The Negative-Acting NMR Regulatory Protein of *Neurospora crassa* Binds to and Inhibits the DNA-Binding Activity of the Positive-Acting Nitrogen Regulatory Protein NIT2[†]

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ABSTRACT: Structural genes of the nitrogen regulatory circuit of the filamentous fungus *Neurospora crassa* are under the control of both positive and negative regulatory proteins. NIT2, the major positive-acting nitrogen regulatory protein, activates the expression of structural genes within the nitrogen circuit. NIT2 binds to upstream activation sites which contain at least two GATA core elements in the promoter regions of the nitrogen-controlled structural genes, and activates their transcription, possibly by way of acidic activation domains. The mechanism by which a putative negative-acting regulator, NMR, mediates nitrogen repression of the various structural genes has remained unclear. In the studies reported here, a direct interaction between the NIT2 and NMR proteins has been demonstrated by the use of two different experimental approaches. The yeast two-hybrid system was used to show NIT2−NMR-specific binding *in vivo*; an independent *in vitro* assay for protein−protein binding also demonstrated a specific interaction between NIT2 and NMR. Two separate regions of the NIT2 protein, both of which appear to exist as α-helices, make direct contact with the NMR protein. One of these α-helical regions occurs within the zinc finger DNA-binding domain of NIT2. Mutant NIT2 proteins with amino acid substitutions in the zinc finger motif do not bind to NMR. Mobility shift experiments revealed that the NMR protein inhibits NIT2 DNA binding *in vitro*. We suggest that NMR carries out its negative regulatory role by directly binding to NIT2, and thereby blocking the function of NIT2 by inhibiting its DNA-binding activity.

In the filamentous fungus *Neurospora crassa,* nitrogen metabolism is a highly regulated process which enables the most efficient usage of nitrogen resources available to this lower eukaryote. The nitrogen regulatory circuit of *Neurospora crassa* contains a number of structural genes which encode enzymes required for the assimilation of different secondary nitrogen compounds when preferred nitrogen sources, e.g., ammonium or glutamine, are not available (Marzluf, 1993). Inorganic nitrate is an excellent secondary nitrogen source, but its utilization requires the *de novo* synthesis of nitrate reductase and nitrite reductase. Similarly, synthesis of a set of catabolic enzymes is needed for the utilization of various purines, such as hypoxanthine, xanthine, or uric acid. The expression of the genes which encode these pathway-specific catabolic enzymes appears to be controlled by both positive and negative regulatory proteins (Marzluf, 1993).

A major positive regulatory protein, NIT2, mediates nitrogen derepression and is required for expression of genes within the nitrogen circuit of *Neurospora crassa.* Mutation of *nit-2* leads to a great reduction or complete lack of expression of many genes that are directly involved in nitrogen assimilation. The amount of the *nit-2* gene transcript changes only moderately under different metabolic conditions, increasing approximately 3−4-fold during nitrogen limitation as compared with repressing conditions (Fu & Marzluf, 1987b). Moreover, there is no evidence for *nit-2* autoregulation at the transcriptional level, nor is *nit-2* transcription controlled by either of two potential regulatory factors, the *nmr* (nitrogen metabolic regulation) gene product or the enzyme glutamine synthetase (Fu & Marzluf, 1987b). DNA sequence analysis revealed that *nit-2* has a long open reading frame interrupted by two short introns and encodes a protein of 1036 amino acids (Fu & Marzluf, 1990d). The native NIT2 protein has been identified in nuclear extracts of *Neurospora* and its properties compared with that of NIT2 expressed in *Escherichia coli.* One interesting feature of NIT2 is the presence of a Cys_2/Cys_2-type zinc finger motif which identifies it as a member of the GATA-binding family of proteins, which are widespread in many species, including various fungi, *Drosophila,* plants, chickens, and several mammals. The zinc finger region of NIT2 and other GATA proteins functions in sequence-specific DNA binding; extensive studies of NIT2 DNA binding have revealed a consensus binding sequence, GATA (Fu & Marzluf, 1990a). Detailed studies have demonstrated that high-affinity NIT2-binding sites contain at least two closely spaced GATA core elements, and suggests that cooperative binding exists between different NIT2-binding sites (Feng et al., 1993; Chiang & Marzluf, 1994). Upstream of the zinc finger motif, the NIT2 protein of *Neurospora* and the homologous protein of *Aspergillus,* AREA, both contain two highly acidic regions, which appear to function as activation domains to turn on structural gene expression.

In addition to nitrogen derepression, activation of structural genes which specify enzymes of particular nitrogen metabolic

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pathways requires induction, which is mediated by pathway-specific positive regulatory proteins. For example, the pathway-specific NIT4 protein and NIT2 are both required to turn on the expression of the *nit-3* and *nit-6* genes, which encode enzymes for nitrate assimilation (Exley et al., 1993; Fu & Marzluf, 1987a). NIT4 is a DNA-binding protein of the GAL4 class and contains a single Cys_6/Zn_2 type of finger structure (Yuan et al., 1991). It is not yet clear how the NIT2 and NIT4 proteins together function to carry out their positive function.

Another important putative nitrogen regulatory gene, *nmr*, appears to act in a negative fashion to repress synthesis of nitrate reductase and the various other nitrogen metabolic enzymes. In nmr^+ strains, the expression of nitrogen-related structural genes, e.g., nitrate reductase, is strongly repressed by primary nitrogen sources, such as glutamine and ammonia. In *nmr* mutant strains, these nitrogen catabolic genes are expressed in a constitutive fashion, even in the presence of high concentrations of primary nitrogen sources (Dunn-Coleman et al., 1981; Premakumar et al., 1980).

The *nmr* gene has been cloned and appears to encode a protein of 54 854 daltons with a very limited homology with the yeast regulatory gene ARGRII (Young et al., 1990). Transformation assays showed that 16% of the NMR protein at its carboxyl terminus is dispensable for its function. NMR does not appear to have any DNA-binding activity nor to directly inhibit the transcription of *nit-2* or the nitrogen-controlled structural genes, such as *nit-3*. A proposed mechanism to explain the opposing actions of NMR and NIT2 is that these two proteins directly bind to one another, and that NMR in some fashion prevents NIT2 gene activation during conditions of nitrogen repression (Young et al., 1990). The availability of both the *nit-2* and *nmr* genes provided an exceptional opportunity to look into the relationship between this pair of counteracting nitrogen regulatory proteins.

The yeast two-hybrid system has proved to be an excellent and sensitive system for studying protein−protein interactions *in vivo*, and many important protein interactions have been uncovered with this system (Fields & Song, 1989). We present here results obtained with this method, and further confirmed with additional *in vitro* experiments, which imply that a highly specific protein−protein interaction occurs between NMR and NIT2.

MATERIALS AND METHODS

Strains, Plasmids, and Growth and Transformation Procedures. The yeast strain Y153CHR (MATα leu2−3,112 ura3−52, trp1−901, his3- 200, ade2−101,gal4 gal80 URA3::GAL-lacZ, LYS2::GAL-HIS3) and two shuttle vectors, pACT and pJS246, derivatives of pSE1107 and pAS1, respectively, were used in this study (Durfee et al., 1994). In all yeast transformation experiments, Leu, Trp, and His were omitted from the amino acid supplemented SC medium for selection of cotransformants. In transformations involving only one hybrid construct, a second vector lacking an insert was included so the same selective conditions could be maintained. Protein−protein interactions were monitored through the reporter gene Z (which encodes β-galacotosidase) which has five GAL4 binding sites in its promoter. The yeast GLN3 protein and NIT2 have homology in their DNA-binding motifs but otherwise are different, and there is no indication for a yeast protein homologous to NMR. Furthermore, negative controls in the assays for interacting proteins via the yeast two-hybrid system were included to eliminate interference by yeast proteins.

The full-length *Neurospora crassa nit*-3, *nit*-2, and *nmr* genes were employed, and the entire genes or various fragments of them were subcloned into the two yeast shuttle vectors. To clone *nit-2* into pJS246, the *Eco*RI fragment of *nit-2* (Fu & Marzluf, 1987b) was first cloned into the *Eco*RI site of pRSETC, followed by cutting out its *Nhe*I−*Xmn*I fragment (which encodes NIT2 residues 1−683) and cloning it into pJS246 which had been cut with *Nhe*I and *Sma*I. In order to clone *nmr* into pJS246, the *Nru*I−*Eco*RV fragment of pNMR (Young et al., 1990) which encodes NMR residues 46−488 was first cloned into pRSETC (Qiagen) at its *Bgl*II site which had blunt-ended with mung bean nuclease; an *Nhe*I−*Pvu*II fragment from this construct was then cut out and ligated with pJ246 which had been cut at *Nhe*I and *Sma*I. A *Spe*I−*Xho*I fragment of pNit4, which encodes residues 1−975 of NIT4 (Yuan et al., 1991), was ligated with pJS246 which had been cut with *Nhe*I and *Sal*I. To clone *nit-3* into pJS246, the *Xho*I DNA fragment of pNit3 (Fu & Marzluf, 1987a), which encodes amino acids 8−650 of nitrate reductase was cloned into pRSETC, after which a *Nhe*I−*Pvu*II fragment that contains *nit-3* was cut out and ligated with pJs246 which had been cut with *Nhe*I and *Sma*I.

To clone the *nit-2* gene into pACT, the *Eco*RI fragment which encodes the full-length (1036 residues) NIT2 protein was filled in with Klenow enzyme and cloned into pACT cut with *Bam*HI and blunt-ended with mung bean nuclease. When ligated, this construct had an altered reading frame; it was then cut with *Xho*I and ligated with the *nit-3 Xho* fragment (encodes residues 8−650) to reestablish the proper reading frame. All constructions were checked to ensure correct reading frames had been obtained by nucleotide sequencing, using primers corresponding to sequences immediately upstream of the linker regions of pACT and pJS246. All other constructions used in this study were derived from those described above using similar approaches, primarily by using various restriction sites to create deleted forms.

The yeast transformation procedure was modified from Ito et al., (1983). A single colony of Y153CHR was inoculated into 10 mL of YPD medium (Durfee et al., 1994) and shaken at 30 °C overnight, after which it was transferred into 100 mL fresh YPD medium and incubated at 30 °C. When the OD_{600} reached 0.8, the cells were harvested by centrifugation at 2500 rpm for 5 min. The cell pellet was washed in 20 mL of 0.1 M LiOAc, resedimented, suspended in 20 mL of 0.1 M LiOAc, incubated 1 h at 30 °C, and then collected and resuspended in 1 mL of 0.1 M LiOAc. For cotransformation, 2−3 μg of each of the two fusion constructions plus 10 μg of salmon sperm DNA as carrier was incubated with 100 μL of the competent yeast cells for 10 min; then 500 μL of 40% PEG in 0.1 mM LiOAc was added, and the mixture was incubated at 30 °C for 1 h. The cells were subjected to a heat shock at 42 °C for 5 min and then mixed with 600 μL of sterilized water, and sedimented at 2500 rpm in a microcentrifuge. The cell pellet was washed again with 1 mL of sterile water, centrifuged, and resuspended in 100 μL of water. The cells were then spread onto plates of sc medium (Durfee et al., 1994) supplemented with amino acids excluding Trp, Leu, and His.

Filter Paper β-Galactosidase Assays. Filter paper assays for β-galactosidase were conducted 3 days after transformation when colonies were clearly visible. Transformed yeast colonies were transferred to H-bond filter paper (Amersham), which was placed into liquid nitrogen for 10 s. The yeast cells on these filters were lysed by placing them at room temperature for 5 min. These filters, with the colonies facing up, were then placed into a petri dish containing 0.3 mL of Z buffer containing 1 mg/mL X-gal (Durfee et al., 1994). Colonies containing β-galactosidase developed a brilliant blue color at different times depending upon the amount of enzyme activity. In this study, typical positive colonies turned intense blue within 1 h, whereas colonies lacking β-galactosidase activity remained white. β-Galactosidase activities were measured quantitatively for some transformants. Candidate transformants were inoculated into 10 mL of SC medium and incubated in a 30 °C water bath shaker overnight. The cells were then collected by centrifugation in a 1.5 mL Eppendorf tube. The cells were lysed by freezing and thawing, after which 1 mL of buffer z containing 1 mg/mL ONPG was added to each tube, which was incubated at 30 °C for 30 min. Cell debris was removed by centrifuging at top speed in a microcentrifuge for 10 min, and the OD_{420} of the supernatant was measured. β-Galactosidase activity is reported in Miller units. One Miller unit = $(OD_{420})1000/(V)(T)(OD_{600})$, where V = volume of culture in milliliters, t = elapsed time in minutes, and OD_{600} is the optical density of the overnight culture.

In Vitro Assays for Protein−Protein Binding. An *in vitro* protein−protein interaction assay was done with purified fusion proteins. pGEX-2T (Pharmarcia) and pRSET (QIAGEN) vector systems were used to express fusion proteins at high levels in *E. coli* strains Xl1-blue (Stratagene) and BL21(DE)3 (Fu & Marzluf, 1990b), respectively, as hosts. DNA fragments encoding NMR were fused in-frame to the coding sequence for GST (in pGEX-2T), and *nit-2* DNA fragments were fused to the sequence encoding the histidine tag (in pRSET). For convenience in subcloning, a sequence encoding 21 additional amino acid residues upstream of the NIT2 zinc finger was included in the constructs with five genes which specify mutant NIT2 proteins. In independent experiments, it was shown that this short stretch of amino acids had no effect on either *in vivo* or *in vitro* binding experiments (not shown). The NMR fusion constructions were transformed into XL1-blue; a single fresh colony was used to inoculate 5 mL of LB medium. After overnight culture, the 5 mL inoculum was transferred to 200 mL of fresh LB medium. When the OD_{600} of this culture reached 0.5, IPTG was added to the final concentration of 0.5 mM. The cells were allowed to grow for another 3 h, and then harvested. NIT2 fusion constructs were transformed and induced in a similar way except that the host strain used was BL21(DE)3. NMR−GST fusion proteins were purified by glutathione−agarose affinity chromatography. NIT2−histidine-tagged fusion proteins were purified with a Ni−NTA−agarose resin according to the manufacturer's suggested procedure. In the assay for *in vitro* protein−protein interaction, 50−100 μL of the glutathione−agarose resin saturated with the NMR−GST fusion protein (10−20 μg) was incubated with 2 μg of purified NIT2 protein in 1 mL of PBS buffer (1 L contains 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of $KHPO_4$, pH 7.4) at 4 °C for 2 h. The resin was then washed twice with PBS buffer containing 1% Triton, and then the GST−NMR fusion protein and any associated proteins were eluted with 200 μL of Tris-HCl (50 mM, pH 8.0) buffer containing 10 mM glutathione. Ten microliters of each eluate was electrophoresed in a SDS−PAGE gel. After the proteins were transferred to nitrocellolose filters by Western blotting, NIT2 proteins were detected by immunodetection with an anti-NIT2 antibody. Polyclonal anti-NIT2 antibody was obtained by inoculation of rabbits with a 247 amino acid segment of the NIT2 protein which includes its DNA-binding domain. Detection was by enhanced chemiluminesence (ECL kit from Amersham) following the instructions of the manufacturer. The primary anti-NIT2 antibody was diluted 500-fold in all experiments, and the second antibody was donkey anti-rabbit antibody conjugated with horseradish peroxidase. After addition of substrate, a 5 s exposure of the filter to Kodak X-ray film was used.

Gel Band Mobility Shift Assays. A 160 bp *Bam*HI−*Sty*I DNA fragment from the *N. crassa lao* promoter region which contains NIT2-binding sites was labeled with [^{32}P]-dATP at its *Bam*HI site by the end-filling method and used in mobility shift assays. The *Kpn*I−*Eco*RI fragment of *nit-2* which encodes the zinc finger DNA-binding motif was cloned into pGEX-2T to yield a NIT2−GST fusion protein. The *Nru*I−*Eco*RV DNA fragment of the *nmr* gene, which encodes the entire NMR protein except for the N-terminal 46 residues, was similarly cloned into pGEX-2T to encode an NMR−GST fusion protein. The constructs were transformed into Xl1-blue, and the fusion proteins were induced and expressed as described above. The fusion proteins were then purified and used in mobility shift assays. The NIT2 and NMR proteins were incubated together at different ratios on ice for 30 min; then the mixture was incubated with the ^{32}P-labeled *lao* DNA promoter fragment (30 000 cpm) in a buffer containing 12 mM HEPES, 50 mM KCl, 3 mM $MgCl_2$, and 2 mM DTT at room temperature for another 30 min (Hope & Struhl, 1985). After this, the mixture was loaded onto a 4% native PAGE gel and run in 0.25× TBE buffer. The gel was then exposed to X-ray film.

RESULTS

In Vivo Interaction between NIT2 and NMR Proteins. The full-length NMR protein was fused to the GAL4 DNA-binding domain, and the entire NIT2 protein was fused to the GAL4 activation domain. Neither of these two constructions activated β-galactosidase expression when they were individually transformed into the yeast host strain; the transformed colonies showed no color change at all with the filter enzyme assay. In contrast, when these two constructs were both transformed into the yeast host together, the transformants turned blue within 1 h with the filter enzyme assay (Table 1). As negative controls, cotransformation was also used to test several other pairs of fusion proteins for protein−protein binding. The NIT2/NMR pair was the only combination that showed positive interaction. Moreover, NMR showed no association with NIT3, nor did NIT2 display binding with NIT4, indicating that neither NMR nor NIT2 tends to generally bind with other proteins. These results suggest that a specific protein−protein interaction occurs between NIT2 and NMR.

Specific Regions of NIT2 Interact with NMR. In order to rule out the possibility that nonspecific interactions between

Table 1: Detection of Possible Protein−Protein Interactions between Members of the *Neurospora c.* Nitrogen Regulatory Circuit

fused to GAL4 DNA-binding domain	fused to GAL4 activation domain	β-galactosidase activity
NIT2	−	−
NIT3	−	−
NIT4	−	−
NMR	−	−
NIT4	NIT2	−
NMR	NIT2	++++
NMR	NIT3	−
NIT4	NIT3	−

[a] Vectors encoding the indicated full-length *Neurospora* protein that fused the DNA-binding domain or the activation domain of the yeast GAL4 protein were constructed as described under Materials and Methods. These constructs were transformed individually or cotransformed into yeast cells as indicated. Yeast colonies were assayed for β-galactosidase, the reporter enzyme, using the filter paper assay with X-gal. ++++, strong β-galacosidase activity; −, no detectable activity. These results were verified with cell-free enzyme assays of individual transformed colonies.

NIT2 and NMR gave a false positive result, different segments of the NIT2 protein were tested to detect specific regions that were responsible for the NIT2−NMR interaction. Different segments, corresponding to the entire amino-terminal region of NIT2 upstream of the zinc finger motif, gave negative results for binding to the full-length NMR protein with the yeast two-hybrid assay. In sharp contrast, with the same assay, a specific region of the NIT2 protein, beginning at the zinc finger and extending to the carboxyl terminus, yielded strong positive results for interaction with NMR (Figure 1).

This region of NIT2, encompassing the zinc finger and the downstream residues, was then subjected to a detailed analysis by testing various segments of it as shown in Figure 1. Two stretches of amino acids were found to interact with NMR. One interacting segment covers the zinc finger DNA-binding motif itself. A second region that interacts with NMR contains the carboxyl-terminal 30 amino acid residues.

One Region of NMR Interacts with NIT2. Different segments of the NMR protein were similarly tested to determine whether a specific region of NMR was responsible for its interaction with NIT2. An NMR region containing 170 amino acids displayed a positive result for binding to NIT2, as indicated in Figure 2. Interestingly, one construct which encodes a larger protein which included the 170 residue segment was negative for interaction in the yeast two-hybrid system. This may be due to a failure of the truncated NMR protein to fold into a correct conformation.

In Vitro Analysis of the Interaction between NIT2 and NMR. Various segments of the *nit-2* gene which encode different regions of the NIT2 protein tested above in the yeast two-hybrid system were cloned into the pRSET vector to readily purify the corresponding NIT2−histidine tag fusion protein for *in vitro* tests. Similarly, different segments of the *nmr* gene were cloned into pGEX-2T to express and purify NMR−GST fusion proteins. The fusion proteins were expressed in *E. coli*, purified, and tested *in vitro* for specific

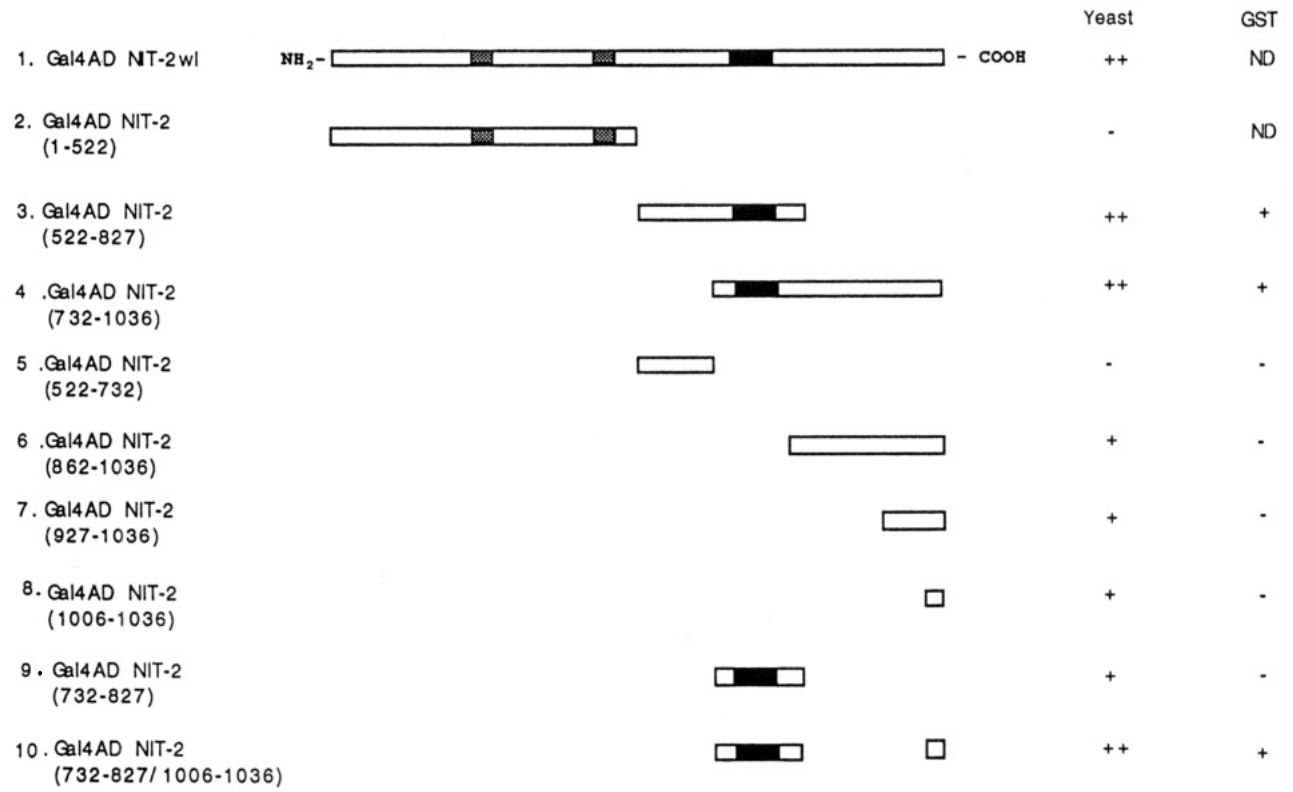

FIGURE 1: Localization of NIT2 segments which interact with NMR. The NIT2 amino acid residues fused to the GAL4 activation domain in various plasmid constructs are identified and shown diagrammatically. Solid boxes represent the NIT2 zinc finger, and striped boxes represent the two acidic domains of NIT2. Each plasmid was cotransformed into the host yeast strain along with the plasmid encoding the NMR−GAL4 DNA-binding domain fusion protein. The column labeled Yeast represents results from the yeast two-hybrid system; ++ and + colonies turned intense blue within 1 h with the filter paper assay, with ++ > +. In the cell-free assay for β-galactosidase activity, ++ transformants possessed 100 ± 30 Miller units, and + transformants, 30 ± 10 Miller units; −, no β-galactosidase activity detected. The identical NIT2 protein regions were also each expressed in *E. coli* and tested for binding with NMR using the *in vitro* assay. The column labeled GST represents results from the *in vitro* experiments, conducted as shown in Figures 3 and 4. +, NMR−NIT2 binding observed *in vitro*; −, no NMR−NIT2 interaction was detected; ND, not done.

FIGURE 2: NMR amino acid residues fused to the GAL4 DNA-binding domain in various plasmid constructs are identified and shown diagrammatically. Each construct was cotransformed into the yeast host along with the plasmid containing the NIT4−GAL4 activation domain fusion. The column labeled Yeast shows the results obtained with the yeast two-hybrid system . +, colonies turned blue within 1 h in the filter paper assay; +/−, colonies turned blue only after 5 h; −, no β-galactosidase reporter activity detected. The column labeled GST shows results of the *in vitro* assay for protein−protein binding of NIT2 to NMR as shown in Figures 3 and 4. +, strong interaction; +/−, moderate interaction; −, no interaction detected; ND, not done.

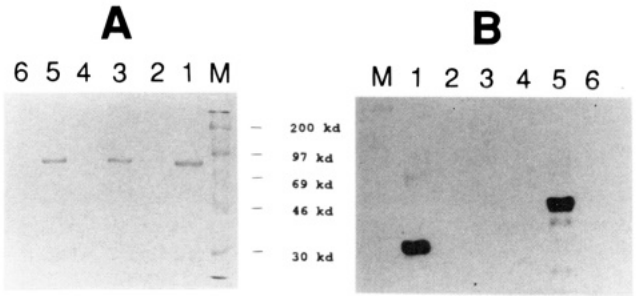

FIGURE 3: NIT2/NMR *in vitro* interaction assays. (Panel A) SDS−PAGE gel analysis of protein samples from the *in vitro* NIT2/NMR interaction assay eluted from the glutathione−agarose affinity resin. Proteins added to the resin differed in the experiment shown in each lane. Lanes: 1, GST−NMR and NIT2 fragment (residues 617−827); 2, NIT2 fragment (residues 617−827) only; 3, GST−NMR only; 4, GST only; 5, GST−NMR and NIT2 fragment (residues 732−1036); 6, NIT2 protein fragment (residues 732−1036) only; M, molecular weight markers. (Panel B) Western blot. The samples shown in panel A were transferred to a nitrocellulose filter and analyzed with anti-NIT2 antibody. Lanes are exactly the same as in panel A.

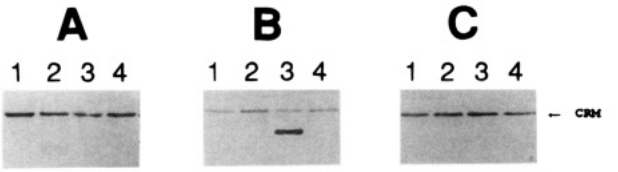

FIGURE 4: Western blots of specific NIT2/NMR *in vitro* interaction experiments. Eluates from the glutathione−agarose resin incubated with GST or GST−NMR plus NIT2 proteins were electrophoresed in SDS gels, blotted, and analyzed using anti-NIT2 antibody. Panel A, GST; panel B, GST−NMR (residues 46−488); panel C, GST−NMR (residues 46−358). Different NIT2 proteins were added to the affinity resin-immobilized GST or GST-NMR proteins. In each panel, lanes: 1, NIT2 (residues 522−732); 2, NIT2 (residues 732−827); 3, NIT2 (residues 732−1036); 4, NIT2 (residues 862−1036). The band labeled CRM identifies a nonspecific cross-reacting material.

NIT2−NMR protein−protein binding as described under Materials and Methods.

The NMR−GST fusion protein is bound by the glutathione−agarose column, but the NIT2 protein will not be retained by the affinity column unless it is bound by the immobilized NMR−GST protein. Two NIT2 protein regions (residues 617−827 and 732−1036) were bound by NMR−GST (Figure 3, lanes 1 and 5) but not to the glutathione resin alone (Figure 3, lanes 2 and 6). A truncated NMR protein (residues 46−488) that lacked 46 amino acids at its amino terminus showed positive interaction with NIT2; however, a second deleted form of NMR (residues 46−358) completely failed to interact with NIT2 (Figure 4C). The two separate regions of NIT2 which gave positive results for interaction with NMR in the yeast two-hybrid assay did not show obvious interaction with NMR in the *in vitro* assay when tested individually (Figure 4B, lanes 2 and 4). However, when a NIT2 protein containing both of these two regions was tested, a strong interaction with NMR *in vitro* was readily detectable (Figure 4B, lane 3). The zinc finger region fused with a 100 residue stretch immediately upstream exhibited some binding to NMR, but the upstream region by itself did not give a positive result in either the *in vivo* or the *in vitro* assay (Figure 4B, lane 1). This upstream region may have some stabilizing effect on the zinc finger domain.

Mutation Analysis of the Zinc Finger Region That Functions in NMR Binding. Five *nit-2* mutations that result in a total loss of NIT2 protein DNA-binding activity (Fu & Marzluf, 1990c) lead to amino acid substitutions in the zinc finger region, which was shown above to interact with NMR (Figure 5). These mutant *nit-2* genes were introduced into the vectors used both for the *in vivo* yeast two-hybrid system and for the *in vitro* system for protein−protein binding described above to test their effects, if any, upon the NIT2−NMR interaction.

Four of these mutant NIT2 proteins with substitutions in the zinc finger were totally deficient or showed a very significant reduction in the interaction with NMR. NIT2 mutations 1, 4, and 5 interacted very weakly with NMR, and the cotransformed colonies turned blue only after overnight incubation with Z buffer containing X-gal in the filter paper assay. In comparison, colonies cotransformed with NMR and wild-type NIT2 or NIT2 mutation 2 turned blue within 1 h. Significantly, NIT2 mutation 3 completely eliminated the interaction between NIT2 and NMR, as no

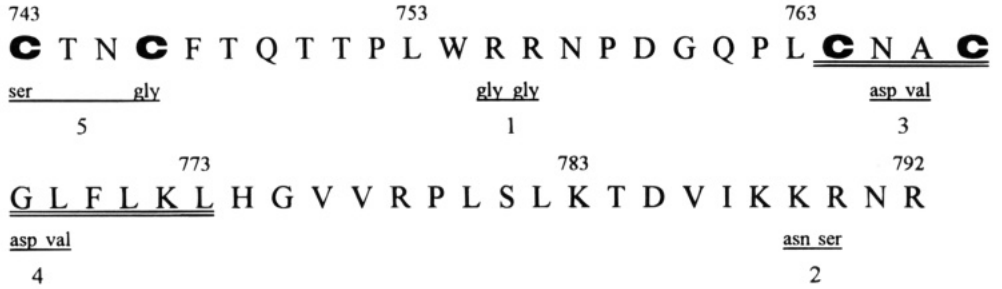

FIGURE 5: Mutations with amino acid substitutions in the NIT2 zinc finger domain. Fifty amino acids which include the NIT2 zinc finger and downstream basic region (residues 743−792) are shown. The four cysteine residues that define the zinc finger are shown in boldface, and residues constituting an α-helix are double-underlined (residues 764−773). Five independent mutant NIT2 proteins are labeled 1−5. Two amino acids were substituted in each mutant protein and are shown under the original residues, underlined and identified by mutant number. This same designation of mutants was used in Figure 6.

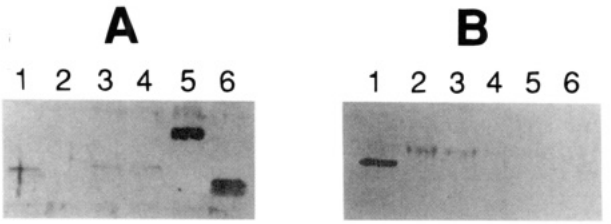

FIGURE 6: 6. *In vitro* analysis of NIT2 mutant proteins for the NIT2/NMR interaction. Wild-type or mutant NIT2 proteins plus the GST−NMR protein were added to the glutathione−agarose affinity resin. Eluates from the affinity column were electrophoresed and analyzed by Western blot using anti-NIT2 antibody. (Panel A) Lanes 1−4 contain NIT2 mutant proteins consisting of residues 711−827 + residues 1006−1036 with mutations 1, 3, 4, and 5, respectively; lane 5, NIT2 wild-type protein, residues 711−1036; lane 6, NIT2 wild-type protein, residues 732−827 + residues 1006−1036. (Panel B) Lane 1, NIT2 wild-type protein, residues 711−1036; lanes 2−6, NIT2 residues 711−827 of wild-type and mutations 1, 3, 4, and 5, respectively, as defined in Figure 5. NIT2 proteins visualized by immunodetection appear as weak bands in lanes 1, 3, and 4, and as intense bands in lanes 5 and 6 of panel A, and lane 1 of panel B. The weak bands in lanes 2 and 3 of panel B are artifactual.

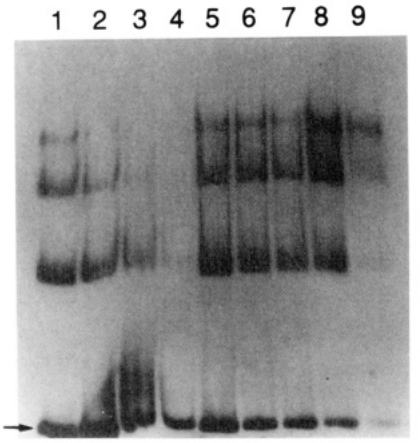

FIGURE 7: NMR inhibition of NIT2 DNA binding. The *Bam*HI−*Sty*I DNA fragment of the *lao* promoter labeled with P32 was used as the probe. In all experiments, the GST−NIT2 fusion protein (with or without different amounts of the GST−NMR, GST, or NMR−His proteins) was incubated with the *lao* promoter DNA fragment and subjected to mobility shift analysis. Lanes: 1, GST−NIT2 protein only; 2−4, plus 0.3, 0.63, and 1.25 mol of GST−NMR protein/mol of NIT2−GST, respectively; 5−7, plus 1, 2, and 3 mol of GST protein/mol of NIT2−GST, respectively; 8 and 9, plus 1 and 2 mol of a truncated NMR−His tag fusion protein/mol of NIT2−GST, respectively. Beta scanning was employed to determine the amount of shifted DNA probe/unshifted probe, and yielded the following ratios: Lanes 1−9, 1.0, 0.4, 0.35, 0.30, 1.1, 1.5, 1.7, 3.8, and 3.1, respectively. A clear inhibition of NIT2 DNA binding by NMR is evident. The unshifted DNA fragment is identified by an arrow; the upper shifted bands all represent NIT2−DNA complexes.

color change could be detected even after 2 days of incubation of the colonies with substrate.

The results obtained with *in vitro* assays with these same NIT2 mutant proteins were consistent with those obtained with *in vivo* assay. Thus, none of the mutant 3 NIT2 protein and only a trace of mutant 1, 4, and 5 proteins were bound by the GST−NMR fusion protein, as revealed by Western blots (Figure 6). In contrast, in parallel assays, two forms of the wild-type NIT2 protein were strongly bound by GST−NMR (Figure 6).

These *in vitro* experiments also provide evidence that a short α-helical region of the NIT2 zinc finger motif, identified by homology with the nuclear magnetic resonance structure of the closely related GATA-1 protein (Omichinski et al., 1993), interacts with NMR. The mutations (3 and 4) which had the strongest effect and almost completely eliminated any NIT2−NMR binding had amino acid substitutions within this α-helical region. We also performed a computer analysis with the IBI Putshell program on another stretch of the NIT2 protein. The last 14 residues of NIT2 at its carboxyl terminus were predicted by the Chou−Fasman analysis to form an α-helical structure. Thus, both regions of NIT2 which appear important for specific protein−protein binding to NMR appear to have an α-helical structure. Both the zinc finger and the carboxyl-terminal interaction regions of NIT2 are required for NIT2−NMR protein−protein binding *in vitro*; the zinc finger region alone is not bound *in vitro* by NMR (Figure 6, panel B).

The NMR Protein Inhibits NIT2 Sequence-Specific DNA Binding. The NIT2 protein binds to the *lao* gene promoter DNA fragment as visualized with distinct retarded bands in mobility shift assays. Three retarded DNA-protein bands are observed due to the presence of two NIT2-binding sites in the *lao* promoter fragment (Xiao & Marzluf, 1993). When the purified GST−NMR fusion protein was incubated with NIT2 before the target DNA was added, a clear decrease in the amount of the NIT2−DNA complexes, represented by the three retarded DNA fragments, was obvious (Figure 7). Moreover, the extent of inhibition of formation of the NIT2−DNA complexes was proportional to the amount of NMR protein which was added, and almost total inhibition of NIT2 DNA binding occurred when the molar ratio of NMR/NIT2 was 1.25 (Figure 7, lane 4). The GST protein alone had no effect on the DNA-binding ability of NIT2 (Figure 7). A truncated NMR−GST fusion protein failed to inhibit NIT2

DNA binding when present at an equimolar concentration with NIT2; when this truncated NMR−GST protein was present at a higher concentration, it appears to result in some loss of the DNA fragment, probably due to nonspecific DNA binding and a smearing throughout the gel (Figure 7, lanes 8 and 9).

DISCUSSION

NIT2 is the major positive-acting regulatory protein of the *Neurospora crassa* nitrogen metabolism circuit. It possesses a zinc finger motif which constitutes its DNA-binding domain and which is required for its trans-activation of multiple unlinked structural genes which specify nitrogen catabolic enzymes. Although the *nmr* gene was cloned and sequenced and its protein product predicted, it has not been possible to establish its role, if any, in nitrogen regulation. The *E. coli*-expressed NMR protein did not exhibit DNA binding nor did it bind glutamine, the metabolite responsible for nitrogen repression (Young et al., 1990). Furthermore, a mutant, *meaB*, of *Aspergillus nidulans* displays a similar constitutive phenotype as does NMR, but is suspected to encode a membrane protein which affects the transport of nitrogenous compounds (Caddick, 1992). However, the NMR protein could not be detected via immunoassays in membrane fractions or other subcellular fractions of *Neurospora*, suggesting it is not a structural protein, since they usually exist in relatively large amounts. Moreover, different nitrogen repression mechanisms may exist in *Neurospora* and *Aspergillus*. The *nit-2* gene can complement an *area*⁻ *Aspergillus* mutant strain, but the transformants possessed a partially derepressed phenotype (Davis & Hynes, 1987).

The results of this study provide evidence which indicates that NMR is indeed a negative-acting regulatory protein. NMR was demonstrated to interact directly with the NIT2 protein by both *in vivo* and *in vitro* assays. The specificity of the interaction was particularly striking because the binding of NMR could be attributed to two discrete regions of NIT2, both of which are postulated to form α-helical structures. Moreover, mutant NIT2 proteins with amino acid substitutions in the zinc finger region completely eliminated or severely reduced NIT2−NMR binding *in vivo* and *in vitro*. The finding that NMR can interfere with the DNA-binding activity of NIT2 suggests a plausible mechanism for its action as a negative regulator. An alternative possibility is that NMR binding at the NIT2 DNA-binding motif might shield a nuclear localization signal in the adjacent basic region and thereby prevent entry of NIT2 into the nucleus under conditions of nitrogen repression. A similar relationship between positive and negative factors is well established in the case of GAL4 and GAL80 regulatory proteins in the galactose utilization pathway of *Saccharomyces cerevisiae* (Ma & Ptashne, 1987; Lue et al., 1987). Other examples of an interaction between negative and positive factors with regulatory effects exist; e.g., the function of another important transcriptional regulator, Yin-Yang, can be inhibited by association with c-myc (Shirvastava et al., 1993).

In this study, two separate regions of the NIT2 protein were demonstrated to interact with NMR. One region is a short α-helical segment within the NIT2 zinc finger, whereas the other corresponds to a stretch of amino acids at the carboxyl terminus, which also is predicted to form an α-helix. Amino acid residues within the zinc finger motif that were identified as critical for NMR binding are also known to be essential for the DNA-binding activity of NIT2. This feature naturally led to the prediction that NMR might block NIT2 DNA binding, and this possibility was confirmed by the results of mobility shift assays. It is perhaps surprising that two distinct α-helical regions of the NIT2 protein appear to be involved in the interaction with NMR, although it is possible that these two helices actually lie near to each other in the tertiary structure of the protein.

It is also noteworthy that several lines of indirect evidence indicate that a NIT2/NMR interaction also occurs *in vivo* in *Neurospora* and plays a negative regulatory role. Random mutagenesis of the *nmr* gene identified one broad region of the NMR protein within which amino acid substitutions resulted in a constitutive mutant phenotype. This region includes amino acid residues 42−300 (Jarai & Marzluf, 1990). A comparison of the amino acid sequences of the NMR protein from different fungal species revealed that one region was highly conserved; this region (residues 210−260) occurs within the sensitive region identified by the random mutagenesis study (Young & Marzluf, 1991). In the work reported here, only one segment of NMR showed a positive, although weak, interaction with NIT2, and it is also located within this very same region (residues 113−253). An interesting point that deserves mention is that in the mutagenesis study described above, it was concluded that there existed no extremely sensitive region of NMR protein within which amino acid substitutions affected NMR function. The experiments reported here failed to detect particular regions of NMR that can account for the specificity or strength of its interaction with NIT2. This feature may be due to the fact that NMR must interact with two discontinuous regions of NIT2, and may also indicate that a correct folding of NMR is required for its binding specificity and a native conformation is only achieved with the full-length NMR protein. The importance for nitrogen control of one of the regions of NIT2 identified here that interacts with NMR has received strong support from a separate study which demonstrated that deletion of the final 30 amino acids of NIT2 resulted in a significant loss of nitrogen repression of the *nit-3* structural gene (Pan et al., unpublished). This segment of NIT2 corresponds to the carboxyl-terminal α-helical region which specifically binds to NMR.

The results reported here, which demonstrate a specific interaction between the positive-acting NIT2 and the negative-acting NMR regulatory proteins, provide important new information about the operation of the nitrogen control circuit in *Neurospora*. However, the precise way in which nitrogen repression and derepression occurs is still not clear; one important missing element is the mechanism by which the nitrogen-repressing metabolite glutamine is sensed. Glutamine might be bound by the carboxyl-terminal region of NIT2, by NMR, or, alternatively, by a site provided by the NIT2−NMR complex.

Our finding of the interaction between the zinc finger domain of NIT2 with NMR may have broader structural and functional meaning beyond its function in nitrogen regulation in *Neurospora crassa*, particularly since additional functions have been found associated with the DNA-binding domain of other trans-acting factors (Geisberg et al., 1994; Liu & Green, 1994). Regulatory proteins of the GATA family are found widely distributed in many species and share with NIT2 a highly conserved zinc finger DNA-binding motif.

Thus, it seems possible that the DNA-binding activity of other GATA proteins may be modulated by proteins which recognize the α-helical region of the finger motif.

REFERENCES

Caddick, M. X. (1992) in *Molecular Biology of Filamentous Fungi* (Stahl, U., & Tudzynski, P., Eds.) pp 141−152, VCH Press, Weinheim.

Chiang, T. Y., & Marzluf, G. A. (1994) *Biochemistry 33*, 576.

Davis, M. A., & Hynes, M. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3753.

Dunn-Coleman, N. S., Tomsett, A. B., & Garrett, R. H. (1981) *Mol. Gen. Genet. 182*, 234.

Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y. Z., Kilburn, A. E., Lee, W. H., & Elledge, S. J. (1994) *Genes Dev. 7*, 555.

Exley, G. E., Colandene, J. D., & Garrett, R. H. (1993) *J. Bacteriol. 175*, 2379.

Feng, B., Xiao, X., & Marzluf, G. A. (1993) *Nucleic Acids Res. 21*, 3989.

Fields, S., & Song, O. K. (1989) *Nature 340*, 245.

Fu, Y. H., & Marzluf, G. A. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8243.

Fu, Y. H., & Marzluf, G. A. (1987b) *Mol. Cell. Biol. 7*, 1691.

Fu, Y. H., & Marzluf, G. A. (1990a) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5331.

Fu, Y. H., & Marzluf, G. A. (1990b) *J. Biol. Chem. 265*, 11942.

Fu, Y. H., & Marzluf, G. A. (1990c) *Mol. Microbiol. 4*, 1847.

Fu, Y. H., & Marzluf, G. A. (1990d) *Mol. Cell. Biol. 10*, 1056.

Geisberg, J. V., Lee, W. S., Berk, A. J., & Ricciardi, R. P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2488.

Hope, I. A., & Struhl, K. (1985) *Cell 43*, 177.

Ito, H., Futuda, Y., Musata, K., & Kimura, A. (1983) *J. Bacteriol. 153*, 163.

Jarai, G., & Marzluf, G. A. (1990) *Mol. Gen. Genet. 222*, 233.

Liu, F., & Green, M. (1994) *Nature 368*, 520.

Lue, N. F., Chasma, D. I., Buchman, A. R., & Kornberg, R. D. (1987) *Mol. Cell. Biol. 7*, 3446.

Ma, J., & Ptashne, M. (1987) *Cell 50*, 137.

Marzluf, G. A. (1993) *Annu. Rev. Microbiol. 47*, 31.

Omichinski, J. G., Clore, G. M., Schaad, O., Felsenfeld, G., Trainor, C., Appella, E., Stahl, S. J., & Gronenborn, A. M. (1993) *Science 261*, 438.

Premakumar, R., Sorger, G. J., & Gooden, D. (1980) *J. Bacteriol. 144*, 542.

Shirvastava, A., Salequie, S., Kalpana, G. V., Artandi, S., Goff, S. P., & Calame, K. (1993) *Science 262*, 1889.

Xiao, X. D., & Marzluf, G. A. (1993) *Curr. Genet. 24*, 212.

Young, J. L., & Marzluf, G. A. (1991) *Biochem. Genet. 29*, 447.

Young, J. L., Jarai, G., Fu, Y. H., & Marzluf, G. A. (1990) *Mol. Gen. Genet. 222*, 120.

Yuan, G. F., Fu, Y. H., & Marzluf, G. A. (1991) *Mol. Cell. Biol. 11*, 5735.

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