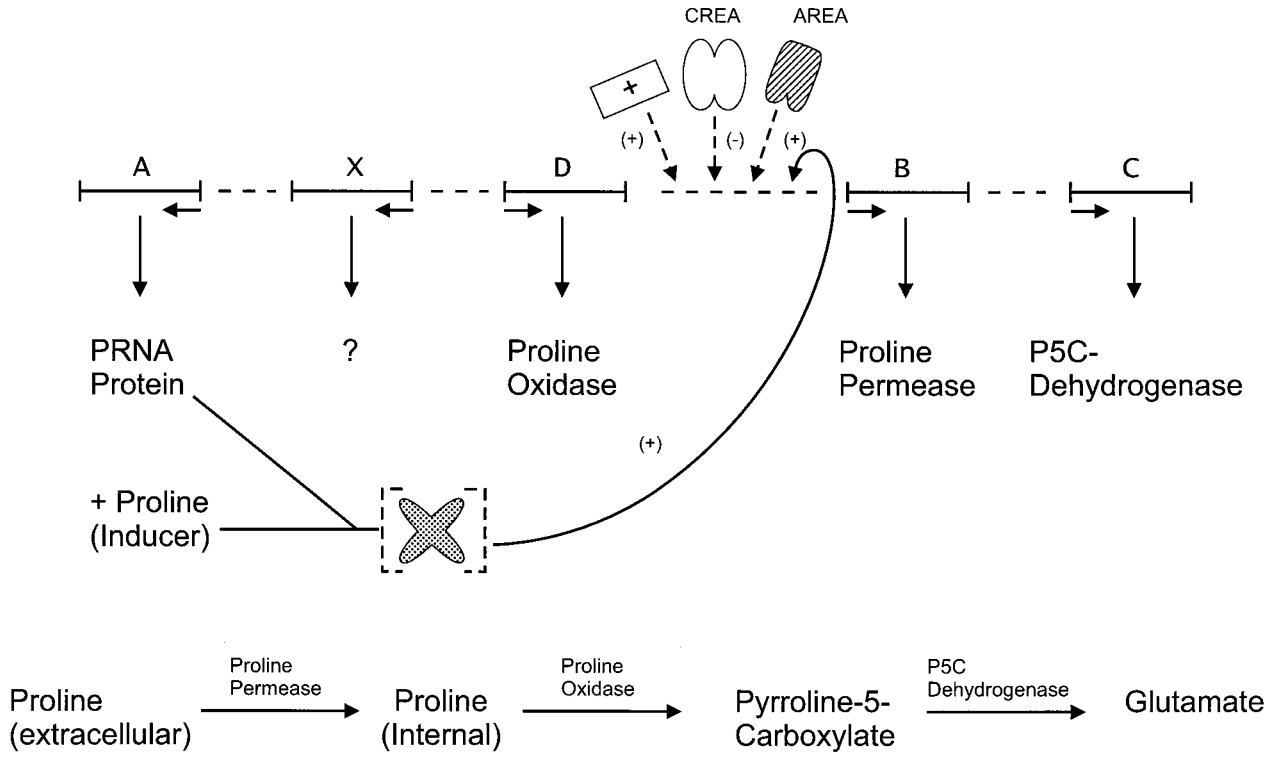

FIG. 2. The *prn* gene cluster and pathway for proline catabolism in *A. nidulans*. The *prnX* gene is controlled in parallel with the other *prn* genes, but its function is unknown. An extensive regulatory region is located between the *prnD* and *prnB* genes. *prnA* encodes the pathway-specific factor with a Cys_6/Zn_2 type of DNA binding motif which binds its cognate element in the regulatory region. This region also contains elements recognized by the positive-acting AREA protein and the negative-acting CREA proteins, which mediate N and C repression/derepression, respectively. An unidentified factor shown as (+) is postulated to activate *prn* gene expression when CREA is in an inactive form, signaling C derepression. Each of the *prn* genes is transcribed separately; the arrow beneath each gene defines its direction of transcription.

tion regions of Put3p. A hybrid protein containing Put3p residues 72 to 979 (lacking only the Put3p DNA binding domain and part of its dimerization domain) responded to the addition of proline (32). However, a hybrid protein containing only the carboxy-terminal 89 residues of Put3p strongly activated reporter expression but did not show any response to proline, which implies that the carboxy-terminal tail does not recognize the presence of proline. A central domain of the Put3p protein appears to serve as the activation region, and mutation of a conserved glycine residue in this central region results in a *PUT3* mutant protein which retains wild-type DNA binding activity but which is incapable of gene activation (32). A critical observation was the finding that when the carboxy-terminal tail of Put3p is deleted, the truncated protein activates *PUT* structural gene expression in the absence of proline (32). This outcome has led des Etages et al. (32) to suggest that the carboxy terminus of the native PUT3 protein binds to a central domain of the protein, masking the activation function of the latter. In the presence of proline, the Put3p protein is visualized to undergo an intramolecular reorganization which exposes the central acidic region, converting it into an active state to promote transcription. Although the *PUT3* protein may contain a proline binding site, it is unknown whether Put3p can bind proline (32). An alternative possibility, i.e., that a repressor protein analogous to Gal80p binds to Put3p and prevents transcription of *PUT1* and *PUT2* when proline is absent, seems unlikely because of the inability to obtain a *gal80*-like mutant in the proline system. Although Put3p does not itself appear to sense the nitrogen status of the cell, it may interact at the promoters of the *PUT* genes with one or more proteins, e.g., Gat1p/Nil1p, which can sense nitrogen.

REGULATION OF ACETAMIDASE EXPRESSION

A particularly well understood case of multiple control signals converging upon a single structural gene is provided by the extensive work of Hynes and his colleagues with *amdS*, which encodes acetamidase of *A. nidulans* (1, 60, 62, 71). Acetamide serves as a nitrogen and carbon source for *Aspergillus*. Expression of *amdS* is highly regulated and requires a derepression signal and at least one of several possible induction signals. Derepression occurs by limitation for either nitrogen or carbon, mediated by the globally acting AREA and CREA proteins, respectively (60). Induction by acetate of *amdS* and of several other genes encoding acetate utilization enzymes is mediated primarily by *facB*, which encodes a protein with a Zn_2/Cys_6 DNA binding domain at its amino-terminal end and several possible acidic activation domains (62). Expression of *facB* itself is subject to carbon catabolite repression and to acetate induction. Another protein encoded by the *amdA* gene, which contains two N-terminal Cys_2/His_2 zinc finger motifs, also mediates a minor degree of acetate induction of *amdS* (71). The *amdS* gene can also be induced by omega amino acids, e.g., Γ-aminobutyrate, via the AMDR protein, which also contains a Zn_2/Cys_6 DNA binding domain at its amino terminus (1).

The *amdS* promoter is complex and modular. It contains distinct binding elements for each of the AREA, CREA,

FACB, AMDR, and AMDA proteins and also contains a CCAAT sequence which is essential to achieve a proper basal level of expression (120). The CCAAT element serves as a binding site for a protein, designated ANCF, which has been detected in mobility shift experiments. Moreover, Hynes and Davis have predicted that *amdS* is controlled by at least one additional unknown factor which also binds at a recognition site in the promoter region (60). An understanding of the multiple possible DNA-protein and protein-protein interactions which occur at the *amdS* promoter and affect the basal transcription apparatus will yield considerable insight into the molecular mechanisms that allow a multiplicity of controls to govern the expression of a single gene.

REGULATION OF OTHER NITROGEN CATABOLIC PATHWAYS

The utilization of various other secondary nitrogen sources, such as individual amino acids, is also highly regulated by similar derepression and induction signals to those described above. The biosynthesis and catabolism of arginine in *S. cerevisiae* are controlled by the ARGRII protein in combination with ARGRI and ARGRIII (94). ARGRII, a protein of 880 amino acids, contains an N-terminal Cys_6/Zn_2 zinc cluster and acts as a bifunctional factor, controlling the expression of enzymes involved in both the synthesis and degradation of arginine (123). Amino acid transport, L-amino acid oxidase, and phenylalanine-ammonia lyase are examples of activities whose expression in *Neurospora* requires induction, nitrogen derepression, and a functional *nit-2* gene product (39, 98, 99, 126). Extracellular proteins can serve as the sole source of nitrogen, carbon, or sulfur for *N. crassa*. The expression of a single structural gene that encodes an extracellular alkaline protease is turned on by distinct signals that indicate a limitation for N, C, or S (55). The response to sulfur catabolite derepression is mediated by CYS3, a regulatory protein with a bZip DNA binding motif, which controls an entire set of sulfur catabolic genes (51). Synthesis and secretion of the alkaline protease not only require derepression but also are completely dependent upon the presence of an extracellular protein; a peptide derived from the external protein appears to provide an essential inductive signal (37). Similarly, *A. nidulans* expresses neutral and alkaline proteases when subjected to multiple derepression states, including nitrogen limitation (19). In *N. crassa*, an extracellular alkaline RNase is synthesized upon limitation for either N, C, or P and its expression requires a functional *nit-2* or *nuc-1* product to respond to nitrogen or phosphorus starvation, respectively (69). The promoters which govern the structural genes encoding these extracellular proteases and nucleases must contain multiple elements that allow a response to several independent derepression signals and to requisite inductive signals, as well as the ambient pH (3, 114).

GLOBALLY ACTING NITROGEN REGULATORY GENES

Major positive-acting regulatory genes, *areA* in *A. nidulans*, *nit-2* in *N. crassa*, and *GLN3* in *S. cerevisiae*, mediate global nitrogen repression and derepression (46, 64, 80, 108). It now is obvious that numerous other fungi possess a homologous factor for nitrogen control and other, related GATA-like factors (Table 2). The molecular cloning and characterization of *areA*, *nit-2*, and *GLN3* and the homologous genes of other fungi, such as *nre* of *Penicillium chrysogenum* (54) and the *nut1* gene of *Magnaporte grisea* (44), represented a dramatic breakthrough in our understanding of nitrogen regulation in the fungi. These regulatory proteins are all members of the GATA family of transcription factors, and all possess a remarkably similar DNA binding domain which consists of a single Cys_2/Cys_2-type zinc finger motif with a central loop of 17 amino acids and an immediately adjacent basic region (Fig. 3). These nitrogen regulatory factors all appear to bind to DNA elements which have the core sequence GATA (hence the term "GATA factors"). However, it is important to note that these organisms possess other GATA-binding factors which serve other regulatory functions; e.g., URBS1 acts in iron regulation in *Ustilago maydis* (122), and two GATA factors, WC1 and WC2, function together in blue-light signal transduction in *N. crassa* (7, 70). In *Dictyostelium*, the cell fate gene *stka*$^+$, which functions in the programming of spore cell determination, encodes a nuclear protein of the GATA family (14). One can readily predict that additional GATA factors, possibly with various regulatory activities, will be identified in the fungi; *S. cerevisiae* possesses at least four GATA factors, encoded by the *GLN3*, *DAL80*, *GAT1*/*NIL1*, and *DEH1* genes (18, 20, 106). PCR technology has very recently been used to isolate two new genes encoding GATA factors in *Penicillium* (53) and the two homologous genes in *Neurospora* (40). One of these new GATA factors possesses two zinc fingers, similar to URBS1 of *U. maydis*, but its cellular function is still unknown (40, 53). The second newly identified gene, found in both *Penicillium* and *Neurospora*, encodes a GATA factor with a single zinc finger which has similar features to the yeast DAL80 protein, suggesting a possible function in nitrogen control. If correct, it will demonstrate a greater commonality in nitrogen regulation between yeast and the filamentous fungi.

GATA proteins serve as transcription regulatory factors in widely different organisms, e.g., *Caenorhabditis elegans* (104), tobacco (29), and vertebrates, where multiple GATA factors serve various tissue-specific control functions (2, 85, 124). These GATA factors in the higher metazoan species have two zinc fingers; the carboxy-terminal finger, which is responsible for sequence-specific DNA binding (78, 127), shows a high degree of homology to the single zinc finger of AREA, NIT2, GLN3, and NRE (Fig. 3). The best-characterized member, GATA-1, regulates erythropoietic cell lineage development and function (85, 124). The amino-terminal zinc finger of the mammalian GATA-1 protein appears to promote dimerization as well as functional interactions with Sp1 and EKLF proteins

TABLE 2. Positive- and negative-acting fungal GATA factors

GATA factor	No. of zinc fingers	Regulatory function[a]	Organism
AFAREA	1	(+) Nitrogen	*Aspergillus fumigatus*
AREA	1	(+) Nitrogen	*Aspergillus nidulans*
AREA	1	(+) Nitrogen	*Gibberella fujikuroi*
AREA	1	(+) Nitrogen	*Metarhizium anisopliae*
DEH1	1	Unknown	*Saccharomyces cerevisiae*
GAF2	2	Unknown	*Schizosaccharomyces pombe*
DAL80	1	(−) Nitrogen	*Saccharomyces cerevisiae*
GLN3	1	(+) Nitrogen	*Saccharomyces cerevisiae*
GAT1/NIL1	1	(+) Nitrogen	*Saccharomyces cerevisiae*
NRE	1	(+) Nitrogen	*Penicillium chrysogenum*
NIT2	1	(+) Nitrogen	*Neurospora crassa*
NUT1	1	(+) Nitrogen	*Magnaporthe grisea*
SREP	2	Unknown	*Penicillium chrysogenum*
UNK1	1	Unknown	*Neurospora crassa*
URBS1	2	(−) Siderophore	*Ustilago maydis*
WC-1	1	(+) Light	*Neurospora crassa*
WC-2	1	(+) Light	*Neurospora crassa*

[a] (+), positive; (−), negative.

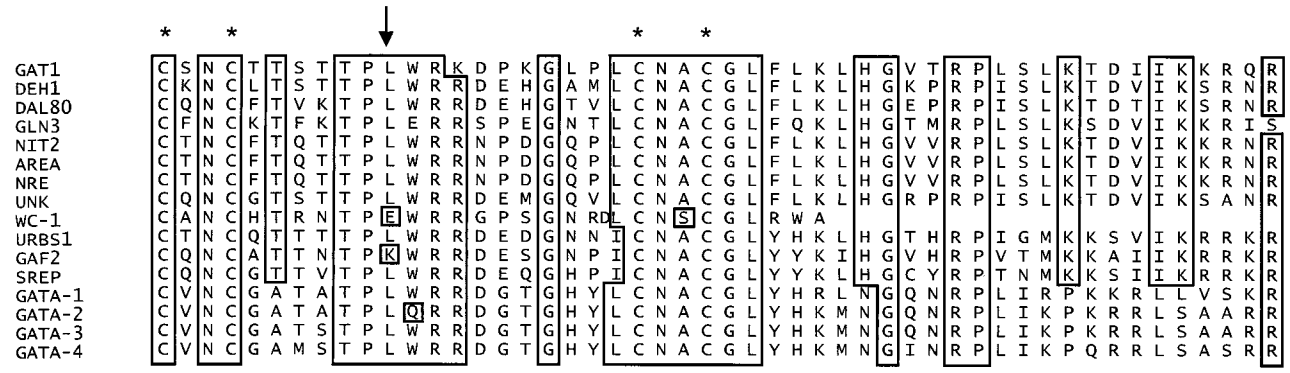

FIG. 3. DNA binding domain of various GATA factors. GAT1, DAL80, GLN3, and DEH1 are from *S. cerevisiae*; AREA is from *A. nidulans*; NIT2, WC-1, and UNK are from *N. crassa*; NRE and SREP are from *P. chrysogenum*; URBS1 is from *U. maydis*; GAF2 is from *Schizosaccharomyces pombe*; and GATA-1, GATA-2, GATA-3, and GATA-4 are vertebrate GATA factors. Only the carboxy-terminal zinc finger is shown for the vertebrate GATA factors and for SREP, URBS1, and GAF2, all of which have two similar fingers. The other fungal proteins have only a single zinc finger motif. Note that WC-1 is unusual, with 18 residues rather than the typical 17 amino acids in the central loop. Most of the fungal factors shown are involved in some aspect of nitrogen regulation except for WC-1 and WC-2 (light regulation) and URBS1 (iron regulation); the physiological function of SREP and of UNK is not yet established. The asterisks (*) indicate the cysteine residues involved in zinc ion chelation; the arrow identifies the leucine residue whose substitution in AREA and NIT2 led to altered promoter recognition (see the text).

(25, 79). Similarly, GATA-2 interacts via its DNA binding domain with the bZip proteins JUN and FOS (63).

The *A. nidulans* AREA, *P. chrysogenum* NRE, and *N. crassa* NIT2 proteins are composed of 876, 834, and 1,036 amino acids, respectively; AREA is 65 and 42% identical to NRE and NIT2, respectively. Amino acid identity is extremely high in the 50-residue sequence which constitutes the DNA binding domain (Fig. 3), and the *Neurospora nit-2* gene complements an *Aspergillus areA* mutation (30). Homology is also high in the amino terminus of AREA and NIT2, which is dispensable for the activation function but is required for nitrogen repression (65). Many segments of the AREA and NIT2 proteins are dispensable for function in gene activation. An internal region of AREA protein composed of only 223 amino acids is sufficient to turn on many but not all of the target genes (64). This truncated AREA protein consists mainly of the DNA binding protein plus an amphipathic acidic region, which may serve as an activation domain (64). Similarly, a truncated NIT2 protein which consists primarily of the DNA binding motif and either of its two acidic regions functions in gene activation (86).

DNA BINDING BY GATA FACTORS

The central region of the *N. crassa* NIT2 protein, which includes the zinc finger DNA binding domain, was expressed in *E. coli* as a LacZ/NIT2 fusion protein (49). This fusion protein displayed sequence-specific DNA binding to recognition elements located upstream of several nitrogen-regulated structural genes, *nit-3* (nitrate reductase), *alc* (allantoicase), and *lao* (L-amino acid oxidase). Most mutations which lead to substitutions for conserved amino acids within the zinc finger or the downstream basic region of NIT2 or AREA completely lack DNA binding activity in vitro and are nonfunctional in vivo (50).

The genetics of *areA*, which were elegantly developed by Arst and his colleagues (4, 23, 64, 65, 87), are very sophisticated; most *areA* mutants have a null phenotype, i.e., show a reduction or complete loss of most nitrogen catabolic enzymes, as expected for loss of function of a positive-acting regulatory factor. Certain rare *areA* mutants have more complex effects: some nitrogen enzymes are missing, whereas others are expressed even during nitrogen repression conditions. Of special interest are mutations which affect the specificity of the NIT2 or AREA proteins so that they differentially affect the expression of target genes. The *areA-102* mutant protein has valine substituted for the highly conserved leucine$_{526}$, centrally located in the zinc finger loop, and shows elevated expression of certain activities, e.g., acetamidase and histidase, and strongly reduced expression of others, e.g., formamidase and the xanthine-uric acid permease (64). The *areA-30* mutation (obtained by reversion of *areA-102*) has a methionine at residue 526 and displays a sharply contrasting phenotype; i.e., it has an elevated level of the xanthine-uric acid permease and decreased amounts of acetamidase and histidase (64). Substitution of the corresponding NIT2 residue, leucine$_{753}$, by either methionine, alanine, glutamate, aspartate, or valine resulted in NIT2 proteins which were functional in vivo. However, each showed a particular pattern of expression of nitrate reductase, allantoicase, and L-amino oxidase, which was paralleled by differences in DNA binding to each of these different promoters (126). Substitutions of the adjacent conserved residue, tryptophan$_{754}$, by several other amino acids all resulted in a nonfunctional NIT2 protein (126). Similarly, NIT2 proteins with substitutions of the neighboring conserved residues Arg$_{755}$ and Arg$_{756}$ lacked all detectable function (50). These results clearly demonstrate that the sequence of the central loop of the zinc finger domain of these GATA factors contributes significantly to the affinity of DNA binding and show that the amino acid which occupies the seventh position (Leu$_{753}$ in the wild type) is important in distinguishing between recognition elements in the promoters of several different structural genes.

The three-dimensional structure of a complex between the DNA binding domain of the chicken GATA-1 protein and its cognate DNA site has been determined by nuclear magnetic resonance spectroscopy (84). The DNA binding domain is composed of two antiparallel β-sheets and an α-helix, followed by an extended loop. The helix and loop connecting the two β-sheets bind in the major groove of the target DNA, primarily via hydrophobic interactions (84). The conserved leucine residue of GATA-1, corresponding to Leu$_{753}$ of NIT2 and Leu$_{526}$ of AREA, interacts with DNA within the major groove, whereas the conserved tryptophan and arginine residues, which correspond to Trp$_{754}$ and Arg$_{755}$ of NIT2, are both involved in maintaining the structural integrity of the zinc binding region (84). These structural studies of the GATA-1/DNA complex illuminate the importance of the central loop

structure of the zinc finger of the GATA proteins and help to explain the role of Leu_{753} in the selectivity of DNA binding.

AREA AND NIT2 RECOGNITION ELEMENTS

The global *trans*-acting AREA and NIT2 regulatory proteins are responsible for selectively turning on many different unlinked but coregulated genes. Individual structural genes can be expressed at markedly different levels or with different kinetics, which may be due at least partly to a different organization of AREA or NIT2 recognition elements in the promoters of target genes. Native NIT2 binding sites in the upstream promoter regions of different genes differ markedly in their number, orientation, location, and nucleotide sequence. With some important exceptions, most NIT2 binding sites contain two or more closely spaced copies of the core element, GATA, recognized generally by the entire family of GATA binding proteins. Most single GATA sequences represent very weak binding sites for NIT2. Two (or more) GATA elements located within 30 bp of each other, facing in the same or opposite directions, constitute a strong NIT2 binding site (15, 17). Such natural elements can point toward or away from the transcriptional start site of the various structural genes regulated by NIT2 (126). Mutation of any of the bases of the GATA core greatly reduces or eliminates NIT2 binding, with one exception; i.e., the sequence GATT retains approximately 50% of the binding affinity of the parental GATA element (15). Several different DNA footprinting techniques demonstrated that all of the G, A, and T nucleotides in the GATA core sequence and in the complementary TATC sequence on the opposite DNA strand are in intimate contact with the bound NIT2 protein (43).

Most of the high-affinity NIT2 binding sites contain at least two closely spaced GATA sequences, a feature which suggests the possibility that some form of cooperative DNA binding occurs. However, at least some strong NIT2 binding sites contain only a single GATA element, e.g., the site at approximately −1.1 kb in the *nit-3* promoter; the characteristic that confers high-affinity binding to single element sites is still unknown but may reflect special flanking sequences (16). It is also noteworthy that at least some structural genes which are not involved in nitrogen metabolism and which are not controlled by NIT2 nevertheless possess what inspection identifies as sites for strong binding by NIT2 in vitro; i.e., they contain closely spaced paired GATA elements. Such cryptic sites almost certainly have no physiological function in vivo (unless other unknown GATA factors are involved), suggesting that additional properties, such as chromatin accessibility, may define functional sites. Similarly, as described above, only certain of the AREA recognition elements in the *niiA-niaD* intergenic control region defined by in vitro binding actually have a physiological function in vivo (93). The promoters of structural genes usually possess multiple control elements, and the fact that loss of a single recognition site will not totally eliminate gene expression apparently explains the rarity of *cis*-acting control site mutations in fungal genes.

EXPRESSION AND MODULATION OF AREA AND NIT2

Regulatory genes which specify *trans*-acting factors are themselves frequently subject to autogenous regulation and/or control by other factors which act at different levels, including transcription, mRNA stability, translation, posttranslational modification, and direct protein-protein interactions. It now appears that the expression of the globally acting *areA* and *nit-2* regulatory genes is indeed controlled at many of these steps.

The *A. nidulans areA*$^+$ gene is highly expressed during nitrogen derepression conditions, yielding three size classes of mRNA, of approximately 3.9, 3.6, and 3.2 kb; *areA* expression is greatly reduced during nitrogen repression (65). Eight different *areA* transcription start sites were identified by reverse transcription-PCR and range from the nearest site at −15 to the most distal site at −800 (+1 represents the ATG initiation codon). It appears that the various mRNAs are functionally redundant, since the use of strains with deletions or point mutations which eliminate any of these mRNAs does not affect *areA* function in vivo. Thirteen GATA sequences—potential AREA binding sites—occur upstream of the *areA* coding region. The four most proximal GATA elements (α, β, γ, and δ) are clustered in a region that contains five closely spaced transcription start sites which give rise to the smallest (3.2-kb) *areA* mRNA (65). A point mutation which eliminates the function of the α GATA element greatly reduces or even totally abolishes the 3.2-kb mRNA, which is also missing in an *areA* mutant that lacks the AREA protein. These results imply that synthesis of the 3.2-kb mRNA is controlled by positive autogenous regulation. In contrast, the 5′ upstream region of the *N. crassa nit-2* gene lacks GATA sequence elements, and there is no convincing evidence that its expression is controlled by autogenous regulation (15).

Arst and Sheerins (5) used a genetic analysis with *areA* to demonstrate that the consensus context for a strong initiation codon for protein synthesis, i.e., one which prevents leaky scanning, in *Aspergillus* is GXX AUG C/UC. In mutants or deletions which lack the correct AUG initiation codon (AUG-1), several internal AUGs serve as initiation codons, and in some cases translational reinitiation can occur. However, in vivo translation of the wild-type *areA* mRNA does not appear to allow leaky scanning and thus is not expected to yield different forms of an AREA protein other than the full-length (876-amino-acid) product.

The *areA* transcript has a 3′ untranslated region of 539 nucleotides within which a sequence of 159 bases has 76% identity to a sequence 3′ of the coding region of the *P. chrysogenum nre* gene (87). In this highly conserved region, the *areA* UTR contains a perfect direct repeat of 28 nucleotides with a 6-nucleotide overlap. Deletion of a single copy of this tandem repeat had no effect; however, a strain with a deletion in the *areA* gene that removed both copies of the 28-nucleotide repeat was significantly derepressed for nitrogen-related activities, indicating that at least one copy of this element is essential for proper modulation of AREA function (87). The 3′ UTR acts at the level of mRNA stability, and the turnover rate of the *areA* transcript depends upon the nitrogen status of the cells. The wild-type *areA* mRNA has a half-life of 40 min during nitrogen-derepressed conditions, but its half-life is only 7 min under nitrogen-repressed conditions. In contrast, an *areA* transcript which is deleted for part of the 28-nucleotide tandem repeats has a half-life of approximately 25 min under both nitrogen-repressed and nitrogen-derepressed conditions (87). The molecular mechanism which underlies this differential *areA* transcript stability is unknown but may involve novel protein factors which recognize the tandem repeat elements. The *N. crassa nit-2* transcript does not appear to contain sequences closely related to these *A. nidulans* nucleotide repeat elements, and it is unknown whether the *nit-2* transcript is subject to nitrogen-controlled differential turnover.

When the carboxy-terminal region downstream of the DNA binding domain of the AREA and NIT2 proteins is deleted, both proteins retain strong *trans*-activation function but their

activity becomes largely insensitive to nitrogen catabolite repression. This indicates that the carboxy terminus of AREA and NIT2 plays an important role in nitrogen regulation (76, 107). This region of the proteins could contain a binding site for the nitrogen-repressing metabolite, glutamine, or could be involved in binding another protein which plays a role in nitrogen repression. Since the carboxy terminus is not required, it was surprising that *areA-2*, a frameshift mutation that has a normal DNA binding domain but results in replacement of the wild-type 122 C-terminal amino acids with a mutant peptide of 117 residues, lacked almost all *trans*-activation function, e.g., was completely devoid of nitrate reductase activity (88). An extensive analysis of reversions demonstrated clearly that the mutant *areA-2* peptide, which has a +20 charge compared with +3 for the wild-type carboxy terminus, was responsible for the severe deleterious effects (88). The protein specified by the *areA-2* mutant is somewhat reduced in binding to DNA fragments with GATA core sequences and shows approximately 500 times greater nonspecific DNA binding than does the wild-type protein. This result demonstrates that the highly positively charged tail of the mutant protein has a dramatic effect on AREA DNA binding specificity; this highly charged region may also interfere with AREA function by acting as a transcriptional repressor (88). These characteristics appear to explain the nonfunctionality of the *areA-2* protein and its partial dominance to *areA*$^+$. This rigorous study should also warn us to be cautious when interpreting results obtained with proteins with attached foreign sequences as well as with fusion proteins.

Modulation of AREA and NIT2 function involves two highly conserved regions of these proteins, a motif within their DNA binding domains and their carboxy-terminal tails. First, certain amino acid substitutions in an α-helical region of the zinc finger DNA binding domain give rise to mutant NIT2 proteins that function in turning on nitrate reductase gene expression but are largely insensitive to nitrogen repression (86). Similarly, substitutions for residues in an adjacent extended loop structure give rise to functional AREA proteins which are partially derepressed (88). Second, deletions of the carboxy terminus of both AREA and NIT2 result in functional proteins which are partially insensitive to nitrogen repression, e.g., turn on nitrate reductase even in the presence of high concentrations of ammonia or glutamine (87, 88). For both AREA and NIT2, much of the carboxy terminus is dispensable for function (or its loss has a very slight phenotypic result). Mutant AREA and NIT2 proteins with internal deletions that remove more than 100 amino acids of the C terminus show normal nitrogen control, provided that the final 12 amino acids at the very carboxy terminus are retained. These studies demonstrated that the 12-amino-acid carboxy-terminal tail acts as a motif that plays an important role in establishing nitrogen repression and thus in modulation of AREA and NIT2 function.

FUNCTION OF THE *NEUROSPORA* NMR REGULATORY PROTEIN

In *N. crassa*, mutations of a gene designed *nmr* (for nitrogen metabolic regulation) result in derepression of nitrate reductase and other nitrogen-controlled activities in the presence of sufficient ammonia or glutamine to completely repress their expression in *nmr*$^+$ strains (92, 116). In the *nmr* mutants, synthesis of nitrate reductase is largely insensitive to nitrogen catabolite repression but still requires induction by nitrate and functional NIT2 and NIT4 proteins (92, 102, 117). This phenotype suggests that *nmr* is a negative-acting regulatory gene and might encode a repressor protein or somehow modulate the activity of the positive-acting NIT2 protein. The *nmr* gene encodes a protein of 488 amino acids that has no distinctive characteristics such as obvious DNA binding or protein kinase motifs (128). The NMR protein expressed in *E. coli* did not display any DNA-binding activity nor did it bind glutamine; however, these results must be viewed with reservation because the expressed protein required denaturation before it could be solubilized (128).

Several lines of evidence now indicate that the NMR protein functions as a negative regulator by binding to the NIT2 protein and somehow modulating the *trans*-activation function of the latter, possibly by interfering with DNA binding. Direct interactions between the NMR and NIT2 proteins have been demonstrated by two different experimental approaches and by genetic analysis. Use of the yeast two-hybrid system showed that a specific interaction occurs between NIT2 and NMR (125). In vitro assays independently demonstrated protein-protein interaction between NIT2 and NMR. Two distinct short regions of the NIT2 protein, both predicted to exist as α-helices, appear to be recognized by the NMR protein (126). One of these regions corresponds to an α-helix that lies adjacent to the extended loop structure within the zinc finger DNA binding domain (Fig. 4). Mutant NIT2 proteins with amino acid substitutions near the carboxy-terminal end of this α-helix or nearby residues in the extended loop fail to bind to NMR in both assays and, moreover, display a derepressed phenotype in vivo (86). The NIT2 carboxy terminus, consisting of approximately 12 amino acid residues predicted to form an α-helix, is the second region that displays specific binding to NMR (125). Significantly, NIT2 proteins which lack this terminal region or which have a proline substituted for a leucine residue within this α-helical tail are largely insensitive to nitrogen repression in vivo, and the mutant NIT2 proteins fail to bind to NMR in both the yeast two-hybrid and in vitro assays (86). In vitro mobility shift assays suggested that NMR may interfere with NIT2 DNA binding (125). These results provide persuasive evidence that the NMR protein exerts a negative regulatory action by binding directly to the NIT2 protein and somehow blocking the *trans*-activation function of NIT2 during conditions of nitrogen repression. In this context, it is intriguing that the homologous DNA binding domains of the mouse GATA-1 protein mediate self-association (25) and specific physical and functional interactions with Sp1 and EKLF proteins (79). Similarly, GATA-2 interacts via its DNA binding domain with the bZip proteins JUN and FOS (63). Thus, it is becoming increasingly evident that the DNA binding domains of GATA factors can participate in specific interactions with other regulatory proteins, thereby giving rise to important regulatory responses.

It is significant that the deletions or mutations of the corresponding regions of the *A. nidulans* AREA protein that were implicated as NMR recognition motifs in *N. crassa* (125), i.e., the extended loop of the zinc finger or the carboxy tail, result in an N-derepressed phenotype (87, 88). However, mutants with a nitrogen-derepressed phenotype similar to *nmr* have not as yet identified an *nmr* homolog in *A. nidulans* despite a wealth of genetic analysis. One possible candidate, the *meaB* gene, whose mutation results in mutants that are resistant to methylammonium and show derepression of certain nitrogen-related activities, has been cloned and characterized (90). The protein encoded by the *meaB* gene has features suggesting that it may serve a control function, perhaps in amino acid uptake or cellular compartmentation of metabolites. However, the MEA protein is clearly not a homolog of the *N. crassa* NMR protein (90). Preliminary results suggest that when it is expressed in *A. nidulans*, the *Neurospora* NMR protein is functional (89). The *tamA* gene of *A. nidulans* has been implicated

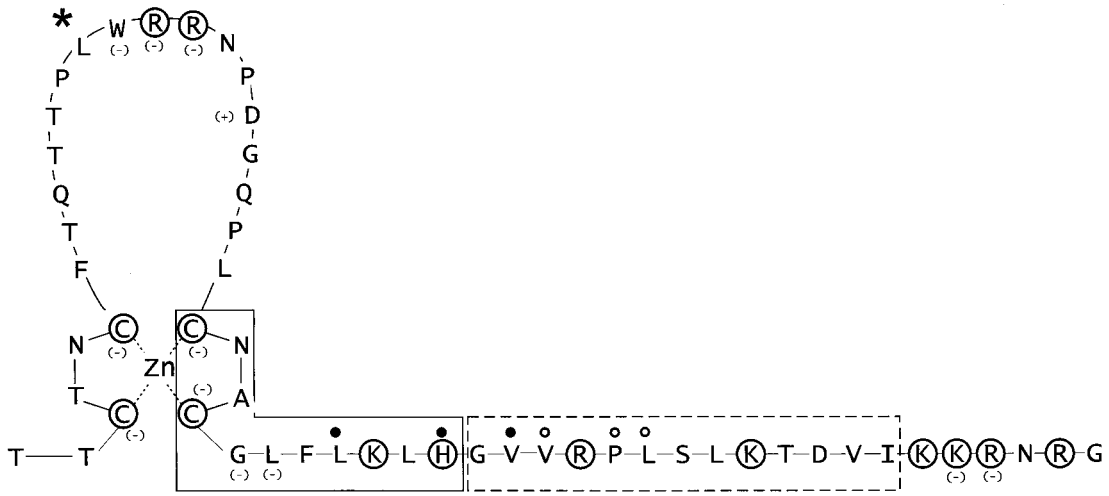

FIG. 4. Structural aspects of the Cys_2/Cys_2 zinc finger motif of AREA and NIT2, modeled from the solution structure of the homologous GATA-1-DNA complex (84). The residues enclosed by the solid line constitute the major α-helix; the residues boxed by the dashed line represent an extended loop. Basic amino acids and the four Cys residues which chelate Zn are circled. Solid circles designate residues in NIT2 which, when substituted, resulted in an N-derepressed phenotype and eliminated NIT2-NMR protein-protein binding; open circles identify residues in AREA which, when substituted, resulted in a N-derepressed phenotype (87). The asterisk (*) identifies the leucine residue which, when mutated, gives rise to altered promoter specificity and DNA binding activity in AREA and in NIT2 (see the text). (−) and (+), residues whose substitution (individually or in pairs) gave rise to nonfunctional or functional NIT2 proteins, respectively (50, 126).

in nitrogen control, and although the exact role of the TAMA protein is unknown, it is not similar to NMR (31).

RECOGNITION OF ENVIRONMENTAL NITROGEN CUES

Glutamine appears to be the critical metabolite which exerts nitrogen catabolite repression (13, 91). Ammonia leads to strong nitrogen repression in these fungi but is not itself active, since it does not cause repression in mutants lacking glutamine synthetase (91). Intracellular glutamine, or possibly a metabolite derived from it, leads to repression, but the cellular location of the glutamine pool responsible for this control response, e.g., cytoplasmic, vacuolar, or nuclear, is unknown. An extremely important but still unknown feature is the identity of the element or signal pathway system that senses the presence of repressing levels of glutamine. It is conceivable that the AREA, NIT2, GLN3, and similar global regulators themselves bind glutamine or that an accessory protein such as URE2 in *S. cerevisiae* or NMR in *Neurospora* or even a complex such as a NIT2-NMR heterodimer recognizes glutamine. However, it is also possible that an as yet unidentified factor(s) detects glutamine and conveys the repression signal to the global activating proteins. Thus, an important goal for future research is the creative use of genetic and biochemical approaches to identify the signaling system that recognizes and processes environmental nitrogen cues.

NITROGEN METABOLIC REGULATION AND FUNGAL PATHOGENESIS

The globally acting nitrogen regulatory protein exerts control over a major area of cellular metabolism, which includes many catabolic activities, including multiple proteases, which have been implicated in fungal pathogenesis. Nutritional limitations of various types, particularly nitrogen deprivation, appears to have a link to pathogenesis and other fungal morphogenetic switches (66, 112). Thus, it seems possible that loss of a major nitrogen regulatory factor will significantly reduce the virulence of plant- or animal-pathogenic fungi. Two lines of evidence imply that an *areA* null mutant of *Aspergillus fumigatus* is significantly reduced in growth in lung tissue compared with an otherwise isogenic $areA^+$ strain (57). First, in neutropenic mice inoculated with *A. fumigatus* disrupted for the *areA* gene, the onset of disease symptoms was significantly delayed compared with that in mice inoculated with the parental $areA^+$ strain. Second, analysis of fungal colonies recovered from the lungs of mice which had been inoculated with an unstable *areA* mutant gene demonstrated that the proportion of $areA^+$ revertants was approximately 60%, compared with only about 5% of colonies grown on standard medium with ammonium as the nitrogen supply. These results suggest that the *areA* mutants are at a selective disadvantage for growth in the lung and imply that utilization of available nitrogenous sources within the lung tissue requires derepression of certain nitrogen catabolic enzymes (57). A relationship between nitrogen assimilation and the yeast-hypha morphological change in *Candida albicans* that involves a decrease in glutamine synthetase activity just prior to the morphological transition has been observed (123).

The *nut1* gene of the rice pathogen *Magnaporthe grisea* encodes a protein with 59% amino acid identity to AREA and NIT2, respectively. A null mutant obtained by disruption of *nut1* was incapable of utilizing nitrate but was still pathogenic on rice, although the lesions produced were somewhat reduced in size (44). Lau and Hamer (66) have isolated mutants altered in nitrogen metabolism (via chlorate resistance) in a strain of *M. grisea* that is pathogenic for barley. Mutants deficient in nitrogen source utilization of the various classes described in Table 1, including *nut1* mutants, all were as fully pathogenic as the starting parental strain. However, mutants with mutations in two novel genes, *npr1* and *npr2*, were isolated and showed a pleiotropic loss of nitrogen source utilization and loss of pathogenicity. *npr1* and *npr2* are each new genes which appear to act as wide-domain regulators; genetic crosses demonstrated convincingly that they are not allelic to each other or to *nut1* and also showed that the effects on nitrogen metabolism and upon pathogenicity result from the same mutational event (66). Moreover, unlike the wild type, mutants with mutations in *npr1*

TABLE 3. Pathway-specific regulatory factors which mediate the induction of structural genes encoding enzymes of specific nitrogen catabolic pathways

Pathway-specific factor	Inducer	Catabolic pathway	Type of DNA-binding domain	Genus	Reference
AMDA	Acetate	Acetate	Cys_2/His_2 finger	*Aspergillus*	71
AMDR	ω-Amino acid	Acetamide	Cys_6/Zn_2 finger	*Aspergillus*	1
FACB	Acetate	Acetate	Cys_6/Zn_2 finger	*Aspergillus*	62
NIRA	Nitrate	Nitrate	Cys_6/Zn_2 finger	*Aspergillus*	9
NIT4	Nitrate	Nitrate	Cys_6/Zn_2 finger	*Neurospora*	129
PRNA	Proline	Proline	Cys_6/Zn_2 finger	*Aspergillus*	96
PUT3	Proline	Proline	Cys_6/Zn_2 finger	*Saccharomyces*	97
UAY	Urate	Purine	Cys_6/Zn_2 finger	*Aspergillus*	109

and *npr2* both fail to express the *mpg1* gene during either nitrogen or carbon deprivation. *mpg1* encodes a hydrophobin protein that plays a role in pathogenesis, and *mpg1* disruptant strains are not pathogenic (112). However, the *npr1* and *npr2* mutants have a more severe loss of pathogenicity than does the *mpg1* disruptant, suggesting that they may control a number of genes which function in the pathogenic process. Despite the extensive genetic analysis carried out with *A. nidulans* and *N. crassa*, genes homologous to *npr1* and *npr2* have not yet been detected. The molecular cloning and characterization of the *npr1* and *npr2* genes will provide extremely important information about nitrogen regulation and its link to pathogenicity in *M. grisea*.

The interaction between the fungal pathogen *Cladosporium fulvum* and tomato includes a hypersensitive defensive response of the plant upon recognition of the products of fungal avirulence genes, e.g., *avr9* (34). The AVR9 elicitor is a 28-amino-acid peptide with a cysteine knot motif (33, 34). Of particular interest is that a fusion gene controlled by the *avr9* promoter is expressed in the *A. nidulans areA*$^+$ strain but not in *areA* mutants under N derepression (33), again demonstrating a connection between nitrogen metabolic regulation and fungal pathogenicity.

PATHWAY-SPECIFIC REGULATORY FACTORS

In activation of expression of at least the majority of target genes, the globally acting proteins AREA, Gln3p, and NIT2 do not function alone but function only in combination with one or more pathway-specific factors which convey induction signals. Each of these pathway-specific regulatory proteins appears to act in a positive fashion and provides the mechanism that allows the selective activation via induction of a specific set of genes which encode the enzymes of a particular pathway from a vast array of nitrogen catabolic structural genes (Table 3). Each pathway-specific regulatory protein is believed to achieve an active form upon binding a specific inducer, although in most cases this concept has not yet been demonstrated experimentally and remains a significant research objective. In the case of NIRA and NIT4, which are involved in nitrate induction, the enzyme nitrate reductase itself may sense the presence of the inducer nitrate and interact in some fashion with the specific factors (23, 76). Many of the specific factors involved in nitrogen catabolic gene expression, e.g., the *Aspergillus* FACB, NIRA, PRNA, and UAY proteins, the *S. cerevisiae* PUT3 protein, and the *Neurospora* NIT4 protein, are members of the large GAL4 family of regulatory proteins that possess a single Cys_6/Zn_2 type of binuclear zinc cluster, which to date has been found only in fungal organisms. However, some exceptions occur; e.g., the *Aspergillus* AMDA protein contains two of the more conventional Cys_2/His_2 zinc fingers (62). Many of the pathway-specific factors are themselves constitutively expressed, apparently at low levels, although some exceptions occur, since the *facB* gene is subject to acetate induction and carbon catabolite repression (62).

Expression of the structural genes which encode the nitrate assimilatory enzymes in *Aspergillus* and *Neurospora* provides a well-documented case involving pathway-specific control. Their expression requires nitrogen derepression signaled by a globally acting factor (AREA or NIT2) and also has an absolute requirement for nitrate induction mediated by a pathway-specific factor, NIRA or NIT4, respectively. The *N. crassa* NIT4 protein is composed of 1,090 amino acids and contains at its amino terminus a GAL4-like Cys_6/Zn_2 binuclear zinc cluster followed by a spacer region and a coiled-coil motif that mediates the formation of a homodimer, the form that is responsible for sequence-specific DNA binding (see above). The isofunctional *N. crassa* NIT4 and *A. nidulans* NIRA proteins have approximately 60% amino acid identity in their amino-terminal 600 residues, including 90% identity in their 50-residue DNA binding motifs (Fig. 5). However, the carboxy-terminal halves of NIT4 and NIRA differ completely from one another (9, 10). NIT4 and NIRA bind to DNA with similar or identical specificity (45, 93), and the *Neurospora nit-4*$^+$ gene can substitute for *nirA* when introduced via transformation into a mutant host (56).

Large segments of some regulatory proteins, e.g., the *S. cerevisiae* GAL4 and GCN4 proteins and the *A. nidulans* AREA protein, can be deleted with retention of strong activation potential (58, 65, 72, 88). In such cases, this has helped to identify activation domains, such as the acidic regions of GAL4 and VP16 and the glutamine-rich segments of SP1, within which specific hydrophobic residues play a major role (24, 72). The carboxy-terminal half of NIT4 appears to function in gene activation and contains glutamine-rich, glycine-rich, and polyglutamine segments and an acidic, leucine-rich tail (41). Deletion of nearly any portion of the NIT4 protein results in loss of function when tested via transformation of the appropriate construct into *Neurospora*, presumably due to instability or improper folding of the protein (41). However, fusion of different regions of NIT4 to the GAL4 DNA binding domain demonstrated that NIT4 contains three different regions which acted as activation domains in yeast. An acidic and leucine-rich segment of 28 amino acids at the NIT4 C terminus tail alone displayed strong activation potential in yeast and, interestingly, showed substantial alignment of potentially critical large hydrophobic residues found in the VP16 and GAL4 acidic and the SP1 glutamine-rich activation domains (24). The *trans*-activation activity of the entire C-terminal half of NIT4 is greater than the additive contributions of the three subdomains, implying that a synergistic effect occurs when these three regions are all present (41).

It is important to appreciate that the globally acting proteins AREA, NIT2 and Gln3p, and presumably the homologous

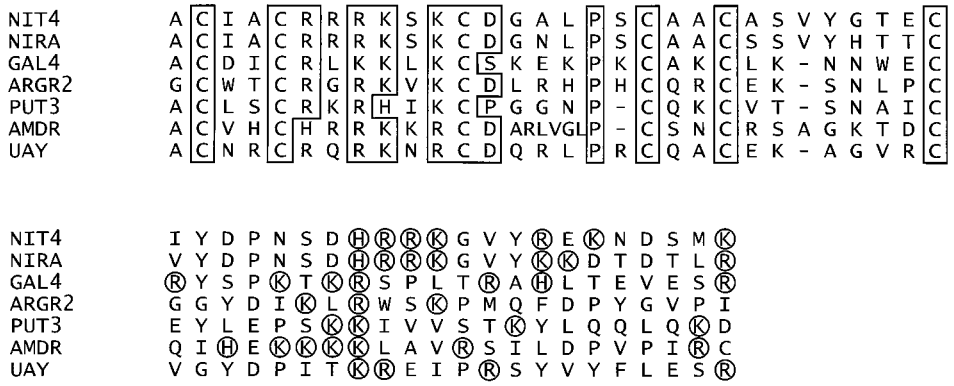

FIG. 5. Amino acid sequence of DNA binding domains of fungal pathway-specific nitrogen regulatory proteins compared with the GAL4 factor that controls galactose metabolism in yeast. All these proteins possess a single N-terminal Cys_6/Zn_2 binuclear finger and adjacent basic region, which is critical for sequence-specific recognition. NIT4 and NIRA, nitrate assimilatory factors in *N. crassa* and *A. nidulans*, respectively; ARGR2, arginine metabolism control in *S. cerevisiae*; PUT3, proline metabolism in *S. cerevisiae*; AMDR, acetamidase control in *A. nidulans*; UAY, purine catabolism in *A. nidulans*. Conserved residues are boxed, and basic amino acids in the adjacent region C-terminal to the zinc cluster are circled.

GATA binding proteins in other fungi, cooperate with multiple positive-acting, pathway-specific regulatory proteins to turn on specific sets of nitrogen catabolic genes, depending upon the availability of substrates and a need for nitrogen. Each pathway-specific factor is a DNA binding protein which recognizes elements in the promoters of the catabolic genes encoding the enzymes of a particular pathway and mediates their activation in response to a particular inducer. An important goal is to compare the pattern of binding sites in the respective promoters for the various pathway-specific factors and their relationship to the binding sites for the global regulatory proteins. Another extremely interesting aspect is whether the global and pathway-specific factors interact directly with each other via protein-protein binding or promote their cooperative DNA binding. For example, the two positive-acting proteins, AREA and UAY, involved in purine catabolism in *A. nidulans* may interact directly with each other to turn on structural-gene expression. Alternatively, it is conceivable that the globally acting and pathway-specific proteins bind to their respective promoter elements and act relatively independently and that the formation of a stable transcription complex requires that they contact different members of the basal transcriptional apparatus. Limited genetic evidence suggests that some type of direct interaction occurs between the AREA and NIRA proteins. A gain-of-function mutation, *nirA^d-106*, in the pathway-specific gene resulted in a significant loss of sensitivity to nitrogen repression, i.e., a function mediated by AREA, but still required nitrate induction (23, 115). This is an excellent example in which genetic experiments carefully executed nearly two decades ago provide insight into potential molecular interactions that now are just being opened to direct investigation. In vitro protein-protein binding assays have recently demonstrated a specific interaction between the comparable *N. crassa* proteins NIT2 and NIT4; in fact, when various regions of NIT2 were tested as GST fusion proteins, it was revealed that the zinc finger region of NIT2 was responsible for binding to NIT4 (42); this is not surprising, since a number of recent reports have shown that the DNA binding domains of various regulatory proteins also function in protein-protein interactions (25, 63, 79). An important goal now is to obtain unequivocal evidence showing whether the AREA-NIRA and NIT2-NIT4 interactions actually have a physiological function in vivo and whether the globally acting proteins show similar interactions with other pathway-specific factors.

CONCLUSIONS AND FUTURE DIRECTIONS

In the past decade, great advances have been made in our understanding of a global regulatory circuit within many fungi which directs the preferential use of primary nitrogen sources when available but also permits the selective utilization of many different secondary N sources when necessary. Responses within the nitrogen regulatory circuit are mediated by global *trans*-acting factors, which bind DNA via a single zinc finger motif at elements containing a GATA core sequence. It is now apparent that the fungi contain multiple GATA-binding proteins which may participate in various regulatory phenomena, e.g., responses to light or to nitrogen or iron deprivation; thus, a major question arises as to how the cell distinguishes among these similar factors. The selective expression of the genes within a particular nitrogen catabolic pathway also requires specific induction, mediated by pathway-specific regulatory proteins which are most often members of the fungal family of proteins with an N-terminal Cys_6/Zn_2 binuclear-cluster DNA binding domain. Some structural genes encoding catabolic enzymes are regulated in an extremely complex fashion, responding to multiple systemwide factors that signal limitation for N, C, S, or P, the external pH, and perhaps other required metabolites, as well as to one or multiple inductive signals. Their promoters must be extremely modular, and a precise description of the multiple DNA-protein and protein-protein interactions which allow integrated responses to different nutritional requirements and environmental conditions will represent a major contribution in understanding complex gene regulation in both higher plants and animals. Paramount among the challenges for future work is the need to understand postulated interactions between the globally acting proteins and the pathway-specific factors which result in turning various genes from "off" to a high level of expression. A number of other mysteries still cloud our vision of the fungal nitrogen control systems, including the nature of the key macromolecules or signal transduction system that recognizes the repressing nitrogen compound, glutamine or a metabolite derived from it, and processes this information to modulate the transcriptional activation steps. A major future goal is to investigate the molecular mechanisms which interconvert the global regulatory factors into active and inactive forms; similarly, although it is generally believed that the pathway-specific factors are activated upon binding specific inducers, the precise

steps in this process are largely unexplored. The tremendous recent strides in our understanding of nitrogen regulation in fungi, due largely to the powerful combination of genetics, biochemistry, and molecular approaches and the isolation and characterization of regulatory and structural genes and their protein products, now provide the framework that will allow tests of long-standing hypothesis. In the next millennium, these approaches will certainly provide an information explosion, yielding unexpected discoveries and significant new insights.

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