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NIT-2, the major positive regulatory protein of the *Neurospora crassa* nitrogen control circuit: DNA binding, nuclear localization, and interaction with NMR.

Xiao, Xiaodong, Ph.D.

The Ohio State University, 1994
NIT-2, the major positive regulatory protein of the *Neurospora crassa* nitrogen control circuit: DNA binding, nuclear localization, and interaction with NMR

Dissertation

Presented in Partial Fulfillment of the Requirement for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

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* * * * *

The Ohio State University

1994

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G. A. Marzluf
To My Family
ACKNOWLEDGEMENT

I express sincere appreciation to Dr. George A. Marzluf for giving me this research opportunity, and for his guidance and insight throughout the research. Thanks go to the other members of my advisory committee for their suggestions and comments. Gratitude is expressed to former and present members of Dr. Marzluf's lab for sharing their ideas and techniques with me. I offer sincere thanks to my wife, Yang Feng, for your persistent support and encouragement.
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PUBLICATIONS

   substitutions in the zinc finger of NIT-2, the nitrogen
   regulatory protein of Neurospora crassa, alter promoter

2. Feng, B, X. D. Xiao, and G. A. Marzluf. 1993 Recognition
   of specific nucleotide bases and cooperative DNA binding
   by the trans-acting nitrogen regulatory protein NIT-2
   of Neurospora crassa.  Nucleic Acids Res. 21: 3989-
   3996.

FIELDS OF STUDY

Major field: Biochemistry
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Biology of *Neurospora crassa*

*Neurospora crassa* is a eukaryotic organism and a member of the fungal class Ascomycetes. It has been referred to be as the pink bread mold because of its pink colored macroconidia growing on bakery products (1).

*Neurospora crassa* is heterotrophic. It can use a variety of substances as its nutrition source. Acetate, glycerol, monosaccharides, and some polysaccharides can be used as carbon sources, and nitrate, nitrite, ammonia, glutamine and other amino acids can be used as nitrogen sources. Besides nitrogen and carbon sources, sulfur, phosphorus, salts and biotin are required for growth. The nutrition requirements of *Neurospora* are relatively simple, and growth conditions can be modified by alternating the ingredients of the media supplied to *Neurospora* (2). This has been proven to be valuable in the study of *Neurospora* metabolism.

*Neurospora crassa* has both sexual and asexual life cycles (3). In its sexual life cycle, conidia of one mating type (A or a) are spread over the surface of a culture of the other mating type. The parental nuclei divide many times before
nuclear fusion happens, which is followed by two meiotic and one mitotic divisions, giving rise to 8 ordered haploid ascospores. A unique phenomenon observed with Neurospora within the sexual cycle, named RIP (repeat induced point mutation), will be described later.

The asexual life cycle begins with the haploid ascospores. The ascospores germinate and develop into a vegetative culture that is composed of multinucleate, branched filaments segmented by incomplete walls. A system of this culture is called a mycelium, from which two kinds of conidia can develop, microconidia and macroconidia. The conidia can then germinate and become vegetative cultures, and so continue the asexual cycle. Macroconidia are multinucleate and used to inoculate vegetative cultures; microconidia are smaller and uninucleate. Microconidia have poor viability and are not used for vegetative culture, but they are useful for genetic studies (4).

Neurospora has a relatively small genome. Each haploid nucleus contains seven chromosomes, and each of them has a size that is no larger than that of E. coli (5). The vegetative growth of Neurospora also shares similarities with E. coli. This makes Neurospora a relatively easy system to investigate. At the same time, cytologically, Neurospora has many of the features of eukaryotic cells. Among them the most important ones are the presence of nuclei, mitochondria and endoplasmic reticulum (6). The use of Neurospora provides a
valuable opportunity to study complicated processes within eukaryotic cells in a simple system.

The advantage of working with Neurospora has long been recognized. Through several decades of study, thousands of Neurospora mutants concerning different characteristics have been isolated. New mutants can be made with great ease. This provides extra advantages for working with Neurospora gene regulation.

Transformation in Neurospora

It is important to introduce genetic material, usually DNA, into a living organism to examine the function of the gene encoded by this DNA, and the effects of this gene on many other genes. This problem was easily solved with prokaryotes. E. coli cells which are competent for receiving exogenous DNA can be easily prepared by treating them with simple inorganic chemicals, such as CaCl₂. Besides chemical treatment, electroporation was also proven to be very efficient and a simple method for E. coli transformation.

Compared to E. coli, transformation in eukaryotic cells is more complicated. In mammalian cells, electroporation, microinjection and cell fusion are frequently used for transfection. In Fungi, like Neurospora crassa, the transformation process is rendered more difficult because of the existence of a cell wall which must be removed before the cells can be made competent. Transformation in filamentous
Fungi like Neurospora includes several important steps: (1) removal of cell wall, (2) introduction of genetic material into spheroplasts, and (3) regeneration of cells.

In overcoming the first problem, the first success came from the transformation with an Neurospora inositol-requiring mutant (inl), as DNA isolated from the wild type (inl\(^+\)) strain was able to confer inositol independence to the mutant strain (7,8). It was thought that the inositol mutant was particularly competent to take up DNA because of its greater porosity when starved of inositol.

The real breakthrough came when Hutchinson and Hartwell found that glucanase was able to dissolve the Saccharomyces cerevisiae cell wall, and the resulting spheroplasts could be stabilized with 1 M sorbitol (9). This approach was later used to prepare N. crassa spheroplasts. Akins and Lambowitz later identified Novozyme 234 as particularly good for Neurospora spheroplast preparation, and this method has been used widely (10).

Unlike yeast, in which exogenous genetic materials integrate into the host genome by homologous recombination, genetic material introduced into Neurospora cells integrates mostly by random insertion, only a small percentage integrates by homologous recombination.

The regeneration of spheroplasts is straightforward. Appropriate maintenance of an osmotic stabilizer and selection for nutrition, and/or antibiotic resistance will produce
Akins and Lambowitz (10) developed a general method for cloning *Neurospora crassa* genes. They based their method on the facts that (1) high efficiency transformation was available by introducing Novozyme into producing spheroplasts, and (2) sib selection was proven to be practical in cloning *Neurospora* genes. Using a *N. crassa* genomic library constructed in plasmid pRAL1, they were able to clone genes complementing auxotrophic mutants, nic-1 and in1. Vollmer and Yanofsky (11) went further in cloning *Neurospora* genes by constructing a *Neurospora* genomic DNA library in a cosmid vector, which carries larger DNA insertions than conventional plasmids, so as to increase the cloning efficiency. Since then, many important genes have been cloned.

Other than complementation, other less popular yet also efficient approaches have been used in cloning fungal genes, techniques such as complementation of *E. coli* mutants (12), screening of genomic libraries by hybridization with DNA probes (13), screening of cDNA libraries by using antibodies against proteins of interest (14), and chromosome walking.

Repeat Induced Point Mutation

One unique feature of *Neurospora* that adds to its power for molecular biology is the RIP phenomenon. It was found that in *Neurospora*, duplicated DNA sequences are altered in the sexual phase, whether they are physically linked or not. RIP
occurs between the stage of fertilization and nuclear fusion. Proximity of the duplicated elements influences the severity of alterations by the RIP process. The nature of the change is exclusively G-C to A-T point mutations. Changes occur mostly at sites where adenine is 3' of cytosine, such that C-G is changed into A-T (15, 16, 17). Methylation may play an active role in the RIP process. But it is not necessarily the cause, in that some constitutively methylated DNA sequences go through crosses unaltered.

This method provides great convenience in studying gene function in Neurospora. Specific segments of genes can be accurately mutated by transforming Neurospora to duplicate certain DNA segments, then crossing the transformants with Neurospora of another mating type. A fraction of the resulting ascospores will be mutant defective of function encoded by the DNA segments that were duplicated.

Gene regulation in Neurospora Crassa

Because of the relative simplicity of the Neurospora crassa genome and the many other advantages of working with Neurospora mentioned above, the gene regulation pattern of several metabolic circuits have been studied to a great extent. Its resemblance to higher eukaryotes in gene regulation has made this model system more valuable.
The phosphorus acquisition system of Neurospora includes a number of genes. Some of the genes encode enzymes that are required for growth when preferred phosphorus sources are limited, enzymes like repressible alkaline phosphatase (pho2) (18) and repressible acid phosphatase (pho3) (19). Several regulatory proteins are also involved. Nuc-1 is the major positive regulatory protein (20). Mutation of nuc-1 leads to Neurospora's inability to produce enzymes involved in phosphorus acquisition. Preg is believed to be the negative regulator, mutation of which leads to the constitutive expression of those repressible structural genes. Nuc-2 is another positive regulator whose function is to suppress the negative regulator preg. Recent results showed that nuc-1 is a DNA binding protein having a HLH DNA binding motif (21).

The sulfur regulatory circuit is similar but has significant differences from the phosphorus circuit. The sulfur circuit contains a set of unlinked structural genes such as sulfate permease (cys-14) (22), choline sulfate permease and aryl sulfatase (23). Those genes are coregulated by cys-3, the major positive regulatory protein, and scon-1 and scon-2, two negative regulatory proteins (24). Cys-3 was cloned and its function studied (25). The CYS-3 protein is composed of 236 amino acids. A Leucine zipper structure and an adjacent upstream basic region confer on CYS-3 specific DNA binding ability, which was proven to be essential for its regulatory function (26). CYS-3 exists as a dimer, and
Dimerization is important for its DNA binding ability (27, 28). CYS-3 was identified from the *N. crassa* nuclear extract. One interesting observation is that CYS-3 protein was totally missing in nuclear extract from a *Neurospora* strain with a *cys-3* point mutation, suggesting autoregulation of *cys-3* (29). Additional indirect evidence suggesting autoregulation of *cys-3* is that CYS-3 can bind to a sequence within its own promoter region. How *scon-1* and *scon-2* work negatively remains unknown.

Quinic acid can be used by *Neurospora crassa* as a carbon source. Genetic and biochemical studies led to the recognition of genes involved in this process (30). As in other metabolic circuits, the quinic acid metabolism pathway includes structural genes, such as 5-dehydroquinate hydrolase (*qa-2*), quinate: NAD+ oxidoreductase and shikimic acid dehydrogenase encoded by a bifunctional gene (*qa-3*), 5-dehydroshikimate dehydratase (*qa-4*), and two presumptive *qa* structural genes, *qa-x* and *qa-y*, whose exact functions are unknown (31). Recent studies using gene replacement suggested that *qa-x* may be a quinic acid permease gene. The expression of these structural genes are under the control of regulatory gene *qa-1P*, which encodes a positive regulatory protein and *qa-1S*, which is a negative regulator (32).

A unique feature of the *N. crassa* quinic acid metabolic pathway as compared to other metabolic pathways in *Neurospora* is that the *qa* genes are physically adjacent and form a gene cluster (33), excluding *qa-y*. DNA binding sites for *qa-1P* have
been identified within the qa-x-qa-2 intergenic region (34). This pattern of gene organization has been shown to be highly conserved in many Neurospora species.

Despite the differences between the organization of genes and the number of genes involved in each metabolic pathway, the way in which each pathway responds to variable growth conditions shares great similarities. Each pathway has at least one major positive regulatory protein which is capable of DNA binding, and usually at least one negative regulator whose function is largely unknown, and a number of structural genes who have DNA binding site(s) within their promoter regions for their respective positive regulator.

The Nitrogen Metabolic Circuit in Neurospora crassa

The nitrogen metabolic circuit is one of the best investigated regulatory circuits in Neurospora crassa. Study of this circuit is supported by a strong background knowledge of its genetics. Recent introduction of the molecular biology approaches to the study of this circuit has improved significantly our understanding of the process of gene regulation in Neurospora.

The nitrogen metabolic circuit of N. crassa includes a number of structural genes, which enable Neurospora to utilize secondary nitrogen sources, such as nitrate, nitrite, purines, amino acids, etc. when preferred nitrogen sources, e.g., glutamine or ammonia, are not available. Genes include
nitrate reductase (nit-3) (36,47), nitrite reductase (nit-6) (37), L-amino acid oxidase (lao) (38), and allantoicase (alc) (39). The expression of these genes requires the activation function of the positive regulatory protein NIT-2. NIT-4 is a pathway specific regulatory protein that is needed specifically for nit-3 and nit-6 expression. NMR, on the other hand, is a negative regulatory protein whose function is to suppress the expression of the structural genes in the presence of sufficient preferred nitrogen sources (35) (Figure 1).

Nit-2 was cloned and its function studied (40). NIT-2 appears to be a protein composed of 1036 amino acids. The cloned nit-2 gene can complement the nit-2 mutant strain. The most significant feature of NIT-2 is its cys2cys2 single zinc finger motif that is highly homologous to that of GATA-1 from chicken (41). It has been found that this zinc finger domain is important for NIT-2's DNA binding ability, as mutations within the zinc finger motif destroy NIT-2's DNA binding ability, and the mutant NIT-2 protein could not transform nit-2 mutant strain (42,43,44). This suggests NIT-2's DNA binding ability is essential for its positive regulatory function. Another important structural feature of NIT-2 is its two acidic domains upstream of its zinc finger region. It has been demonstrated that deletion of the two acidic domains eliminates NIT-2's activation function, but detailed studies are needed to investigate the function of the individual
acidic domains.

Nit-4 was identified as a pathway specific regulatory protein whose mutation would lead to N. crassa's inability to use nitrate and nitrite as nitrogen sources. Nit-4 was also cloned and its function studied (45). Sequence analysis showed that NIT-4 contains 1090 amino acids. NIT-4 appears to possess a Zn(II)$_2$Cys$_6$ binuclear-type zinc finger which is homologous to that of GAL-4 from yeast (46,48). Its DNA binding ability has been demonstrated. NIT-4 contains two regions close to its C-terminus that are rich in glutamine. A similar structural motif was also found in the mammalian regulatory protein SP1, and has been shown to be important for SP1's activation function (49).

NMR is a negative regulatory protein of the N. crassa nitrogen circuit. Mutation of nmr leads to the constitutive expression of nitrogen related structural genes, even in the presence of preferred nitrogen sources (50,51). The nmr gene was cloned and it was found to encode a putative protein with 488 amino acids (52). No specific structural motif has been identified in the NMR protein that could suggest the way NMR exerts its negative regulatory effect. NMR protein does not bind to glutamine, and no evidence suggested that NMR could form dimers or oligomers. The effort to localize NMR within native N. crassa cells, which could be valuable in assessing NMR's function, was not successful (53). The lack of understanding of the negative action of nmr represents a major
uncertainty in studies of nitrogen regulation.

Nitrogen Metabolism in Other Organisms

The nitrogen metabolic regulation pattern of *Neurospora crassa* is shared by other species, e.g., *Aspergillus nidulans*. In *Aspergillus nidulans*, the nit-2 counterpart, area, has a zinc finger motif that is 98% homologous to nit-2 (54); AREA has two acidic domains upstream of its zinc finger domain. These two acidic domains and several other regions whose function is not known, are homologous to different extents to NIT-2. These conserved regions may be structurally or functionally important. Recently, the nit-2 counterpart from *Penicillium*, nre, has been identified (Haas et al. in preparation). The homology between nit-2, area and nre is very significant, suggesting the similar regulatory roles they have. It is interesting to note that the nitrate reductase gene from tomato carries NIT-2 binding sites within its promoter region (55), suggesting the existence of nit-2 like plant gene, and possibly the existence of a nitrogen metabolic circuit similar to that of *Neurospora* in tomato and other plants.

The Nit-4 counterpart in *Aspergillus* is nirA (56). NIRA and NIT-4 show homology through the N-terminal half, including a zinc cluster region, but are strikingly different throughout their carboxyl termini. Unlike NIT-4, NIRA lacks a glutamine rich region, yet a hybrid composed of the N-terminal
517 residues of NIT-4 and 397 C-terminal residues of NIRA functions as a substitute for NIT-4 in Neurospora.

The nitrate reductase (niaD) and nitrite reductase (niiA) genes of Aspergillus are the counterparts of nit-3 and nit-6 from Neurospora crassa (57). The organization of niaD and niiA is different from that of nit-3 and nit-6, as niaD and niiA are physically closely linked. The intergenic region between niaD and niiA gene contains binding sites for both AREA and NIRA. In Neurospora, the nit-3 and nit-6 are not linked and each has its own promoter.

In the yeast Saccharomyces cerevisiae, nitrogen metabolism is different from that of Neurospora (58). No nitrate reductase has been found in S. cerevisiae, and in yeast more DNA binding factors are involved in nitrogen regulation. Three kinds of DNA elements have been identified within the promoter regions of yeast nitrogen metabolic structural genes. They are UAS (upstream activation sequence), UIS (upstream induction sequence), and URS (upstream repression sequence) (59,60). The DAL80 protein, which is the negative regulator of the yeast purine metabolism system, was shown to recognize a specific GATA sequence. So far we do not have knowledge of any negative regulator from the Neurospora nitrogen metabolic circuit that is capable of binding to DNA (61,62).
GATA Binding Regulatory Proteins

It is generally recognized that transcriptional regulatory proteins function by binding to the upstream DNA sequences of the genes under their control. Different DNA binding motifs have been identified, including helix-loop-helix, leucine zipper, homeo box and HMG (high mobility group). Another large group of DNA binding factors share one common feature, and contain a zinc ion in their DNA binding motif. They are generally referred to as zinc finger proteins. So far five different families of zinc finger proteins have been found, depending mainly on the way they chelate zinc. The representatives for each family are TFIIIA from Xenopus oocytes, which contains repetitive (9) Cys₂His₂ zinc finger motifs (63); glucocorticoid receptor from mammals has two Cys₂Cys₂ zinc coordination sites (64); GAL4 from yeast has a Zn(II)Cys₆ zinc finger motif which is frequently called a zinc cluster (65); GATA-1 from human has a Cys₂Cys₂ single zinc finger (66); MyT1 identified from glial cells possesses a Cys₂CysHis zinc finger motif (67).

NIT-2 with its Cys-x₂-Cys-(x₁₇)-Cys-x₂-Cys zinc finger motif, belongs to the group represented by GATA-1. Surprisingly, many members of this family recognize the same core DNA sequence, GATA.

GATA, as a cis-element, was first defined in analyses of globin genes. This DNA sequence was found to be necessary for the expression of chicken globin genes, human 3'-β-globin
gene, and overexpression of human τ-globin gene in response to a mutation causing the hereditary persistence of fetal hemoglobin syndrome (68). Later it was found that other than globin genes, the expression of other erythroid-expressed, nonglobin genes also requires the presence of the GATA element (69).

As expected, a nuclear factor capable of recognizing the GATA sequence was isolated and its gene cloned from mammalian cells, the gene now known as GATA-1 (70). What was unexpected was that by doing cross-hybridization screening and cDNA cloning, more GATA factors were found, and they were named GATA-2, GATA-3 and GATA-4 (71,72,73). The distribution of the above-mentioned GATA factors within different tissues differ from each other, suggesting different roles they may have in development. It has been established that GATA-1 is essential for erythroid development of both primitive and definitive lineages (74).

An important question that needs to be answered is how GATA factors themselves are controlled and what exact genes, including possible regulatory factors, are under their control. As genes are often controlled by more than one factor, the exact roles GATA factors may have on the expression of other genes may be complicated, and much more work is needed to understand this.
It is interesting that the DNA binding motif possessed by the members of GATA factor family has been conserved throughout the course of evolution. Other than the GATA factors found in mammalian cells, GATA factors with a similar DNA binding motif have been identified in fungi, plants, drosophila, and C. elegans, suggesting the wide range of function GATA proteins have (76).

In fungal species, many GATA factors have been found. Area and nit-2 are the major positive regulatory proteins in the nitrogen metabolism circuit of Aspergillus and Neurospora respectively. Dal80 is the negative regulatory protein in the yeast purine catabolic pathway. Urbs1, a gene recently identified from Ustilago maydis, encodes a protein that has a zinc finger motif similar to GATA-1 (75). Even though these proteins bind to the same core DNA sequence, their functions are variable. It is not clear what defines their specific roles in addition to their specific DNA binding. It is suggested that factors associated with these GATA factors may contribute to their specificity. The different expression pattern of GATA factors may also limit their function to certain tissues (77). One other possibility is that the minor difference between the finger motifs of different GATA factors may enable them to differentially recognize GATA sequence with different flanking sequences. As more regulatory factors are found that are functionally and structurally related to GATA factors, the overall picture of GATA factors will become
clear.

Yeast Two Hybrid System

Fields and Song developed an in vivo approach to detect protein-protein interactions in yeast (94). This approach takes advantage of the fact that most regulatory proteins are modular, and their functional domains are separable. These individual domains can function independently, but cannot activate gene expression unless they are connected. GAL4 is the major positive regulatory protein of the lactose catabolic pathway in yeast, Saccharomyces cerevisiae. Its DNA binding and activation domains are well defined (102). The yeast two hybrid system includes two shuttle vectors which allow genes of interest to be fused with the GAL4 DNA binding domain and activation domain separately. The two vectors with different fusion constructions can then be cotransformed into yeast strain carrying a beta-galactosidase (β-gal) gene under the control of GAL4. If the genes of interest encode proteins that are capable of interacting with each other, the two functional domains of GAL-4 will be connected, and β-gal will be activated. Simple enzyme activity assays can thus detect interactions between proteins. This method provides an in vivo assay, with which even weak protein interactions can be readily detected. Moreover, if a gene fused with GAL4 DNA binding domain contains protein motifs that can act as activation domains, this single construction can activate β-
gal expression efficiently. This has been referred to be as a one hybrid system.

Goals of this research

One of my research goals was to examine the residues constituting the zinc finger region, so as to understand if changes of the amino acids within this region may lead to changed DNA binding specificity, and to identify which residues are important for DNA binding. NMR resolved structures of zinc finger families represented by TFIIIA and the glucocorticoid receptor but not GATA proteins are available. My study was designed to help to build a model that describes how NIT-2 interacts with DNA. Several residues within the NIT-2 zinc finger loop region were studied by introducing mutations in the positions of these residues, and the function of the altered NIT-2 proteins was analyzed by enzyme assays and in vitro EMSA (electrophoretic mobility shift assay). My results showed that changes of two conserved residues had different effects on NIT-2 function, suggesting different structural roles. A less conserved residue was not important for NIT-2 function.

My second goal was to study NIT-2 as a whole protein. Previous studies of NIT-2, mostly of its DNA binding characteristics, were carried out with a NIT-2/β-gal fusion protein. It was desirable to examine the full length protein. I was able to express full length NIT-2 in E. coli, but the
protein obtained in this way was insoluble and was not functional. In an effort to examine full length NIT-2 and study its important features, I identified NIT-2 in N. crassa nuclear extracts. Characteristics that are important in understanding NIT-2's regulatory role were examined with the native protein present in nuclear extracts. One question which puzzled researchers involved in the study of fungal nitrogen regulation is how negative regulation is exerted on the circuit when preferred nitrogen sources are available. In Neurospora, even though a negative regulatory gene, nmr, was identified, studies of it provided little clue as to how nmr negatively regulates the nitrogen circuit. My study revealed that NMR interacts directly with the NIT-2 protein, and this interaction probably blocks NIT-2's DNA binding ability in vivo.
CHAPTER II
MATERIALS AND METHODS

Materials

The Escherichia coli strain, XL1-blue (Stratagene) was used as a host for subcloning and protein expression. Bl21 (DE3) was used specifically for protein expression. Plasmid pBluescript was from Stratagene. Plasmid pRSET was from QIAGEN. Plasmid pSL1180 was developed by Jurgen Brosius and used extensively for subcloning in yeast two hybrid system.

Neurospora wild type strain and nit-2 mutant strain (allele nr 37) were obtained through the Fungal Genetics Stock Center, Kansas City, Kan. Nif-2 RIP mutant was prepared by H. G. Pan.

Yeast strain Y153CHR and two shuttle vectors, pJS246 and pACT used in yeast and E. coli transformation were developed by Durfee et al (78).

Growth Conditions of Neurospora for Growth Test and Enzyme Assay

To prepare mycelia for enzyme assay and growth tests, flasks containing Vogel's complete media were inoculated with conidial suspensions of wild type or mutant strains and grown
at 30°C for 16 hrs. The mycelia were collected and washed with sterilized water, and put into Vogel-N media supplemented with 20 mM KNO₃, 10mM hypoxanthine, or 5 mM phenylalanine respectively for enzyme assay of nitrate reductase, allantoicase or L-amino acid oxidase, and for growth test under these three different conditions.

Nitrate reductase, Allantoicase and L-amino acid oxidase assays

For nitrate reductase assay, a method described by Garrett (79) was used. Mycelia (0.5 g) were ground with 0.5 g of sand plus 2 ml of 0.1 M KPO₄, pH 6.8, 1 mM β-mercaptoethanol, 0.5 mM EDTA, 1% NaCl in a mortar. The suspension was spun in an Eppendorf centrifuge for 5 minutes. The supernatant was then collected for enzyme assay. One hundred ul of supernatant was assayed in a volume of 0.5 ml containing 50 mM KPO₄, pH 7.75, 50 mM NaN₃, 25 mM Na₂SO₃, and 0.1 mM FAD. This mixture was first incubated at 30°C for 10 min, and then NADPH was added to a final concentration of 0.02 mM. The reaction was allowed to proceed for 20 min and stopped by adding 100 ul of 25% barium acetate. The reaction mixture was centrifuged and the precipitate removed. One half ml of 1% sulfanilamide in 20% HCl, 0.5 ml of 0.13% N-(1-napthyl)-ethylene diamine dihydrochloride, and 1.5 ml water were added to the supernatant. The absorbance at 540 nm was read for each sample against a zero time blank.
L-amino acid oxidase activity was assayed according to the protocol described by Friedeman (80) with some modifications. Mycelia prepared as before (1 gram) were resuspended in 5 ml of cold 0.05 M potassium phosphate (pH 6.1) buffer and ground in a mortar. The supernatant was collected after centrifugation and brought to 95% saturation with solid ammonium sulfate and incubated on ice for 30 min. The mixture was then centrifuged and the pellet resuspended in 1 ml of cold 0.05 M potassium phosphate buffer, pH 6.1 and dialyzed against 500 vol of the same buffer, giving the crude enzyme extract. The assay mixture contained 2 mM L-leucine in 200 mM potassium phosphate buffer pH 7.6. After addition of 0.4 ml of the crude extract to bring the whole mixture to 1 ml, the reaction was allowed to proceed for 40 min at room temperature. After this, 1 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl was added and incubation was continued for another 5 min. Finally, 2 ml of absolute ethanol was added slowly followed by 5 ml of 2.5 M NaOH. After 5 min, the OD 550 was determined.

Allantoicase assay was done according to the method of Reinert and Marzluf (81). One half gram of mycelia was ground with 0.5 ml of 0.1 M Tris-HCl buffer, pH 7.1 and 0.4 gram of sand in a mortar. The mixture was then transferred to 1.5 ml Eppendorf tube and 0.5 ml more Tris-HCL buffer was added. After centrifugation, the supernatant was ready for enzyme assay. One hundred ul of crude extract was added to a test
tube containing 5.7 umoles of potassium allantoate and 100 umoles Tris-HCl, pH 7.1 to a final volume of 1.0 ml. After incubation at 30°C for 10 min, 1.0 ml of 0.5 N NaOH was added to stop the reaction. One min later, 4 ml of 0.4 M Phosphate buffer, pH 7.0 and 1 ml of freshly made 0.33% phenylhydrazine-HCl were added to the mixture which was held at room temperature for at least 5 min, and then cooled on ice. Five ml of cold conc. HCl and 1 ml of 50 mM potassium ferricyanide solution were added to develop color. After 15 min at room temperature, the OD
535
 was determined. Identical samples with zero reaction time were used as blanks.

Plasmid Isolation from E. coli

The procedure for small scale isolation of plasmid is the same as the well established protocol (82). A single colony was used to inoculate 5 ml LB (1% tryptone, 0.5% yeast extract, and 1% NaCl) for overnight growth at 37°C in a water bath shaker. The culture was then collected by centrifugation and the supernatant discarded. One hundred ul of solution I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 15% sucrose) was added to the E. coli pellet to resuspend it completely. Two hundred ul of Solution II (0.2 N NaOH, 1% SDS) was then added to lyse the cells. After 5 min at room temperature, 150 ul of solution III (3 M sodium acetate, pH 5.2) was added to the mixture and the mixture was put on ice for 5 min. Five min later, the mixture was spun in an Eppendorf centrifuge, and the
supernatant was saved. Two and half volumes of absolute ethanol was added to the supernatant and after two minutes at room temperature, the plasmid DNA was collected by centrifugation at top speed in a microcentrifuge. The DNA pellet was washed once with 70% ethanol and air dried or speed vacuum dried. Plasmid DNA obtained with this process is good for restriction digestion. If the plasmid DNA is needed for DNA sequencing, Neurospora or yeast transformation, the DNA was further purified by treating with RNase 1, then extracting once with phenol and chloroform.

If a large amount of plasmid is needed, the above described procedure could be scaled up easily without changing the quality of plasmid DNA. In some of my experiments, PEG (polyethylene glycerol) was used to precipitate DNA instead of using ethanol. No significant difference was noticed.

Large Scale DNA Isolation from Neurospora crassa

A large amount of Neurospora genomic DNA was needed in my study for PCR cloning of certain DNA fragments. The procedure used was as described by Metzenberg (83). Wild type conidia were inoculated to Vogel complete media to 0.2 at A420 and allowed to grow for 18 hrs. The mycelia were collected and ground in mortar and pestle using a few glass beads. The homogenate was suspended in 100 ml of extraction buffer (250 mM Li:EDTA, 0.5% Triton, and 250 ug/ul pronase). This
suspension was incubated at 30°C for 18 hrs with gentle shaking. This mixture was then centrifuged at 5000 rpm (GSA rotor) for 15 min to remove glass beads and cell debris. Two and a half vol of cold absolute ethanol was added and the sample was centrifuged at 10,000 rpm for 10 min. The pellet was dissolved in 40 ml of low salt buffer (1 mM NaCl, 1 mM Tris-HCl, 0.1 mM Na₂EDTA, pH 7.4). Six volumes of ethanolic perchlorate was added to this solution at room temperature, and the mixture was put on ice for at least 30 min. The DNA was then collected by centrifugation at 10,000 rpm (SS-34 rotor) for 10 min and redissolved in 28 ml of low salt buffer and 15 ml of high salt buffer (100 mM NaCl, 25 mM Tris-HCl, 2 mM Na₂EDTA, pH 7.4) containing 300 ug/ml preboiled RNase A. This RNase A digestion was allowed to proceed at 37°C for 1 hr. The mixture was then extracted with phenol and chloroform once. Two and a half vol of ethanol was added to supernatant from last step and the mixture was chilled for 0.5 hrs. The DNA pellet was obtained by centrifugation at 10,000 rpm (SS-34 rotor) for 10 min. The DNA was stored as a dry pellet until use or was dissolved in sterile TE (pH 8.0). The concentration of DNA was determined by taking the OD₂₆₀ of a sample diluted from the original stock.

Preparation of Yeast DNA

To recover plasmids from yeast cells, the procedure as described was used (84). Transformants were inoculated to 5 ml
of SC media with appropriate selective pressure. After overnight growth at 30°C in a water bath shaker, yeast cells were sedimented in Eppendorf tubes. Two tenths ml of buffer containing 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1% SDS was used to resuspend the cell pellet, and approximately an equal volume of glass beads (0.45 mm diameter) was added to each tube. Two hundred ul of phenol was then added to each tube and vortexed vigorously for 1 min. The aqueous solution was separated from phenol and glass beads by centrifugation and transferred to another tube. The supernatant was reextracted with phenol and chloroform once, and yeast DNA was collected by ethanol precipitation as described before. Yeast DNA resulting from this procedure was rather impure and failed to give transformants when conventional E. coli transformation was done. But electroporation with conventional E. coli competent cells and this yeast DNA gave a large number of transformants.

Preparation of E. coli Competent Cells

E. coli transformation was done according to the protocol of Mandel and Higa (85). E. coli strain stock or competent cells were inoculate into 5 ml of LB medium. After overnight culture in a 37°C water bath shaker, the inoculum was transferrd to 200 ml of fresh LB medium to an OD₆₀₀ of 0.1, and was allowed to continue to grow at 37°C with shaking. After approximately 3 hrs, the OD₆₀₀ was around 0.6. E. coli cells
were collected by centrifugation at 5,000 rpm (GSA rotor). The cell pellet was resuspended in 50 ml of ice cold 0.1 M MgCl₂. After being completely resuspended, the cells were collected again and suspended in 10 ml of ice cold 0.1 M CaCl₂. After this, 100 ml of ice cold 0.1 M CaCl₂ was added to the suspension and the cells were put on ice for 60 min. The cells were then collected and resuspended in 12.5 ml of 0.1 M CaCl₂ plus 2.5 ml of glycerol. After complete suspension, cells were aliquoted in small volumes into Eppendorf tubes and stored at -70°C.

Transformation of *E. coli*

DNA was added to competent cells, which were put on ice for 30 min before they were heat shocked at 42°C for 2 min. The cells were directly spread onto LB-agar plates with appropriate antibiotics for selection. After overnight incubation at 37°C, transformants were clearly visible.

Transformation of *N. crassa*.

*Neurospora* transformation was done as described by Vollmer and Yanofsky (11) with some modifications. Conidia from seven-day old cultures in 500 ml flasks with Vogel complete medium and 1.5% agar were washed with distilled water and collected by filtration through three layers of cheesecloth. The conidia were collected by centrifugation, and were resuspended in 20 ml of water. 150 ml of Vogel's
medium was then inoculated with $2 \times 10^9$ conidia, and germinated with vigorous shaking (250 rpm) at 30°C. After 4-6 hrs, germination was checked with a light microscope. When the germination reached 80%, the conidia were collected and washed several times by repeated resuspension in water and centrifugation. After washing, the conidial pellet was suspended in 10 ml of 1 M sorbitol and put in a sterile 100 ml flask. Twenty mg of Novozyme 234 was added and mixed gently by shaking at a speed less than 80 rpm. Constant checking under microscope was done to guarantee that 90% of the germinated conidia were digested by Novozyme to form spheroplasts. Spheroplasts were put into a 50 ml conical tube and the volume was brought to 50 ml with 1 M sorbitol. The spheroplasts were pelleted gently at 500 rpm on a table top centrifuge. The pellet was then washed two times with 1 M sorbitol solution and once with STC (1 M sorbitol, 50 mM Tris-HCl, pH 8.0 and 50 mM CaCl$_2$). The spheroplasts were finally collected and resuspended in a solution containing 15 ml of STC, 200 ul of dimethylsulfoxide and 4 ml of PTC (40% PEG$_{4000}$, 50 mM Tris-HCl, pH 8.0 and 50 mM CaCl$_2$). The suspension was aliquoted in Eppendorf tubes and stored at -70°C.

For transformation, 1 ug of plasmid DNA in a small volume was mixed with 5 ul of 5 mg/ml heparin in STC. The mixture was put on ice for 5 min, and 100 ul of spheroplasts were added. This mixture was put on ice for 30 min and 1 ml of PTC was added. The mixture was left at room temperature for 20 min and
mixed with top agar (per liter: Vogel's -N, 20 g sorbose, 0.5 g fructose, 0.5 g glucose and 0.8% agar) preincubated at 45°c, and immediately spread onto bottom agar plates (per liter: Vogel's -N, 20 g sorbose, 0.5 g fructose, 0.5 g glucose, and 1.5% agar). Transformed colonies appeared in 2 days after growth at 30° C.

Yeast Competent Cell Preparation and Transformation

Yeast transformation procedure was modified from a method described by Ito (86). A single yeast colony was inoculated into 10 ml of YPD (per liter: yeast extract 10 g, peptone 20 g, dextrose 20 g) and incubated at 30° C in a water bath shaker and grown overnight. The inoculum was then transferred to 100 ml of fresh YPD medium and allowed to continue to grow. After about 6 hrs, the OD₆₀₀ was between 0.8-1.0. The cells were collected by centrifugation at 2,500 rpm. The pellet was resuspended in 20 ml of 0.1 M LiAc and collected again. The cells were then resuspended in 20 ml of 0.1 M LiAc and incubated 1 hr in 30° C shaker. The cells were then collected and resuspended in 1 ml of 0.1 M LiAc and 100 ul samples were aliquoted to Eppendorf tubes.

For yeast transformation, 2-3 ug of DNA, and 25 ug of salmon sperm DNA were mixed with 100 ul of competent cells and incubated for 10 min at 30° C. Then 0.5 ml of PEG buffer (40% PEG4000, 10 mM Tris pH 7.5, and 0.1 M LiAc) was added to the mixture. The resulting mixture was incubated for 1 hr at 30° C
and was followed by heat shock at 42°C for 5 min. To this mixture was added 0.6 ml of water and cells were collected by centrifugation at top speed in a microcentrifuge for several seconds. The cell pellet was washed two more times by water and finally suspended in 100 ul of water. This cell suspension was then spread onto plate with appropriate selection pressure. Transformants appeared in two days after growth at 30°C.

Rapid Screen of E. coli Transformants

The amount of work required to screen large numbers of transformants was reduced by using radioactively labelled probe to do a hybridization screen. The procedure is as follows: E. coli transformed colonies were picked up by pressing nitrocellulose paper against the agar plates containing transformants. At this stage, a mark was made with India ink on both filter and agar plate. The filter with transformants was then laid on top of a drop of denaturing solution (0.5 N NaOH, 1.5 M NaCl) with the colonies facing up. Five minutes later, the filter was laid on top of neutralizing buffer (1.5 M NaCl, 0.5 M Tris.HCl, pH 7.4) for 5 min. The cell debris on the filter was gently scratched off with kimwipes. The filter was then ready for the hybridization screen, and was put into a plastic bag containing 15 ml of hybridization buffer (6xSET, 0.25% dry milk, 0.5% NP40) (10x SET contains: 1.5 M Nacl, 0.3 M Tris-HCl, pH 7.5, 20 mM EDTA)
for 5-10 min as prehybridization. Then randomly labelled probe was added (1,000,000 cpm) to the bag and incubated for 3 hrs in a 65°C water bath. After hybridization, the filter was washed with 3xSET, 2xSET with 0.5% SDS, 1xSET with 0.2% SDS and 0.5xSET at 65°C. The filter was then exposed to X-ray film. Transformants with insertions of interest were easily isolated by aligning the x-ray film with the agar plate.

DNA Fragment Labelling

In my studies, two approaches were used to label DNA fragments. End labelling was used to label probe for EMSA (electrophoretic mobility shift assay) and footprinting experiments. The DNA fragment was cut out with a restriction endonuclease, which left at least one end with a 5' overhang. The DNA fragment was isolated by methods described and dissolved in 37.5 ul of water. To this DNA sample were added 2 ul of dNTP mixture (2.5 mM of each except dATP), 5 ul of restriction digestion buffer 2 (BRL), 3 ul of $^{32}$P-dATP (ICN), and finally 2.5 ul of Klenow fragment (BRL). This reaction was allowed to continue for 30 min at room temperature. The probe was then purified with a spin column.

Random primed DNA labelling was done using random primed DNA labelling kit from Boehringer. The DNA sample to be labelled was solubilized in 23 ul of water and heated to 100°C for 5 min. After the mixture cooled, 2 ul each of dCTP, dGTP, and dTTP, and 15 ul of random primer mixture from the
kit were added to the denatured DNA, followed by the addition of 5 ul $^{32}$P-dATP (ICN), and 1 ul of Klenow fragment (BRL). The reaction continued for 1 hr at 25°C, and was stopped by the addition of 5 ul of stop buffer (0.2 M EDTA, pH 8.0). The DNA probe was then purified with a spin column.

DNA Fragment Isolation

Isolation of small amounts of DNA from agarose gels was easily done by using glass wool. A 0.5 ml microcentrifuge tube was pierced at the bottom by using a 21½ gauge needle. A small amount of sterilized glass wool was put into the bottom of this tube, which was then fitted to a 1.5 ml Eppendorf tube. An agarose gel slice containing a DNA fragment was put into the 0.5 ml tube, and was spun at 8,000 rpm in a microcentrifuge. The solution collected in the 1.5 ml tube was then extracted once with phenol and chloroform, and precipitated with ethanol.

For isolation of large amounts of DNA fragments, gel slices containing DNA were put into dialysis tubing and electroeluted in TAE (40 mM Tris-acetate, 2 mM EDTA) buffer. The TAE solution within the tubing was then collected, extracted once with phenol and chloroform, precipitated with ethanol.
Spin Columns

In order to eliminate the radioactive nucleotides from the labelled DNA probe, spin columns were used. Sephadex G-50 was preswollen by dissolving in STE (100 mM NaCl, 10 mM Tris.HCl, pH 8.0, 1 mM EDTA) and autoclaving. A 1 ml syringe (Becton-Dikinson) was sealed at bottom with glass wool and then filled with the G-50 suspension. This column was then centrifuged in a table top centrifuge at 2,500 rpm for 4 min. This process was repeated until the bed volume reached 0.9 ml. The column was then washed twice with 100 ul of STE and was ready for use. DNA probe from end-labelling or random-labelling was brought to 100 ul by adding STE, and loaded onto the column. After centrifugation at the same speed for the same period of time, the purified probe was collected as the eluate. One ul of this probe was then added to 10 ml scintillation fluid in a vial, and counted on a scintillation counter.

Single-Stranded DNA Preparation

In order to do efficient site-directed mutagenesis, single-stranded DNA containing the target sequence is necessary. The high efficiency mutagenesis method developed by Kunkel (87) was used. The DNA fragment of interest was cloned into phagemid pBluescript. A transformant of the desired clone was used to amplify DNA. This DNA in turn was transformed into E.coli strain CJ236, which is dut⁻, uNG⁻. A colony from the
second transformation was streaked onto a LB plate containing chloramphenical (30 mg/ml) and ampicillin (100 mg/ml). A single colony was picked to inoculate 10 ml of LB containing the same antibiotics and was allowed to grow overnight at 37°C. 1 ml of the overnight culture was used to inoculate 50 ml of 2xYT (4% bactotryptone, 1% yeast extract, 0.5% NaCl) containing the same antibiotics. The culture was grown at 37°C with shaking until it reached OD600 of 0.3. At this time, helper Phage M13K07 was added to around 20 phage/cell. After 1 hr of incubation at 37°C with shaking, 70 ul of 50 mg/ml kanamycin was added and the incubation was continued for another 4-6 hrs. After this, the cell culture was centrifuged at 17,000xg for 15 min twice and the supernatant saved. The supernatant was transferred to a clean polyallomer centrifuge tube and 150 ug of RNase A was added. One fourth volumes of 3.5 M ammonium acetate, 20% PEG-6,000 was added to the supernatant and mixed well before it was put into a 4°C refrigerator for 16 hrs. Phage particles were then collected by centrifuging at 17,000xg for 15 min. The phage particles were resuspended in TE buffer and extracted twice with an equal volume of neutralized phenol, once with phenol/chloroform (1:1:1/24 phenol:chloroform:isoamyl alcohol), and once with chloroform. Back-extracting was done at each step to increase the yield. The aqueous phases were pooled followed by the addition of 1/10 vol of 7.8 M ammonium acetate and 2.5 vol of ethanol. The phagemid was collected by centrifuging at top speed in a
microcentrifuge for 15 min. The resulting DNA pellet was washed once with 90% ethanol and resuspended in 20 ul of TE.

Site-Directed Mutagenesis

The oligonucleotides were phosphorylated before they could be used. Approximately 200 pmol of mutagenic oligonucleotides were dissolved in 30 ul of a buffer containing 100 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 5 mM DTT, and 0.4 mM neutralized ATP. Then, 4.5 units of T4 polynucleotide kinase was added to the reaction mixture, which was incubated at 37°C for 45 min. The reaction was stopped by heating at 65°C for 10 min. The oligonucleotides were ready to use.

Uracil-containing single-stranded DNA prepared as described above and the phosphorylated oligonucleotides were annealed as follows: 200 ng of single-stranded DNA and 6-9 pmol of oligonucleotide were mixed in 10 ul of a buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, and 50 mM NaCl. The mixture was placed in a 70°C water bath and then allowed to cool to 30°C in a 40 min period. The mixture was put into an ice bath and in a sequential manner the following reagents were added: 1.5 ul 10x synthesis buffer (4 mM of each dNTP, 7.5 mM ATP, 175 mM Tris-HCl, pH 7.4, 37.5 mM MgCl₂, 15 mM DTT), 3 ul of 1 unit/ul T4 DNA ligase, 1 ul of T4 DNA polymerase, and 0.5 ug of T4 Gene 32 protein. The reaction was allowed to proceed for 5 min on ice, 5 min at 25°C and 90 min at 37°C before it was stopped by adding 90 ul of stop buffer
(10 mM Tris, pH 8.0, 10 mM EDTA) and frozen. The products of the reaction were then checked on a agarose gel. Ten ul of the reaction mixture was used to transform XL1-blue competent cells. Plasmids isolated from the transformants were checked for the presence of the directed mutation by DNA sequencing.

Double Strand DNA Sequencing

A conventional sequencing procedure requires a single-stranded template DNA. This requires the cloning of the sequence to be sequenced into M13 derived phagemid vector and isolating single-stranded DNA. Throughout my study, a more convenient approach was used, namely, double strand DNA sequencing. Two to three ug of DNA (any conventional plasmid DNA) were dissolved in 20 ul total volume of water. Two ul of 2 M NaOH, 2 mM EDTA was added to the DNA sample, and mixed completely by vortexing at room temperature for 1 min. After 5 min incubation at room temperature, 3 ul of 3 M NaAcetate (pH 5.2) was added and mixed by vortexing. Seven ul of water and 80 ul of ethanol were added immediately. The mixture was chilled in liquid nitrogen, and then centrifuged for 15 min at the top speed in a micricentrifuge. The DNA pellet was washed once with 80% ethanol and dried in a speed vacuum. This DNA preparation was used for sequencing. The following steps were the same as that of single strand sequencing. Sequencing was accomplished by the dideoxy method developed by Sanger (88) with T7 DNA polymerase.
Preparation of PAGE (polyacrylamide) Gels

PAGE gels were extensively used. A typical sequencing gel (6%) was prepared as follows: 75 grams of urea were dissolved in 150 mls of 1xTBE (89 mM Tris-borate, 89 mM Boric acid, 2 mM EDTA) containing 22.5 ml of 40% Acrylamide-Bis mixture (38:2). The mixture was then filtered through Whatmann #1 paper and degassed completely. After adding 0.9 ml of freshly made 10% ammonium persulfate and 41 ul of TEMED, the mixture was immediately used to pour a gel. After about two hrs, the crosslinking reaction was complete and the gel was ready for use. Sequencing gels were run in 1x TBE buffer. Before loading the DNA sample, it was necessary to wash the sample wells to get rid of excessive urea.

A non-denaturing PAGE gel was used for EMSA. It was prepared as follows: 6 ml of 40% acrylamide (acrylamide: Bis 38:2) was mixed with 3 ml of 5xTBE, and the volume of the mixture was brought to 60 ml by adding water. Finally, 0.4 ml of freshly made 10% ammonium persulfate and 40 ul of TEMED were added to the mixture. The gel was immediately poured, and although 30 min was enough time for gel to solidify, a longer time provide to provid a better band pattern. Mobility shift gels were run in 0.25x TBE buffer to stabilize the DNA-protein complex.

Discontinuous SDS-PAGE gels were used to examine protein samples. A typical 7.5% SDS-PAGE gel was prepared as follows: To make 10 ml of separating gel, 4.85 ml of water, 2.5 ml of
1.5 M Tris-HCl, pH 8.8, 0.1 ml of 10% SDS, 2.5 ml of 30% Acrylamide/Bis (29.2/0.8) were mixed together and degassed. After this, 50 ul of 10% freshly made ammonium persulfate and 5 ul of TEMED were added. Seven ml of this mixture was enough for a mini gel. After pouring this mixture into the gel apparatus, the surface was covered with butanol to speed up the polymerization of the gel. Twenty minutes later, the separating gel solidified, butanol was removed by using kimwipes and the stacking gel was made as follows: 3.05 ml of water, 1.25 ml of Tris-HCl, 50 ul of 10% SDS, and 0.67 ml of 30% acrylamide/Bis were mixed. Without degassing, 25 ul of 10% ammonium persulfate and 5 ul of TEMED were added to the mixture, which was immediately poured on top of the separating gel. A comb was inserted into the stacking gel to form sample wells. SDS-PAGE gels were run in a buffer whose 5x stock contains 4.5 g of Tris base, 21.6 g of Glycine, and 1.5 g of SDS in a volume of 300 mls.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) was used in my study to clone the promoter region of lao gene. An EriComp Thermal cycler was used as the heating/cooling source for the enzymatic reactions. One tenth ug of Neurospora genomic DNA was combined with 200 uM each dNTP, 0.1 ug of each of the two primers (MA1: 5'-CAGATACGGCCACACTAGACTC; MA2: 5'-GCTGGCTGATGGAGTGATGAGCT), 10 ul of 10x reaction buffer (1x
contains 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin), and the volume was brought to 100 µl by water. 2.5 units of Taq polymerase were added to the reaction mixture and 100 µl of sterile mineral oil was loaded on top of the reaction mixture to prevent evaporation. The conditions used in amplifying the lao promoter were 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C. After 30 cycles, an additional 3 min at 72°C completed the amplification reaction. The PCR product was analyzed on a 1% agarose gel, and the band corresponding to the expected size was cut out and purified using glass wool. Purified DNA was blunt ended using the same approach as described for end labelling except that T4 DNA polymerase was used in the place of Klenow fragment. The DNA fragment was then ligated to pBluescript already cut with EcoRV. The ligated plasmids were then transformed into XL1-blue competent cells. Transformed colonies were inoculated into 5 ml of LB containing 100 mg/ml Amp. After overnight growth at 37°C in a water bath shaker, the E. coli cells were collected and plasmids were prepared as described. Double strand sequencing approach was used to identify the cloned DNA fragments.

Isolation of Nuclei from N. Crassa.

Neurospora nuclei were prepared as described (89). One 1 of Vogel's medium was inoculated with wild type conidia to an O.D₄₂₀ of approximately 0.5 and grown for 16-20 hrs with vigorous shaking. The mycelial pads were collected by vacuum
filtration, washed with distilled water, and then subjected to other growth conditions as desired, such as repression or induction for another 4 hrs. The mycelia were then collected again, cut into small strips and stored in -70°C freezer until used. The mycelial pad was placed into a bead beater chamber together with 50 gram acid-washed glass beads (0.5mm diameter) and 100ml buffer A (1 M sorbitol, 7% ficoll 400, 20% glycerol, 5mM MgCl₂, 10 mM CaCl₂, 0.5% Triton X-100). The chamber was allowed to stand on ice for sufficient time so that mycelia thawed and could be penetrated by buffer A. The chamber was then put on the bead beater apparatus and homogenized with four 30 seconds pulses at 30 second intervals. The mixture was transferred to a beaker and allowed to settle so that the glass beads could be separated from the rest of the mixture. When there was difficulty for the glass beads to separate, more buffer A was added to the mixture. After settling, the supernatant was carefully decanted into a 400 ml Omni-mixer chamber. The mixture was homogenized at a speed setting of 6.5 in the Omni-mixer for two 15 minute periods with a 10 minute interval. The mixture was kept cold by submerging the Omni-mixer chamber in ice water. Approximately 250 mL of homogenate were usually obtained and centrifuged in a GSA rotor at 2050 rpm for 10 minutes to pellet cell debris. The supernatant was then transferred to a new bottle and centrifuged at 7500 rpm for 45 minutes to obtain a crude nuclear pellet.
Preparation of Nuclear Extract.

In some cases, a high quality nuclear extract was needed. For example, for DNase I footprinting assay, a relatively pure and DNase free nuclear extract preparation was necessary. Ludox gradient was used to prepare a rather pure nuclear fraction from the crude nuclear pellet obtained as described above. If only gel-band mobility shift was to be done with nuclear extract, the Ludox gradient was not necessary.

To isolate nuclei by using a ludox gradient, crude nuclear pellet was resuspended in approx. 10 ml of ice cold Tris-sucrose buffer (1 M sucrose, 50 mM Tris pH 7.5, 5 mM MgCl₂, 10 mM CaCl₂, 1% Triton X-100) with 10-20 strokes of the TriR homogenizer. Three ml of this suspension were carefully layered onto a ludox HS-40 step gradient and centrifuged in a swinging bucket rotor (HB4) at 8500 rpm for 30 minutes.

The Ludox gradient was prepared as follows: Ludox was mixed with an equal volume of 2x Tris-sucrose (no triton) buffer and the pH was adjusted to 7.5 with HCl (Ludox is very basic). This 50% stock was then used to prepare 25% and 12.5% ludox solutions in 1x Tris-sucrose (no triton). Seven ml of each of the above mentioned concentrations of ludox were used to form a step gradient in a 30 ml corex tube. Three to four ml of the nuclear suspension were layered on top of this gradient and centrifuged. After centrifugation, an opaque layer formed between 25% and 50% ludox layers was collected by using a syringe attached to a cannula. Four volumes of 1x
Tris-sucrose was added to the isolated nuclear suspension and centrifuged at 7500 rpm for 20 minutes to pellet the nuclei.

The nuclear pellet was homogenized at 15,000 rpm for 40 seconds in a Virtis 60 homogenizer in 2.5 mls of buffer containing 5 M urea, 3 M NaCl, 10 mM Tris- HCl (pH 7.4), and 1 mM EDTA. The homogenate was centrifuged at 7,000 x g for 15 minutes in a GSA rotor and the supernatant saved. The pellet was re-extracted with 2 mL of the same buffer. The two supernatants were combined and brought to 9%(w/w) PEG by addition of 30% PEG in the urea–NaCl buffer. The mixture was stirred slowly at 4°C for 30 min and centrifuged at 8,000xg for 15 min in the same rotor. The supernatant was dialyzed against buffer A (20 mM Hepes pH 7.9, 50 mM KCl, 1mM DTT, and 0.1% NP-40) overnight. After dialysis, the supernatant was then applied to a 400 ul heparin-agarose column equilibrated with the same buffer. Nuclear extract was eluted with buffer A which contains 500 mM KCl. The eluted protein was diluted with the same buffer to 50 mM KCl and concentrated by Centricon-filters. The nuclear extract resulting from this process is rather pure and relatively DNase free, as there was no indication of endogenous DNase activity in DNasel footprinting assay experiments.

If gel-mobility shift assays were to be done, then the crude nuclear pellet was suspended directly in buffer A containing 50 mM KCl. After the pellet was completely resuspended, the suspension was sonicated to release protein
from the nuclei, centrifuged at 9,000 rpm in a SS-34 rotor for 30 minutes, and the supernatant was collected. The supernatant was then loaded onto a heparin-agarose column as described before. Nuclear extract from this procedure was not as pure as that obtained with the Ludox gradient, but clear shifts could be obtained when an appropriate amount of protein was used. This preparation was also good enough to show protected DNA elements by using depurination interference experiments, instead of the DNaseI footprinting assay, which has a strict requirement for good quality protein.

NIT-2 Protein Expression

In order to study characteristics of NIT-2 protein in vitro, I expressed NIT-2 protein in different forms.

NIT-2 was first expressed as a NIT-2/β-gal fusion protein to study its DNA binding ability (42). In my experiments, in order to study the effects of different mutations within the zinc finger loop region on NIT-2's DNA binding ability, NIT-2/β-gal fusion proteins were also made. Briefly, a KpnI-EcoRI fragment of nit-2 was cloned into pBluescript, within which site-directed mutagenesis was carried out. The same fragments carrying different mutations were then cloned into pSKS 106, which already had nit-2 BamHI-EcoRI cloned in it. By replacing the KpnI-EcoRI fragment within the BamHI-EcoRI fragment, different mutations were introduced into this fusion construction, and nit-2 gene was fused to full length β-gal
gene in reading frame. The mutant proteins were expressed and purified according to the protocol described by Casadaban et al (90).

NIT-2/β-gal fusion protein contains only small portion of NIT-2 protein. In order to study the characteristics of a full length NIT-2 protein, I tried to express the full length NIT-2 in E. coli. The pRSET protein expression vector (QIAGEN) system and E. coli BL21 (DE3) were used in this experiment. Different segments of NIT-2 were cloned into pRSET A, B or C in order for NIT-2 to be in reading frame. Constructions with appropriate nit-2 insertions in correct reading frame were obtained by transforming and screening in E. coli XL1-blue strain, then they were transformed into BL21 (DE3) for protein expression (91). A single fresh colony from the BL21 (DE3) transformation was used to inoculate 5 ml of LB. After overnight growth at 37°C, the inoculum was transferred to 200 ml fresh LB, and allowed to grow under the same conditions. Within 3 hrs, the OD_{600} would reach 0.4 and IPTG was added to the culture to a final concentration of 0.4 mM. Induction was allowed to continue for 3 hrs, and the E. coli cells were collected by centrifuging at 5,000 rpm (GSA rotor) for 5 min. The cell pellet could be stored at -20°C for several days without evident protein degradation.

It was noticed that truncated forms of NIT-2 were not as efficient at DNA binding as the NIT-2/β-gal fusion protein. I found that short segments of NIT-2 could be easily fused with
glutathione-S-transferase and more efficient NIT-2 proteins could be obtained. NIT-2 DNA fragments were cloned into the multiple cloning site of pGEX-2T (Pharmacia) and used to transform XL1-blue. A single colony was used directly for protein expression, as was described for pRSET expression vector.

Different segments of NMR protein were also expressed in either pRSET or pGEX-2T. The expression procedure was the same.

Protein Purification Procedure

NIT-2 proteins expressed in pRSET vector were purified using Ni-NTA (nitrilo-tri-acetic acid) in two different approaches suggested by the manufacturer. For long segments of NIT-2 whose solubility was very low, a denaturing purification protocol was used. The frozen *E. coli* cell pellet from the freezer was thawed at room temperature and resuspended in Buffer A (6 M Gu-HCl, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 8.0) at 5 ml per gram wet weight. The suspension was stirred for 1 hr at room temperature. Cell debris were removed by centrifuging at 10,000xg for 15 min. A 50% slurry of Ni-NTA resin was used to pack a column of 0.5 ml for 200 ml cell culture. The column was equilibrated with Buffer A before the supernatant from the last step was loaded onto it. After loading, the column was washed with 10 column volumes of Buffer A, and 5 column volumes of Buffer B (8 M urea, 0.1 M
Na-phosphate, 0.01 M Tris-HCl, pH 8.0). The column was then washed with buffer C (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 6.3) until no significant amount of protein could be detected in the eluate. Finally, the NIT-2 protein was eluted with 2 ml of Buffer D (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris, pH 4.5). Samples were analyzed by SDS-PAGE gel.

For short segments of NIT-2 proteins that were readily soluble in non-denaturing buffers, protein purification under native condition was carried out. The cell pellet was suspended in Buffer A' (50 mM Na-phosphate, pH 7.8, 300 mM NaCl). Several cycles of freeze and thaw were efficient to break the host cells. Insoluble matter was removed by centrifuging at 10,000 rpm (SS-34 rotor) for 30 min, and the supernatant was collected. Approximately 0.5 ml Ni-NTA resin was equilibrated with Buffer A' before the supernatant from the last step was loaded onto it. After loading, the column was washed with 4 bed volumes of Buffer A', followed by 4 bed volumes of Buffer B' (200 mM ammonium acetate, 300 mM NaCl, pH 6.0). The NIT-2 protein was then eluted with buffer C' (buffer B' with pH 4.5). The eluate was collected and analyzed by SDS-PAGE.

For NIT-2 or NMR that were expressed with pGEX-2T vector, purification was carried out using glutathione-agarose resin according to the protocol described (108). The cell pellet was suspended in 15 ml of PBS buffer (1 liter contains: 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, and 0.24 g KH₂PO₄, pH 7.4). The
cells were broken by sonication for 4 min, as XL1-blue cells were much harder to break than BL21 (DE3) cells. After sonication, the supernatant was collected by centrifugation at 10,000 rpm (SS-34 rotor) for 30 min. Preswollen glutathione-agarose resin was used to pack a 0.5 ml bed volume column, and equilibrated with PBS buffer. The supernatant from the last step was loaded onto the column. After loading the sample, the column was washed with 4 bed volumes of PBS buffer. The fusion protein was eluted by adding 2 bed volumes of elution buffer (50 mM Tris-HCl, pH 8.0, 5 mM reduced glutathione). The eluate was collected and analyzed by SDS-PAGE.

NIT-2 Polyclonal Antibody Preparation

A 30,000 dalton peptide containing the NIT-2 zinc finger region was expressed in BL21(DE3), and purified as described above. Approximately 300 ug of pure NIT-2 protein was obtained and used to inject two rabbits. A booster was given four weeks later, and three weeks later the rabbit serum was collected and tested for reaction with NIT-2 protein.

Western Blots

For Western blot analysis, protein samples were run on discontinuous SDS-PAGE gels first, and then transferred to nitrocellulose filters. A sheet of nitrocellulose filter of the same size as the SDS-PAGE gel was placed on top of the gel, and all air bubbles were removed. They were sandwiched by
3 pieces of 3 MM paper on each side. The protein samples were electrotransferred to the filter in a Bio-Rad Trans-Blot Cell at 53 V for 3 hrs in transfer buffer (39 mM glycine, 48 mM Tris pH 8.3, 0.037% SDS, 20% methanol). After transfer, the filter was immediately placed into the blocking buffer (5% dried milk in TBS-T) (TBS-T contains in 1 liter: 2.42 g Tris, pH 7.6, 8 g NaCl, 3.8 ml 1 M hydrochloric acid, 0.1% Tween 20). The following steps were carried out with ECL western blotting kit from Amersham, according to protocols suggested by the manufacturer. The blocking procedure was carried out for one hr (overnight blocking was also frequently used with no ill effect) and the filter was washed with TBS-T once for 15 min and twice for 5 min. Primary antibody against NIT-2 was diluted 500 times in TBS-T and nitrocellulose paper with the transferred protein samples was incubated for 1 hr in this buffer at room temperature with gentle shaking. The filter was then washed with TBS-T once for 15 min and twice for 5 min, followed by the addition of the secondary antibody. The secondary antibody was anti-rabbit IgG antibody raised in donkey, to which horse radish peroxidase was conjugated. The secondary antibody was diluted 1000 times in all of my Western blot experiments. A 1 hr incubation was usually used with the secondary antibody. The filter was washed with TBS-T once for 15 min and four times for 5 min. The filter was then dried on top of kimwipes with the protein samples facing up. Reagents 1 and 2 from the kit were mixed in equal volumes and gently
poured onto the dried filter. After 1 min, the excessive reagent buffer was dried using a kimwipe, and the filter was wrapped in a sheet of Saran wrap, and immediately exposed to X-ray film. The exposure time varied from 0.5 s-30 s depending on the strength of the fluorescent signal. Immunoblotting with Immunoselect kit from BRL was also used in some early Western blot experiments. ECL was more sensitive and easier to handle.

Electrophoretic Mobility Shift Assay (EMSA)

One of MIT-2's most distinctive functions is its DNA binding ability. Extensive EMSA experiments were done to characterize MIT-2 DNA binding.

EMSA was carried out according to the protocol used by Hope and Struhl (92). DNA probes used in the EMSA experiments were from nit-3, lao, and alc promoter regions; some artificial oligonucleotides were also used. DNA probes used in the EMSA were all end labelled with $^{32}$P; usually 20,000-50,000 cpm of probe were used for each reaction. DNA probe and protein samples were incubated in 25 ul of a buffer containing 12 mM Hepes, 2mM DTT, 3 mM MgCl$_2$, 2 ug poly(dI-dC), and 50 mM KCL at room temperature for 25 min, and the reaction mixture was loaded on a 4% nondenaturing polyacrylamide gel. The gel was run in 0.25xTBE buffer and then either dried or directly exposed to X-ray film. For some experiments, samples were loaded with the gel running. This had no ill effect on the quality of the band pattern. For EMSA with protein samples
that were contaminated with a significant amount of nuclease (mostly from crude protein extract from E. coli or Neurospora), MgCl₂ was omitted from the EMSA buffer.

NIT-2 Stability Test

Various samples of NIT-2 protein were tested for the ability to regain function after heat and detergent denaturation or after being deprived of bivalent cation. Each NIT-2 sample was run on a denaturing SDS-PAGE gel, after which NIT-2 was purified according to a protocol developed by Dr. James Young. The gel slices containing NIT-2 protein were cut out and placed into a small plastic funnel attached to a 7 mm diameter, 75 mm length glass tube. The other end of the glass tube was fit into a Centricon-10 concentrator (Amicon). The two ends of this design were placed in chambers filled with SDS-PAGE gel running buffer, and an electric field of 300 volts was applied overnight. The sample within the Centricon-10 was concentrated by centrifugation at 5,000xg in a SS-34 rotor until the desired volume was obtained. The protein sample was then dialyzed against renaturing buffer (12 mM Hepes, pH 8.0, 50 mM KCl, 2 mM DTT) overnight. After dialysis, the protein sample was used for EMSA experiments.

NIT-2 protein solubilized in renaturing buffer which was proven functional for DNA binding function was incubated with Chelex (Iminodiaceticacid, SIGMA) which was preincubated with renaturing buffer at 4°C for 30 min. The protein sample was
then used for EMSA experiment.

Glutamine Binding Assay

Equilibrium dialysis was used to determine whether NIT-2 might bind glutamine. Concentrated NIT-2 protein samples, either purified *E. coli* expressed protein or nuclear extract was dialyzed against buffer containing 12 mM Hepes, pH 8.0, 50 mM KCl, 2mM DTT. 100 ul of protein were mixed with 10 ul of $^3$H labelled glutamine (Amersham) and put into dialysis tubing. This tubing was then put into 100 ml of the same buffer. After different periods of time at 4°C, samples from inside and outside the tubing were tested for radioactivity in a scintillation counter.

Glutamine-agarose resin (Sigma) was also used to test whether NIT-2 binds to glutamine. The same protein samples (100 ul) as used in equilibrium dialysis experiments were incubated with 20 ul glutamine agarose resin for different time periods at 4°C, then the resin was eluted with the same buffer containing 500 mM KCl. Protein samples from both the eluate and flow through fractions were then analyzed on a SDS-PAGE gel.

Protein Cross-Linking Assay

In order to determine if NIT-2 exists as a dimer or if NIT-2 monomers tend to interact with each other under certain circumstances, and also to find out if NIT-2 and NMR could
interact with each other, cross-linking with glutaraldehyde was carried out.

*E. coli* expressed NIT-2 and NMR were purified and obtained in high concentrations. The purified proteins were then dialyzed against PBS buffer, pH 7.4. Approximately 4 ug of protein was used in each cross-linking experiment. To test for NIT-2 dimerization, glutaraldehyde was added to 10 ul of a NIT-2 protein sample to a final concentration of 0.005%. After incubation on ice for 5, 10, or 30 min, the reaction was stopped by adding an equal volume of SDS-PAGE running buffer, and boiling for 5 min. The protein sample was analyzed by SDS-PAGE gel.

To test for any interaction between NIT-2 and NMR, approximately equal amounts of NIT-2 and NMR were mixed in PBS buffer (4 ug each). After 1 hr incubation on ice, glutaraldehyde was added to the mixture to a final concentration of 0.005%. After incubation on ice for 5, 10, and 30 min, the reactions were stopped and protein samples were analyzed by the same method as described above.

DNaseI Footprinting Assay

DNase I footprinting assays were conducted according to the protocol by Hope and Struhl (92) with modifications. An EMSA experiment was done prior to footprinting assays to determine the amount of protein necessary to bind all of the DNA probes. The amount of protein that was able to shift all
of the DNA probe in EMSA was usually doubled in experiments to do DNasel footprinting.

The appropriate amount of protein was incubated with an end labelled DNA fragment (at least 50,000 cpm) in 25 ul of buffer containing 12 mM Hepes (pH 7.9), 2 mM DTT, 3 mM MgCl₂, 50 mM KCl at room temperature for 25 min, after which DNase I (varying amount) was added. After 2 min, the DNasel digestion was terminated by adding an equal volume of stop buffer (0.5% SDS, 10 mM EDTA, and 0.5 mg/ml yeast tRNA). The DNA was precipitated with EtOH, washed with 80% ice-cold EtOH, resuspended in DNA sequencing buffer, and electrophoresed on a 6% sequencing gel. The gel was then dried and exposed to X-ray film.

Other DNA Footprinting Techniques

Methylation interference footprinting was used to confirm the results of DNaseI footprinting. The DNA probe used in this experiment was methylated before being used as follows (93). 200,000 cpm singly end-labelled probe was suspended in 20 ul TE, and the volume was brought to 200 ul by adding buffer containing 50 mM sodium cacodylate, pH 8.0, 1 mM EDTA. One half ul of DMS (dimethyl sulfate) was added to this mixture, which was incubated at room temperature for 2.5 min. Fifty ul of DMS stop buffer (1.5 M NaAc, pH 7.0, 1 M mercaptoethanol) and 750 ul of absolute ethanol were added, and then the mixture was put into liquid nitrogen and centrifuged at 15,000
rpm for 10 min in a microcentrifuge. The DNA pellet was redissolved in 300 ul of 0.3 M NaAc, pH 5.2 and 900 ul ethanol were added. The probe was precipitated again, washed with ethanol and dried. The DNA was finally redissolved in water at 200,000 cpm/ul.

Depurination interference footprinting was used to determine which base pairs make direct contact with amino acid residues in DNA binding factors. Depurinated DNA was prepared as follows: the DNA was suspended in 30 ul of TE buffer with 10 ug of yeast tRNA as carrier. To this solution, 3 ul of 1 M formic acid was added and the reaction was continued at 37°C for 30 min. 3 volumes of ethanol were then added and DNA was collected as described above.

The methylated (or depurinated) DNA was incubated with NIT-2 protein and the mixture was subjected to a mobility shift. The retarded band, representing DNA/protein complex, and the free probe band were each cut out from the polyacrylamide gel, according to the corresponding image on X-ray film. The DNA in each gel slice was electrotransferred to a DEAE membrane. The membrane was washed vigorously with buffer containing 20 mM Tris-HCL pH 8.0, 1 mM EDTA, 0.2 M NaCl. After washing, the probes were eluted by putting the membrane in 200 ul of elution buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 N NaCl) and heating at 65°C for 1 hr. The supernatant was transferred to a clean Eppendorf tube and centrifuged to remove gel and membrane debris. The
supernatant was then extracted once with phenol and once with chloroform. The DNA probes were finally collected by ethanol precipitation.

For both methylation and depurination experiments, the final steps were the same. The DNA was dissolved in 10 ul of buffer containing 2 mM Naphosphate pH 7.0, 1 mM EDTA, and heated at 90°C for 20 min, after which 0.5 ul of 1 N NaOH was added. The mixture was incubated for another 30 min at 90°C, when 5 ul of sequencing stop buffer was added, and the mixture was heated at 95°C for 3 min. The sample was chilled on ice and loaded on a 6% sequencing gel. After electrophoresis, the gel was dried and exposed to X-ray film.

Analysis of β-Gal Activity of Yeast Transformants

The yeast two hybrid system is a relatively new technique developed by Fields et al. (94). In my study, the yeast two hybrid and one hybrid systems were both used to study protein-protein interactions and protein activation domains respectively. Yeast transformation has been described above. Beta-gal activity of the transformants was tested in order to find out if one protein makes contact with another, or if domains of a protein have activation function.

β-gal activity was analyzed both qualitatively and quantitatively. Qualitative β-gal assays were done by using a filter paper assay. Yeast transformant colonies (four days old) were transferred to Hybond filter paper (Amersham). This
was achieved by pressing the filter firmly against the agar plates containing yeast colonies. The Hybond filter paper with transformants was then put into liquid nitrogen for 10 seconds. Yeast cells were then lysed by simply putting the filter paper at room temperature for 5 minutes. The filter paper was then laid onto a petri dish containing 0.3 ml z buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl, 0.001 MgSO₄, and β-mercaptoethanol, pH 7.0) containing 1 mg/ml X-gal. The side of the filter with colonies faced up. The petri dish was left at room temperature for blue color to develop if there was β-gal activity.

β-gal activity was also measured quantitatively. Candidate transformants were inoculated into 10 ml of SC medium (78) 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 tubes containing the cell pellets were then dropped into liquid nitrogen for several seconds and then allowed to stay at room temperature for 5 min. One ml of buffer z containing 1 mg/ml ONPG was then 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, the OD₄₂₀ of the supernatant was measured. The β-gal activity was shown as Miller units.

\[
\text{Miller unit} = \frac{1000}{(V)(t)(OD_{600})}
\]

\((1)\)
where $v =$ volume of culture in ml, $t =$ elapsed time in minutes, $\text{OD}_{600}$ was the optical density of the original overnight culture.

Southwestern Blot

Southern blot is used for detecting DNA fragments of interest by using a DNA probe. Western blots, on the other hand, are used to detect proteins of interest using antibody. Southwestern is a method utilizing the characteristics of DNA binding proteins. DNA binding factors immobilized on nitrocellulose filter often retain their DNA binding ability, and thus can be detected by using radioactively labelled DNA probe (95). Southwestern was used in my experiments to identify NIT-2 from $N. \text{crassa}$ nuclear extract. Procedure for Southwestern blots are as follows: protein samples were run on a SDS-PAGE gel as described, and then transferred to a nitrocellulose filter as described previously, except that the methanol in the transfer buffer was 10% instead of 20%. The nitrocellulose filter was then put into a square petri dish with 15 ml of HBB (10x HBB contains 250 mM Hepes, pH 7.7, 250 mM NaCl, 50 mM MgCl$_2$) with 1 mM DTT. After 5 min, the filter was put into the same buffer with 6 M guanidine-HCl in addition to the above mentioned buffer. Twenty min later, this buffer was replaced by another buffer which contained half the concentration of guanidine of the previous one, and incubation lasted for 10 min. The concentration of guanidine was cut into
half every 10 min until its final concentration reached 0.19 M (total of 5 changes). The filter was then put into HBB with 1 M DTT for 20 minutes. After the protein renaturing procedure described above, the filter was then preincubated with HBB with 1 mM DTT, 5% dry milk, and 0.05% NP-40 for 60 min, followed by incubation with HBB with 1 mM DTT, 1% dry milk, 0.05% NP-40 for 30 min. After preincubation, the filter was incubated with 250,000 cpm labelled probe in Hyb buffer (20 mM Hepes, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂) with 1% dry milk and 0.05% NP40. The hybridization process was allowed to proceed overnight with gentle shaking. It was important that all steps beginning with renaturation were done at 4°C. After overnight hybridization, the filter was washed several times with Hyb buffer without probe, air dried, wrapped in a sheet of saran wrap and exposed to X-ray film. 2-3 hrs of exposure was adequate.

**In vitro** Protein-Protein Interaction Study Using GST Fusion System.

To test a possible interaction between NIT-2 and NMR *in vitro*, a GST fusion system was used. The gene encoding NMR full length protein was fused to GST, expressed in X11-blue and purified as described previously. Sequences encoding NIT-2 were cloned into pRSET, expressed and purified.
Approximately 50 ul of glutathione-agarose resin was used for each interaction assay. The resin was equilibrated first with PBS buffer, and incubated with purified GST-NMR dissolved in PBS buffer at 4°C for 20 min. After the GST-NMR was absorbed to the resin, purified NIT-2 protein in PBS buffer was added. NIT-2 and NMR proteins were allowed to interact in an Eppendorf tube at 4°C for 1 hr. The resin was separated from supernatant by gentle centrifugation, and washed twice with PBS buffer. Proteins bound to the resin were then eluted as described before. The protein samples were run on a SDS-PAGE gel, transferred to a nitrocellulose filter, and detected with anti-NIT-2 antibody.

To identify NIT-2 from nuclear extract, glutathione-agarose resin with GST-NMR absorbed on it was used as a protein affinity column. The nuclear extract was first dialyzed against PBS buffer, and then mixed with the affinity column. The following steps were as described above. Protein samples were then concentrated with a Centricon -10 and used for EMSA and western blot.

DNA Affinity Column for NIT-2 Protein

A DNA affinity column was used to clone important genes (96). A 150 bp fragment from the promoter region of lao contains 3 separate NIT-2 binding sites composed of a total of 5 GATA core sequences. This DNA fragment with such a high density of GATAs provided an ideal natural material for
preparing a NIT-2 specific DNA affinity column. A total of 500 ug of lao promoter fragment was prepared according to the protocol described in a previous section. This DNA was then coupled to CNBr activated Sepharose 4B. The resin (SIGMA) was put in a 15 ml polypropylene screw-cap tube, and 4 ml of 10 mM potassium phosphate, pH 8.0 was added to give a thick slurry. The DNA was then added to this mixture, and the coupling reaction was carried out at room temperature for 16 hrs on a rotary shaker. After the reaction, the resin was collected on a sintered-glass funnel, washed with water (200 ml) and 1 M ethanolamine-HCl (pH 8.0; 100 ml) and suspended in 1 M ethanolamine to inactivate unreacted CNBr-derivatized Sepharose. The inactivation was carried out at room temperature for 4-6 hrs on a rotary shaker. The resin was collected on a sintered-glass funnel and washed successively with 10 mM potassium phosphate (pH 8.0, 100 ml), 1 M potassium phosphate (pH 8.0, 100 ml), and 10 mM Tris-HCl (pH 7.6) containing 0.3 M NaCl, 1 mM EDTA. The resin was stored at 4°C in 10 mM Tris-HCl (pH 7.6) containing 0.3 M NaCl.

The resin was used to pack a 1 ml column. It was equilibrated with a buffer containing 12 mM Hepes (pH 7.9), 50 mM KCl, 2 mM DTT. MgCl₂ was deliberately omitted from the buffer to inhibit contaminating endonuclease activity. Nuclear extract was dialyzed against the buffer used for equilibrating the column and then loaded onto the column. The column was washed with 10 volumes of the loading buffer. Protein samples
were eluted with the same buffer except that the concentration of KCl was 0.5 M. The protein samples were diluted with loading buffer so that the concentration of KCl was reduced to 100 mM, and the protein was concentrated by using a Centricon-10. The protein samples were then analyzed using EMSA and Western blots.
AMINO-ACID SUBSTITUTIONS IN THE ZINC FINGER OF NIT-2 ALTER
PROMOTER ELEMENT RECOGNITION

Cloning of The lao Promoter Region

NIT-2 is a DNA binding protein of the GATA family of transcription factors, and its DNA binding ability is necessary for its positive regulatory function. Expression of the lao gene, which encodes L-amino acid oxidase, was found to be activated by NIT-2 (97). In order to find out if lao has one or more NIT-2 binding sites within its promoter region, as has been demonstrated for nit-3 and alc, a 700 bp fragment which covers -350 to +350 (translation starting site as +1) region of lao was cloned by PCR using a pair of primers and conditions described in the Materials and Methods section. The PCR product was checked by electrophoresis in an agarose gel. A major DNA band of the expected size (700 bp) was found, and was cloned into pBluescript. DNA sequencing proved that the fragment cloned was from the lao promoter region.
NIT-2 Protein Binds to the Lao Gene Promoter Region

Mobility-shift assays revealed that the NIT-2/β-gal fusion protein bound specifically to the 5' flanking DNA of the lao gene. To determine the number and location of binding sites for NIT-2, EMSA assays were conducted with different segments of the cloned 700-bp DNA fragment. NIT-2 binding was found to occur within a DNA fragment of approximately 100 bp (Figure 2). DNA footprinting experiments (DNase I protection assays) were then employed to precisely identify the NIT-2 binding sites in the lao gene promoter (Figure 3). Two NIT-2 binding sites were identified, a strong binding site at -240 and a second, weaker, binding site at -325, upstream of the L-amino acid oxidase structural gene. Nuclear extracts of the nit-2* strain showed binding of the native NIT2 protein at these same two sites, with even clearer binding to the weaker site at -325 than found with the fusion protein (Figure 4).

Site-Directed Mutagenesis of Nit-2

The NIT-2 protein consists of 1036 amino acids and contains a single Cys2/Cys2-type zinc finger, which, together with a downstream basic region, comprises a DNA-binding domain. Site-directed mutagenesis was employed to create mutants with amino acid substitutions for residues in the 17 amino acid loop of the zinc finger, so that the importance of conserved and nonconserved amino acid residues could be evaluated. It was of particular interest to obtain mutant NIT-
2 proteins which retained DNA-binding activity but whose specificity might be altered by the amino acid substitutions in the zinc finger domain. The mutagenic primers used to introduce amino acid changes were: 5'-CGACCCCA(G,A)(T,A,C)GTGGCGTCGTAACC for amino acid residue 753; 5'-CCCATTTG(A,C,G)(A,T)GCGCGTAAC at amino acid 754; 5'-CGCGGTATACCA(C,A)(C,G)TGGACCAAC at residue 759. Mutants were obtained which led to eight different amino acid substitutions at three positions in the DNA binding domain (Figure 5). Substitutions were obtained for Leu$_{753}$ and Trp$_{754}$, which are conserved residues among several proteins with a homologous zinc finger motif. As a positive control, amino acid substitutions were also obtained for Asp$_{759}$, which is not conserved. Mutants which led to amino acid substitutions for the two adjacent conserved residues, Arg$_{735}$ and Arg$_{736}$, and for residues Asn$_{765}$ and Ala$_{766}$ within the "knuckle" of the zinc finger motif were obtained previously (43).

Transformation Assays

Each of these mutations was introduced into a nit-2 wild type gene and sequenced to verify that the correct construction was achieved. A nit-2 mutant host strain was transformed with an identical amount of each mutant nit-2 gene and with a wild-type gene as a positive control. Five of the eight new mutant NIT-2 proteins retained function, whereas the other three mutants, all changes in Trp$_{754}$, completely failed
to function in vivo and were completely deficient in DNA binding in vitro, as was also found for the substitutions of residues Asn765 and Ala766 (Figure 5) (43).

Enzyme Assays and Growth Tests

The promoters of the structural genes of the nitrogen regulatory circuit each possess NIT-2 binding sites. However, the binding elements found in these promoters are different from each other, which suggested the possibility that mutant NIT-2 proteins which retain function may be altered in their relative affinity for different promoters. Transformants which had received each of the functional mutant nit-2 genes were assayed for their content of nitrate reductase, allantoicase, and L-amino acid oxidase. The specific activities of these enzymes in the different mutants were compared to the wild type strain as shown in Figure 6. It is striking that each nit-2 mutant displays a unique pattern of relative levels of these three enzymes, for the most part being quite different from that of wild type. Mutants L753M and D759S show relatively high activities for all three enzymes, whereas mutants L753E and L753A possess a very low level of nitrate reductase and intermediate amounts of allantoicase and L-amino acid oxidase. In contrast, mutant L753V has very substantial amounts of nitrate reductase and L-amino acid oxidase but a reduced amount of allantoicase.
This same set of transformants was tested for their growth with nitrate, hypoxanthine, or phenylalanine, to determine whether the altered enzyme levels affect growth with each of these compounds as the sole nitrogen source. As revealed in Figure 6, growth upon these alternative nitrogen sources showed a strong positive correlation with the pattern of enzyme levels in each of the functional nit-2 mutants.

NIT-2/β-gal Fusion Protein Expression and Purification

Mutations L753M, L753V, L753E, W754E, and D759S were introduced into expression vector psks106 (42). Each construction was sequenced to confirm that the correct mutation and reading frame was obtained. Fusion proteins were expressed in E. coli, purified by affinity chromatography, and their identity confirmed by using anti-NIT-2 polyclonal antibody (Figure 7). I found that none of these amino acid substitutions in the NIT-2 protein affected its synthesis or stability in E. coli since a comparable amount of each mutant and wild-type NIT-2 fusion protein was obtained.

DNA Binding Activity

Gel band mobility-shift assays were performed with purified wild type and each mutant nit-2/β-gal fusion protein. Each protein was tested for the ability to bind to promoter DNA fragments of the three genes which encode nitrate reductase, allantoicase and L-amino acid oxidase. In order to
maintain comparable binding conditions, an identical amount of each fusion protein was used in these binding assays with the three different promoters. The DNA binding results, shown in Figure 8, demonstrate an excellent correlation with the results of the enzyme assays and growth tests. Thus, as expected, the W754E mutant NIT-2 protein, which lacked function in vivo, failed to bind to any of these promoters. The L753E mutant NIT-2 protein, on the other hand, showed weak binding to all three promoter DNA fragments. The mutant L753V protein showed strong binding to the promoter fragments of L-amino acid oxidase and the nitrate reductase genes, but poor binding to the allantoicase gene promoter.

NIT-2 Binding Site Identification.

In collaboration with Dr. Bo Feng, extensive DNA-protein interaction techniques, including methylation and depurination interference experiments, were employed to study base pairs within and surrounding the strongest binding site of the lao promoter (93). These sensitive footprinting techniques revealed that each base of the GATA core was implicated for direct interaction with the NIT-2 protein; moreover, at least 3 nucleotides in the complementary sequence, TATC, located on the opposite DNA strand also were identified to have intimate contact with NIT-2. The results also revealed that in addition to the GATA core sequences, nucleotides adjacent to GATA were also involved in DNA-protein interaction (Figure 9). This
study provided a more detailed view, extending the results of DNasel footprinting assays, to reveal how individual bases within and surrounding the binding site of the lao promoter made contact with NIT-2 protein. Comparison of the NIT-2 binding sites within the lao promoter, together with binding sites identified from nit-3 and alc promoters, showed that although each binding site contains the core sequence GATA, significant differences exist among these naturally occurring NIT-2 binding sites; they differ in the spacing between the GATA elements, the orientation of GATA sequences relative to the transcription starting sites, and the sequences flanking each GATA element (Figure 10).

**EXPRESSION OF NIT-2 PROTEIN IN E. COLI**

**NIT-2 Protein Expression in E. coli**

In order to study NIT-2 function in *vitro*, it was important to express NIT-2 as an integral protein. The pRSET expression vector system was used in my study to express and purify NIT-2 protein. *E. coli* BL21 (DE3) was used as the host (26). I took advantage of the convenient multiple cloning site of pRSET and many unique restriction sites within nit-2 gene to express different segments of NIT-2, and encountered no problems with expressing any part of NIT-2 protein. Segments of the nit-2 gene which encode regions of the NIT-2 protein
and include its zinc finger were expressed, including a BamH1-EcoR1 DNA fragment (encodes residues 606-1036), a Sal1-EcoR1 fragment (residues 462-1036), and a PstI-EcoR1 fragment (residues 200-1036) (Figure 11). Each of these truncated NIT-2 proteins were shown active in binding DNA fragments which contain GATA sequences (Figure 12). However, the full-length NIT-2 protein which was also expressed in E. coli was found to be insoluble, and thus it was purified by using Ni-NTA affinity chromatography under denaturing conditions (Figure 13). Attempts to renature the whole length NIT-2 protein failed, as it precipitated in non-denaturing buffers.

NIT-2 Protein Purification by Heparin Column

A NIT-2 truncated protein (residues 462-1036) solubilized in heparin column equilibration buffer was loaded onto a heparin-agarose column. The washing and elution steps were exactly the same as in nuclear extract preparation. Samples from fractions of the eluate and flow through were analyzed on a SDS-PAGE gel. Results showed that NIT-2 protein was specifically absorbed by the heparin column under normal sample loading conditions, and was readily eluted by Buffer A containing 500 mM KCl (Figure 14).

Truncated NIT-2 Proteins Are Resistant to Denaturation

NIT-2 truncated proteins which were functional in DNA binding as described above were purified and used to carry
out experiments to study NIT-2's physical characteristics. A NIT-2 protein consisting of residues 606 to 1036 (encoded by the BamH1-EcoR1 DNA fragment) was subjected to denaturing conditions, including heat treatment at 100°C and detergent denaturation. After the NIT-2 protein was subjected to these harsh conditions, it was dialyzed against renaturing buffer, and then was found to bind tightly to a NIT-2 binding site (data not shown). These results demonstrate that this partial NIT-2 protein, which consists of approximately 400 amino acids but lacks the entire amino terminal region, was very resistant to denaturation, or readily renatured possibly due to its highly conserved and stable DNA binding motif.

Chelex Treatment Destroyed NIT-2 DNA Binding Ability

NIT-2 is a member of the GATA family, all believed to be zinc chelating DNA binding proteins. Thus, zinc should be very important for the structural integrity and function of the DNA binding motif. Previous treatments of NIT-2 with high concentrations of EDTA did not affect the DNA binding activity of NIT-2. In my study, the NIT-2 protein was treated with Chelex, a more powerful chelating reagent with a preference for bivalent ions. After treatment with Chelex, the DNA binding activity of the NIT-2 protein was totally eliminated. Attempts to restore NIT-2 DNA binding ability after Chelex treatment by adding zinc ion all failed (Figure 15).
NIT-2 Protein Does Not Bind Glutamine

Equilibrium dialysis experiments were used to test whether the NIT-2 protein binds glutamine. Many conditions were used, but no indication of glutamine binding was obtained. The NIT-2 protein inside the dialysis tubing failed to enrich glutamine. When samples from inside and out of the dialysis tubing were tested for the amount of radioactively-labelled glutamine, no difference was observed (Table 1); thus, these experiments failed to detect any interaction between NIT-2 and glutamine.

Affinity chromatography with a glutamine-agarose resin was also used to test whether NIT-2 binds glutamine. NIT-2 segment (606-1036), solubilized in renaturing buffer as described previously, was incubated with glutamine-agarose at 4°C for different lengths of time, and the resin was eluted with the same buffer containing 500 mM KCl. Protein samples from eluate and flow-through fractions were analyzed on a SDS-PAGE gel; no NIT-2 protein was found within the eluate; rather, all of the NIT-2 was found in the flow-through (data not shown).

EMSA assays with N. crassa nuclear extract and glutamine showed that high concentrations of glutamine included in the EMSA buffer had no obvious negative effect on the DNA binding ability of native NIT-2 protein (Figure 16). This again suggested that NIT-2 does not bind glutamine.
NIT-2 Protein Dimerization

Chemical crosslinking experiments were done to test the possibility that the NIT-2 protein might form homodimers, since dimer formation is a characteristic frequently observed with DNA-binding proteins. Because of its insolubility, an E. coli-expressed full-length protein could not be examined for dimer formation. A truncated NIT2 protein containing residues 606 to 851 (encoded by a BamH1-EcoRI DNA fragment) which contains the NIT-2 zinc finger domain was used for this study, because many homodimeric DNA binding proteins have their dimerization region very close to their DNA binding domain. After the NIT-2 protein was subjected to crosslinking conditions (0.005% glutaraldehyde) and analyzed by SDS-PAGE gel electrophoresis, dimers were observed and small amounts of multimers were also visible. Another NIT-2 protein which was shorter by 30 amino acids, consisting of residues 606 to 821 (encoded by a BamH1-SstII DNA fragment), was similarly tested and also was found to form homodimers. As a negative control, the full-length NMR protein was subjected to identical cross-linking conditions and did not form any dimers, in agreement with previous experiments carried out by Dr. Young (110) (Figure 17).
IDENTIFICATION OF NIT-2 IN *NEUROSPORA CRASSA* NUCLEAR EXTRACTS

Wild-type *Neurospora* nuclear protein extracts were prepared as described in Materials and Methods, and used to determine whether the native NIT-2 protein could be identified based upon sequence-specific DNA binding. A factor in the nit-2* nuclear extracts binds DNA and yields a specific DNA-protein complex with a BamHI-RsaI DNA fragment derived from the promoter region of the L-amino acid oxidase gene (*lao*) in a gel band mobility shift assay (Figure 18). This DNA fragment contains paired GATA elements which have been demonstrated to constitute NIT-2 binding sites. In contrast, no specific complex was detected when the identical nit-2* nuclear extract was tested for binding with a similar-sized DNA fragment (300 bp) that lacked GATA elements (Figure 18). Moreover, a nuclear extract prepared from the nit-2 rip mutant completely failed to show the specific complex with the *lao* promoter as was observed with the nit-2* extract (Figure 19). These results demonstrate that a factor in the nit-2* nuclear extract binds DNA with the specificity expected of NIT-2, and imply that this factor is, in fact, the native NIT-2 protein.

The factor present in NIT-2* nuclear extracts binds to different *lao* promoter binding sites with different affinities, which agrees with a similar pattern displayed with that of an *E. coli*-expressed NIT-2 protein. The *lao* promoter contains two NIT-2 binding sites, each of which possesses two
GATA elements, and a single binding site that contains only one GATA; these distinct binding sites have a different binding affinity for the E. coli-expressed NIT-2 protein (44). When DNA fragments containing these different sites were tested individually, it was found that they differ significantly in binding affinity for the factor present in nuclear extracts (Figure 20); the same pattern of binding site strength was also found with the E. coli-expressed truncated NIT-2 protein. One binding site, which has two GATA elements separated by 3 bp, was clearly a stronger binding site than the other site which contains two GATAs with a spacing of 10 bp; a DNA fragment that contains both of these binding sites is bound with a high affinity by the factor present in the NIT-2\textsuperscript{+} nuclear extract and by the E. coli-expressed NIT-2 protein.

Inhibition of DNA Binding by An Anti-NIT2 Antibody

A polyclonal antibody, raised in response to a E. coli-expressed polypeptide containing the DNA binding region of NIT-2 protein, strongly inhibits specific DNA binding by the NIT-2/\textbeta-Gal fusion protein. This polyclonal antibody also strongly inhibited the specific DNA-binding by the factor present in nuclear extracts (Figure 21). As a negative control, this same antibody was shown not to affect DNA binding by a nuclear factor from another fungal species, Penicillium (data not shown), and this control nuclear factor
was shown not to bind the GATA DNA sequence (112).

Specific Recognition of GATA Elements by Factor Present in Nit-2* Nuclear Extracts

To further investigate whether the DNA binding protein present in wild-type nuclear extracts is the native NIT-2 protein, DNaseI footprinting assays were conducted with DNA fragments derived from the lao promoter. The factor present in the nuclear extract protected nearly the identical region as did the E. coli-expressed NIT-2/β-gal fusion protein (Figure 22). To exclude the possibility that a sequence adjacent to GATA element might be responsible for this protection pattern, a depurination experiment was employed. The results demonstrate that the factor directly recognizes the nucleotides of the GATA sequence (Figure 22). Three NIT-2 binding sites exist within the DNA fragment used for the depurination experiment; accordingly, only partial protection of individual sites, as expected, was observed.

To further characterize the DNA binding factor in nuclear extracts, four DNA fragments derived from the nit-3 gene promoter, that differed only by base substitutions in GATA elements, were used in mobility-shift assays with the nit-2+ nuclear extract. It was found that a single nucleotide change within just one GATA of paired GATA elements that constitute a binding site significantly reduced the DNA-protein interaction (Figure 23).
Detection of NIT2 by Western blots

A Western blot detected a specific protein band of the expected size (110,000 daltons) of NIT-2 in wild type nuclear extracts. In contrast, this protein was not observed in nuclear extracts of the nit-2 rip mutant (Figure 19). The various results presented above together imply that the DNA-binding factor present in nit-2+ nuclear extracts is the native NIT-2 protein itself.

NIT-2 Is Localized Within the Nucleus

Nuclear and cytoplasmic extracts were prepared from nitrate-induced and nitrogen-repressed wild-type cells and examined by both mobility-shift and Western blot experiments. In both nitrate induced (nitrogen-derepressed) cells and in nitrogen-repressed cells, the NIT-2 protein was localized within the nucleus, and could not be detected in the cytoplasm (Figure 24). It is also noteworthy that NIT-2 in nuclear protein extracts is eluted during heparin-agarose chromatography by 300 mM KCl, which is typical of nuclear proteins (Figure 25). The content of the NIT-2 protein was increased about 3 to 4-fold in cells under derepressed conditions, as compared to that observed in nitrogen-repressed cells (Figure 25). This agrees with previously-reported Northern blot experiments which showed that the nit-2 mRNA level increased by 3 to 4-fold in wild-type cells moved from
nitrogen-repressed to nitrogen-derepressed conditions.

DETECTION OF DIRECT INTERACTION BETWEEN NIT-2 AND NMR

In vivo Test for Interaction between NIT2 and NMR Proteins Using the Yeast Two Hybrid System:

The \textit{nmr} gene was cloned into pJS246 to encode the full-length NMR protein fused to the GAL4 DNA binding domain. The \textit{nit-2} gene was cloned into pACT to allow expression of the entire NIT-2 protein fused to the GAL4 activation domain. Neither of these two constructions activated $\beta$-gal 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 for $\beta$-gal. In contrast, when these two constructs were both transformed into the yeast host together, the transformed colonies turned blue within one hour with the filter enzyme assay. As negative controls, several other cotransformations were also done, and NIT-2/NMR was the only pair that showed positive interaction (Table 2). These results suggest that a specific protein-protein interaction occurs between NIT-2 and NMR.

Specific Regions of NIT-2 Interact with NMR.

In order to rule out the possibility that non-specific interactions between NIT-2 and NMR gave a false positive
result, different regions of the NIT-2 protein were tested to detect specific regions that were responsible for the NIT-2-NMR interaction. The entire amino terminal region of NIT-2 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 26, 27).

This region of NIT-2, 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 28. Two stretches of amino acids were found to be responsible for the interaction 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.

A Specific Region of NMR Interacts with NIT-2.

Different segments of the NMR protein were similarly tested to determine whether a specific region of NMR was responsible for interaction with NIT-2. An NMR region containing 170 amino acids displayed a weak positive result for binding to NIT-2, as shown in Figure 29. Interestingly, one construct that 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 the correct conformation.

**In vitro Analysis of Interaction between NIT-2 and NMR.**

Various segments of the nit-2 gene which encode different regions of the NIT-2 protein tested above in the yeast two hybrid system were cloned into the pRSET vector for expression in *E. coli* to obtain the corresponding NIT-2 proteins for *in vitro* tests. Similarly, different segments of the nmr gene, which were fused to the GAL4 DNA binding domain for tests with the yeast two hybrid system, were cloned into pGEX-2T to express and purify fusion proteins. The fusion proteins were expressed in *E. coli*, purified, and tested *in vitro* for specific NIT2-NMR protein-protein binding as described in Materials and Methods.

A truncated NMR protein that lacked its amino-terminal 46 amino acid residues showed positive interaction with NIT2; however, all other deletions of NMR resulted in a loss of its interaction with NIT-2 (Figure 27). 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. However, when a NIT2 protein containing both of these two regions was tested, a strong interaction with NMR *in vitro* was readily detectable. 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 in vitro assay. This upstream region has a weak affinity for the agarose resin (data not shown), but this stretch may also have some stabilizing effect on zinc finger domain. The results of the in vitro studies are shown in Figure 27.

Mutation Analysis to Identify Residues within Zinc Finger Region Critical for NMR Binding

Several nit-2 mutations which give amino acid substitutions in the zinc finger region result in a total loss of NIT2 protein DNA binding activity (Figure 30). These mutant nit-2 genes were introduced into the vectors used for both the in-vivo yeast two hybrid system and for the in vitro affinity system described above to test their effects, if any, upon the NIT2-NMR interaction. Several mutants that encoded NIT-2 proteins with substitutions in the zinc finger had significant effects upon the interaction with NMR; moreover, the results obtained with the in vivo and in vitro assays yielded consistent results (Figure 31). These results indicated that a short α-helical region of the zinc finger motif of NIT-2, identified by homology with the nuclear magnetic resonance structure of the closely related GATA-1 protein (98), interacts with NMR. It was clear that the mutations which had the strongest effect and almost completely eliminated any NIT2-NMR binding had amino acid substitutions within this α-
helix region. I also performed a computer analysis with the IBI Pustell program on the other region at the C-terminus of the NIT-2 protein which displayed binding to NMR. The last 14 residues of NIT-2 at its carboxyl terminus were predicted by the Chou-Fasman analysis to form an alpha helical structure. Thus, both regions of NIT-2 which appear important for specific protein-protein binding to NMR appear to have an alpha-helical structure.

The NMR Protein Inhibits NIT-2 Sequence-Specific DNA Binding.

The NIT2 protein binds to the lao gene promoter DNA fragment as visualized with a distinct mobility shift. When the purified GST-NMR fusion protein was incubated with NIT-2 before the target DNA was added, a clear decrease in the amount of NIT2-DNA complex represented by the retarded DNA fragment was obvious. Moreover, the extent of inhibition of formation of the NIT-2-DNA complex was proportional to the amount of NMR protein which was added. The GST protein alone had no effect on the DNA binding ability of NIT-2 (Figure 32).

AN Activation Function of NIT-2 Zinc Finger Domain

The nit-2 DNA sequence which encodes the region upstream of its zinc finger was cloned into pJS246 to fuse it with the GAL4 DNA binding region. When this individual construction was transformed into yeast, β-gal gene transcription was activated, as revealed by a positive response with the filter
paper assay. This agreed with a previous finding that two acidic domains within this region might be involved in NIT-2's activation function. Unexpectedly, the zinc finger region of NIT-2 alone when fused to the GAL4 DNA binding domain also activated β-gal expression in the yeast one hybrid system. A region with as few as 100 amino acid residues (encoded by a Kpn1-EcoR1 DNA fragment) including the NIT-2 zinc finger fused with GAL4 DNA binding domain was able to activate β-gal activation. The effects of five different mutations which give rise to amino acid substitutions in the NIT-2 zinc finger domain were examined (Table 3). It was found that the effects of these mutations on the interaction of NIT-2 with NMR were different from their effects on the activation function of the zinc finger domain. Mutation #5, which reduced the interaction between NIT-2 and NMR, eliminated the activation function of the NIT-2 zinc finger completely. On the other hand, mutation #3 had almost no negative effect on the activation function of the NIT-2 zinc finger while it eliminated the interaction between NIT-2 and NMR.
In *Neurospora crassa*, the ability to selectively utilize a variety of different nitrogen sources is precisely controlled by an integrated regulatory circuit which includes trans-acting regulatory proteins and small molecular weight repressors and inducers. A major nitrogen regulatory gene, *nit-2*, is central to operation of the entire nitrogen circuit. The NIT-2 global-acting nitrogen regulatory protein contains a single Cys$_2$/Cys$_2$-type zinc finger motif which, together with the adjacent basic region, confers sequence specific DNA binding which is essential for *nit-2* function. Promoters of two structural genes, *alc* and *nit-3* subject to nitrogen control were previously found to possess recognition elements for the NIT-2 protein. The expression of L-amino acid oxidase is controlled by the *nit-2* gene product (97). The results of this work confirmed that the *lao* promoter contains NIT-2 binding sites. The strongest NIT-2 binding site contains two GATA core sequences separated by only 3 base pairs. A second weaker site contains two GATA sequences separated by 10 base pairs. The third and weakest binding site in the *lao* promoter contains only a single GATA sequence. A mutant NIT-2 protein
which results in a reduced expression of L-amino acid oxidase in vivo showed a parallel reduced binding of the mutant NIT-2 protein to the lao promoter in vitro. These results strongly support the suggestion that these binding sites are important functional elements for lao expression in vivo, and likewise imply that the DNA binding ability of NIT-2 is necessary for its regulatory function.

These distinct DNA elements may allow different levels of expression of the three structural genes and may also be important in permitting interaction of the NIT-2 protein with specific trans-acting factors, which differ for each of these three structural genes and are required in each case to mediate their specific induction.

The availability of DNA fragments containing promoters of three unlinked but co-regulated structural genes, lao, alc and nit-3, each of which is recognized by the NIT-2 protein, provided an unusual opportunity to study how a global-acting regulatory protein might bind with subtle differences to related but non-identical cis-acting elements. Of particular interest was the possibility that amino acid residues within the zinc finger are important in DNA binding and in distinguishing between these DNA elements.

Site-directed mutagenesis was employed to change two conserved amino acids and one nonconserved amino acids within the zinc-finger loop region into other residues. Enzyme assays to determine the level of L-amino acid oxidase, allantoicase,
and nitrate reductase in transformants enabled me to directly compare the effects of precise changes in the DNA binding domain of NIT-2 upon function in vivo and differential DNA binding in vitro. As expected, conserved residues were less tolerant to amino acid substitutions than nonconserved residues. However, major differences were found in the effect of substitutions for the two conserved residues. Leu₇₅₃ could be replaced by three hydrophobic residues, methionine, valine, and alanine, which differ significantly in the bulkiness of their side chains, and also by the charged residue, aspartate, and still retained function in vivo and in vitro. On the other hand, changes in Trp₇₅₄ could not be tolerated at all, since identical substitutions for it eliminated any detectable NIT-2 function. Similarly, a NIT-2 mutant protein with substitutions for Arg₇₅₅ and Arg₇₅₆ lacked function in vivo and was completely deficient in DNA binding in vitro (43). Thus, of the four adjacent conserved residues in the central region of the loop structure, only substitutions of Leu₇₅₃ have been found that retained function.

Each of the functional mutant NIT-2 proteins resulted in a unique pattern in vivo of expression of three nitrogen-controlled enzymes, nitrate reductase, allantoicase, and L-amino acid oxidase. One mutant, L753V, was particularly interesting. This mutant showed marked preference for the L-amino acid oxidase and nitrate reductase gene promoters in comparison with the allantoicase gene promoter, and this
response was observed both in vivo and in vitro. The results of these studies allowed the prediction that Leu$_{753}$ and Trp$_{754}$ must be important in maintenance of the folded conformation of the zinc finger motif or the specificity of interactions with bases in the binding site. This prediction was subsequently confirmed when the results of a nuclear magnetic resonance structure of the GATA-1/DNA complex was published (98). This structural study revealed that the leucine residue in the zinc finger of GATA-1 that corresponds exactly to Leu$_{753}$ makes direct contact with a base pair in the binding site; moreover, the Trp residue in the loop structure of GATA-1 that corresponds to Trp$_{754}$ was found to be important for maintaining the conformation of the zinc finger that is essential for DNA binding. The characteristics of the GATA1-DNA structure explained to some extent how residues within the zinc finger loop region could actually determine the specificity or affinity of DNA binding. The results of elegant genetic studies with the closely related regulatory protein AREA of Aspergillus nidulans also supports this interpretation, since area mutants which altered nitrogen source utilization affected residues in the loop of the zinc finger motif (54). Thurkal et al. (99) demonstrated that single amino acid substitutions of specific residues in the first finger of the yeast ADR1 regulatory protein, which contains two Cys$_2$/His$_2$ type zinc fingers, could alter the specificity of base recognition in its DNA binding site.
Moreover, a point mutation leading to a single amino acid replacement within the second zinc finger of the WT1 protein had been found in patients with the human kidney neoplasm Wilms tumor (100).

Even though the core DNA sequence recognized by NIT-2 and other GATA binding factors is a GATA element, it was found that flanking regions might have a modest effect on the interaction between GATA sequence and protein factors. Actually, one specific nucleotide change within the core sequence, from GATA to GATT, has been demonstrated to be compatible with significant although reduced (approximately 50%) NIT-2 DNA binding (114). The changes in strength or specificity of specific sites due to flanking regions and certain changes of the core sequence thus provide the basis for differential regulation of functionally related genes.

Previous work clearly demonstrated that the basic region of NIT-2 immediately downstream of the zinc finger is important for DNA binding. My studies within the zinc finger loop region do not rule out the possibility that the basic region might also contribute to the specificity of DNA recognition, including the ability to distinguish between related recognition elements. The NMR-resolved structure of GATA-1 showed that its basic region wraps around the DNA strand while conserved residues formed an α-helix, which was inserted into the major groove of DNA. Except for the prominence of positively-charged residues, the basic regions
of different GATA factors are less conserved than are the zinc fingers. These features are consistent with the concept that the basic region also contributes to the specificity of DNA binding.

Previous studies have shown that NIT-2 is the major positive regulatory protein in the nitrogen regulatory circuit of *N. crassa*. It controls gene expression by binding to upstream activation sites of many different structural genes, a mechanism that is widely used by many transcription regulatory factors. In some cases, expression of the regulatory genes is highly regulated and can include autogenous control (29). The trans-acting factors are synthesized in the cytoplasm of eukaryotic cells and must be transported into the nuclei to exert their control function. This suggested that NIT-2 is a nuclear protein, and in this study, I have demonstrated that NIT-2 is indeed a nuclear protein. A factor present in nuclear extracts isolated from wild-type *N. crassa* exhibited all of the characteristics expected for the NIT-2 protein. This factor bound to DNA fragments from the promoter regions of two genes that are subject to nitrogen control and that are recognized by a NIT-2/β-gal fusion protein expressed in *E. coli*. This factor binds specifically to DNA fragments containing defined NIT-2 binding sites containing characteristic GATA core sequences, and it failed to bind to DNA fragments which lack GATA core sequences, and thus displays exactly the same specificity
established for NIT-2 with an E. coli expressed protein (42). This factor was absent in nuclear extracts of a nit-2 rip mutant. Western blot analysis carried out with anti-NIT-2 antibodies demonstrated that nuclear extracts of wild-type but not of a nit-2 rip mutant contain a protein of the size predicted for NIT-2. It can be concluded from these combined results that I have identified the native NIT-2 protein and that it is localized, as anticipated, in the nucleus. Nuclear transport of some transcription factors is controlled such that they enter the nucleus only under conditions where they will activate gene expression. The NIT-2 protein was found only in nuclear extracts, and not in the cytoplasm, under both nitrogen limiting and nitrogen repression conditions, although in the latter case the amount of NIT-2 was 3 to 5 fold less than under derepressed conditions.

The NIT-2 protein is present in the nucleus under both nitrogen derepressed and repressed conditions; thus, the entry of NIT2 into the nucleus is not controlled, implying that some other mechanism must exist to completely shut down the positive regulatory function of NIT2 during nitrogen repression. Glutamine has been identified as the probable metabolite which exerts nitrogen repression. It remains to be determined whether glutamine might bind directly to the NIT-2 protein and inhibit its activation function, although results which I present suggest that NIT-2 is not capable of binding glutamine. Alternatively, a separate protein, e.g., NMR, could
be the target site for glutamine, and upon binding glutamine, this hypothetical protein could prevent NIT2 activation by direct protein-protein interaction, similar to the GAL4-GAL80 interaction (101,102). Indeed results presented above demonstrated that NIT-2 and NMR specifically bind to one another both in vitro and in vivo.

It was also deemed important to examine the requirements and specificity of DNA binding by the native NIT-2 protein present in wild-type nuclear extracts, in order to compare its properties with those of the recombinant NIT-2/β-gal fusion protein or truncated NIT-2 proteins expressed in E. coli. The E. coli-expressed NIT-2 proteins have been shown to bind with high affinity to sites containing two or more GATA core sequences, with an optimal spacing of 10 bp (113). A single nucleotide substitution in even one of the paired GATA sequences virtually eliminated all NIT-2/β-GAL fusion protein binding, with one exception being that the sequence GATT allows approximately 50% of the binding observed with GATA (114).

DNA binding specificity with the native NIT2 protein present in wild-type nuclear extracts generally agrees with the results obtained with the E. coli-expressed fusion protein, but some important differences were observed. While it is true that a strong binding site for the native NIT-2 protein must contain two GATA elements, their optimal spacing differs from that observed with the NIT-2/β-gal fusion
protein. A binding site with two GATA sequences separated by 3 bp clearly has a higher affinity for the native NIT-2 protein than does a site with GATA elements separated by 10 bps, which is the opposite of that observed for the fusion protein. These two sites are both from the lao gene promoter region. Moreover, I observed that some sites containing only a single GATA element displayed some affinity for the native NIT-2 protein present in nuclear extracts, whereas the NIT-2/B-Gal fusion protein almost completely failed to bind to such sites. A detailed study has been carried out to examine the binding of the native NIT-2 protein in nuclear extracts to oligonucleotides containing different numbers of GATA elements and with varied spacing between two GATAs (Chiang et al., unpublished results). It was found that the native NIT-2 protein binds with different affinities to sites containing a single GATA element, as shown in Figure 23. These results indicate that whereas the GATA core sequence is essential for NIT-2 binding, the context of this element is also significant in determining the precise strength of the binding site.

One phenomenon observed with mobility shift assays with the nit-2+ nuclear extract and lao promoter was that, even though three NIT-2 binding sites exist within the lao promoter DNA fragment used in this assay, only a single DNA-protein complex was always observed. DNA footprinting experiments showed that at least 2 of these sites are occupied by the NIT-2 protein. These sites were individually tested via mobility
shift assays and shown to have different affinities for NIT-2 binding. I interpret the presence of only a single DNA-protein complex with the DNA fragment containing three separate NIT-2 sites as indicating that the individual sites are occupied by NIT-2 at the same time. In contrast, multiple retarded bands, corresponding to the number of individual binding sites, were observed in mobility shift experiments with the NIT-2/β-gal fusion protein and this same lao promoter DNA fragment. One interpretation for this different behavior of the NIT-2 protein in nuclear extracts and the NIT-2/β-gal protein is that the native NIT-2 binds to two or more sites with high cooperativity. In contrast, the fusion protein lacks this ability, perhaps due to the presence of the large β-Gal mass and/or the fact that it lacks significant portions of the full length NIT-2 protein, which might be responsible for NIT-2 oligomerization following DNA binding. The LEXA protein forms homodimers, but only upon the binding of monomers to adjacent DNA sites (115).

Additional indirect evidence in this context was our observation that a single GATA site in the lao promoter DNA fragment was not detected in DNaseI footprinting, but was clearly observed in depurination footprinting. It is plausible that a cooperative binding of NIT2 with the two high affinity sites, each with two GATA elements, changed the conformation of the intervening DNA sequence so that the single GATA site in this region was not available for NIT-2
occupancy. However, in depurination footprinting, the loss of bases from the DNA fragment may have eliminated this cooperativity, so that the single GATA remained available for NIT-2 binding. It is obvious that an important area for investigation is this postulated interaction of NIT-2 monomers upon DNA binding that leads to a high degree of cooperativity.

It is perhaps surprising that many different regulatory proteins recognize the same DNA element, the GATA sequence. AREA, NRE, and GLN3 are positive-acting nitrogen regulatory proteins of Aspergillus nidulans, Penicillium chrysogenum, and Saccharomyces cerevisiae, respectively, that bind to GATA containing sequences (54, 91, Haas et al. unpublished data). DAL80, a negative regulator of multiple nitrogen catabolic gene in Saccharomyces cerevisiae, also binds to GATA sequences (62). An interesting negative-acting GATA binding protein, urbs1, controls iron sequestration in Ustilago (75). Multiple GATA binding factors have been found in mammals, chickens, and in many other species (71), and are believed to play diverse regulatory roles in various tissues.

NIT-2 is the first GATA binding protein gene to be identified from Neurospora. More recently a second Neurospora GATA binding protein with an entirely different function has been identified (unpublished results).

It is obvious that the GATA binding proteins represent a large family of transcription factors which are widely distributed in eukaryotic organisms; these GATA factors exert
diverse regulatory roles, including both positive and negative
control of transcription. Of special interest is the feature
that although eukaryotic cells may contain multiple
transcription factors which recognize the same GATA sequence
elements, specific integrated regulatory responses occur with
precision.

Significant progress has been made in the study of
positive regulation mechanisms of several metabolic circuits
in Neurospora. The DNA binding ability of several different
positive regulatory factors have been very well studied. These
DNA binding factors include \textit{qa1-f} which controls the quinic
acid metabolic pathway, \textit{Cys-3} from the sulfur metabolic
circuit, \textit{nit-2} from the nitrogen metabolism circuit, and \textit{nuc-1}, which controls genes of the phosphorous metabolic pathway.
Negative regulatory proteins have also been identified which
function in each of these metabolic pathways, but unlike the
positive regulators, their mode of function has generally
remained poorly understood. The genetically-defined negative-
acting factors include \textit{qa1-s} which acts in the quinic acid
pathway, \textit{preg} for the phosphorous pathway, \textit{scon-1} and \textit{scon-2}
for sulfur metabolism, and \textit{nmr}, which appears to be a
negative-acting regulator for nitrogen metabolism pathway.
Although the \textit{nmr} gene has been cloned, sequenced, and its
protein product predicted, it was not possible to establish
its role, if any, in nitrogen regulation. The \textit{E. coli-}
expressed NMR protein did not exhibit DNA binding nor did it
bind to glutamine, the metabolite responsible for nitrogen repression. Furthermore, a mutant, meaB of *Aspergillus nidulans* displays a similar constitutive phenotype as does nmr, but it is suspected to encode a membrane protein which affects the transport of nitrogenous compound (116). 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 mechanism may exist in *Neurospora* and *Aspergillus*. The *nit-2* gene can complement an area- mutant strain, but the transformants possessed a partially derepressed phenotype.

It is not a rare occurrence that one regulatory protein inhibits the function of another regulatory protein by binding directly to it. In the lactose catabolic pathway of the yeast, *Saccharomyces cerevisiae*, the negative regulator GAL 80 binds to the last 30 residues of GAL4 (101), so as to inactivate its positive regulatory function. Also in yeast, PHO80, the negative regulator of the phosphorous metabolic pathway, interacts with the positive regulator, PHO4, and inhibits its function (117). In human beings, c-myc inhibits the transcriptional regulator yy-1 by association with it (103).
The recently developed yeast two-hybrid system has provided a powerful but simple tool to study protein-protein interactions, and many important pairs of interacting proteins have been identified (94). The results presented in the work presented here provide evidence which indicates that NMR is indeed a negative-acting regulatory protein. NMR was demonstrated to interact directly with the NIT-2 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 NIT-2, both of which are postulated to form α-helical structures. The finding that NMR can interfere with the DNA binding activity of NIT-2 suggests a plausible mechanism for its action as a negative regulator.

Two separate regions of the NIT-2 protein were demonstrated to interact with NMR. One region is a short α-helical segment within the NIT-2 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 NIT-2. This feature naturally led to the prediction that NMR might block NIT-2 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 NIT-2 protein appear to be
involved in the interaction with NMR, although it is possible that these two helices actually lie near each other in the tertiary structure of the protein.

It is also important to note that several lines of indirect evidence indicate that a NIT-2/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 mutant phenotype. This region includes amino acid residues 42 to 300 (104). A comparison of the amino acid sequences of the NMR protein from different fungal species revealed that one region was highly conserved, and this region (residues 210 to 260) is included in the sensitive region identified by the random mutagenesis study (105). In the work reported here, only one segment of NMR showed a positive (but weak) interaction with NIT-2 and it is also located within this very same region (residues 113 to 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 NIT-2. This feature may be due to the fact that NMR must interact with two discontinuous regions of NIT-2, 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 of the regions of NIT-2 identified here that interact with NMR has received strong support from another study which has demonstrated that deletion of the final 30 amino acids of NIT-2 resulted in a significant loss of nitrogen repression of structural genes in the nitrogen control circuit (Pan et al., unpublished). This segment of NIT2 corresponds to one of the α-helical regions which specifically binds to NMR.

The results reported here which demonstrate a specific interaction between the positive-acting NIT-2 and 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 NIT-2, by NMR, or, alternatively, by a site provided by the NIT-2/NMR complex.

Our finding of the interaction between the zinc finger domain of NIT-2 with NMR may have broader structural and functional meaning beyond its function in nitrogen regulation in N. crassa, particularly since more functions have been found associated with the DNA binding domain of trans-acting...
Regulatory proteins of the GATA family are found widely distributed in many species and share with NIT-2 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.

The experiments using the yeast one-hybrid system demonstrated that the NIT-2 zinc finger domain could replace the GAL4 activation domain to turn on the expression of beta-galactosidase. Yeast one hybrid system has been used successfully in identifying activation domains in several important genes (106, 109). This can be done due to the fact that activation domain motifs identified are conservative and show little species preference. It is unknown whether the zinc finger of NIT-2 not only is essential for DNA binding but also participates in gene activation in Neurospora, but it is interesting to note that recent studies have shown that DNA binding domains of different regulatory proteins may have additional functions other than just DNA binding. The DNA-binding domains may serve as motifs for interaction with other proteins or they may function as activation regions (107, 111).

Studies reported in this thesis include identification of important residues within the NIT-2 zinc finger region that are necessary for NIT-2/DNA interaction, expression of various NIT-2 protein forms in E. coli, identification of the native
NIT-2 protein in *Neurospora* nuclear extracts, and the detection of an interaction between NIT-2 and NMR. Each of these findings not only has its own biological significance, but also provides critical information and concepts which will allow exploration of new features of nitrogen regulation. This study represents significant progress in the study of the nitrogen metabolism circuit in *Neurospora crassa*, and adds to our understanding of complex and integrated patterns of gene transcription regulation in eukaryotic cells.
Figure 1. *Neurospora crassa* nitrogen metabolic circuit. Relationships between members of this circuit are shown. NIT-2 is shown to bind to promoter regions of each structural gene. NIT-4, on the other hand, binds only to promoters of two structural genes, *nit-3* and *nit-6*. "+" represents activation function, and "−" represents repression function (118).
Figure 1
Figure 2. Identification of NIT-2 binding sites in the lao gene promoter. BamH1-Rsa1 DNA fragment from the 5' upstream region of the lao gene was used as the probe. Lane 1 contained probe only. Lanes 2-6 contained probe plus 0.01, 0.02, 0.04, 0.1, or 0.25 ug of NIT-2/β-gal fusion protein. Two major shifted complexes were observed with this DNA fragment.
Figure 2
Figure 3. DNaseI protection experiment with the \textit{lao} promoter and NIT-2/β-gal fusion protein. DNA fragments were incubated in the presence and absence of the NIT-2/β-gal fusion protein, and were subjected to DNaseI digestion as described in Materials and methods. In lanes 1-4, the \textit{BamH}I-\textit{Rsa}I DNA fragment was \textit{32}P-labelled at the \textit{BamH}I site. Lanes 1 and 2, contain DNaseI treated DNA in the absence of NIT-2 protein. Lanes 3 and 4 contained DNA plus 1 ug of the NIT-2/β-gal fusion protein, plus 2 or 4 units of DNaseI, respectively. Lanes 5-8, the \textit{BamH}I-\textit{Styl} DNA fragment was end labelled at the \textit{Styl} site. Lanes 5 and 6, DNaseI treated DNA only. Lanes 7 and 8, contained DNA plus 1 ug of the NIT-2/β-gal fusion protein, plus 2 or 4 units of DNaseI, respectively. The footprint of the stronger binding site on both strand is indicated, while the weaker one is not.
Figure 4. Summary of mobility shift with different DNA fragments from the lao gene promoter region. ++, strong DNA binding by the NIT-2 fusion protein; +, weaker DNA binding; -, no detectable DNA binding. Two filled circles represent the NIT-2 binding sites, as determined by DNA footprints. The position of the binding sites are -240 and -330 respectively.
Figure 4
Figure 5. Mutations of amino acid within the 17 amino acid loop of the single zinc finger of NIT-2. The finger motif on NIT-2 is compared with that of the AREA protein of A. nidulans, the Gln3 of S. cerevisiae, and with one finger of a mouse GATA-binding protein. The four cysteine residues which define the finger motif and four conserved residues (LWRR) within the 17 amino acid loop are boxed. Site directed mutagenesis was carried out to obtain four amino acid substitutions for the conserved Leu, and three substitutions for the conserved Trp. A single substitution was obtained for the nonconserved residue, Asp. (-), non-functional in vivo; (+) and (++) , functional (increasing) in vivo.
Figure 5
Figure 6 A, B. Enzyme levels and growth tests of wild type and transformants containing the nit-2 mutant genes. A. Enzyme activities. The specific activity of each enzyme in the wild type is assigned 100%, and the activity present in each mutant is shown as a percentage of the wild type. B. Growth tests. The growth of the wild type control on each medium was assigned 100%, and the growth of the mutants is reported as a percentage of the wild type. In panel A, striped bar represents nitrate reductase activity; filled bar represents allantoicase activity; open bar represents L-amino acid oxidase activity. In panel B, different bars represent nitrogen sources used for growth tests. Striped bar, nitrate; filled bar, hypoxanthine; open bar, phenylalanine.
Figure 6
Figure 7. Fusion protein detection by NIT-2 polyclonal antibody. Different mutations within zinc finger loop region were introduced into NIT-2/β-gal fusion protein. After protein expression and purification, protein samples were run on a SDS-PAGE gel. Polyclonal antibody against NIT-2 zinc finger region was used to detect these mutant fusion proteins. Lanes 1-6 contained WT, L753M, L753V, L753E, W754E, and D759S respectively.
Figure 7
Figure 8 A-C. Mobility shift experiments with wild-type and mutant NIT-2 proteins. Wild-type and each mutant NIT-2 protein were expressed in *E. coli* as a NIT-2/beta-gal fusion protein. Gel-band shift experiments were carried out as described in Materials and Methods. A *lao* promoter DNA fragment; B *nit-3* promoter fragment; C *alc* promoter DNA fragment. All gels were loaded with the DNA fragment which had been incubated with the same amount of wild-type and mutant proteins in the same order. Lanes: 1, wild type; 2, L753M; 3, L753E; 4, L753V; 5, W754E; 6, D759S; 7, free probe.
Figure 9. Depurination footprinting of NIT-2 binding site within *lao* promoter. An 80 bp *lao* promoter fragment was used as the probe, and NIT-2/beta-gal was used as the DNA binding factor. Depurination interference was done on both transcribed and nontranscribed strands, and the results are summarized. The magnitude of the effects on binding is shown by the height of the bar above or below a base. The full height of the scale represents 100% interference and bases at the binding site are numbered.
Figure 9

GGAACGTCAGGTTGAGGGTAACGCGGATACTGATCTCTCCCTCCGCCCTCAATCCCTTTGAG
CCTTGAGAACCAACTCCATTTGCGGTATGACATAGAGGGAGGGGAGTAAGGGAACTC

[Diagram with base pairs]
Figure 10 A, B. Naturally occurring NIT-2 DNA binding sites. (A) NIT-2 binding sites in promoter regions of nit-3 (at -150), alc (-150) and lao (-240 and -325), are shown. The nit-3 site has three GATA core elements, while all other sites have two GATA elements. In each case, the spacing between GATA elements and their orientation relative to their respective structural genes is shown. GATA sequences are oriented 5' toward structural genes, ATAG sequences as shown represent 5' GATA sequences on the opposite strand, thus facing away from the structural genes. The element at -240 upstream of lao is a strong NIT-2 binding site, whereas at -325 is a weak binding site. (B) the nucleotide sequence surrounding each GATA core element (shown 5' to 3') for the binding sites of nit-3, alc, and the two lao sites is shown. Although each binding site has at least two conserved GATA elements, considerably variability in their orientation, spacing, and surrounding sequences is evident.
Figure 10
Figure 11 A, B. Expression of segments of NIT-2 in *E. coli*. The *SalI-EcoR*I (encodes residues 462-1036) DNA fragment and the *PstI-EcoR*I (encodes residues 200-1036) DNA fragment were expressed in *E. coli* as described in Materials and Methods and sonicated bacterial cell extracts were analyzed on SDS-PAGE gels. (A) *M*, molecular weight marker. Lane 1, uninduced cell extract. Lane 2 and 5, induced cell extract. Lane 3, *SalI-EcoR*I encoded protein segment solubilized in PBS buffer, pH 9.5. Lane 4, the same protein in PBS buffer, pH 8.0. (B) *M*, marker. Lane 1 and 5, uninduced cell extract. Lane 4, induced cell extract. Lane 2, small portions of *PstI-EcoR*I encoded protein solubilized in PBS buffer, pH 9.5. Lane 3, most of the protein remained in insoluble cell pellet.
Figure 11
Figure 12. Gel band shift with different segments of NIT-2 protein. NIT-2 fragments expressed in *E. coli* were used in EMSA assay. *BamH*I-*Rsal* DNA fragment from *lao* promoter was used as the probe. Lane 1 and 2 contained 1 and 2 ug of full length NIT-2 protein in PBS buffer, pH 9.5. Lanes 3-5 contained 1, 2, and 4 ug of *PstI*-EcoR1 (encodes residues 200-1036) NIT-2 protein fragment in PBS buffer pH 9.5. Lane 6 and 7 contained 1 and 2 ug of *SalI*-EcoR1 (encodes residues 462-1036) NIT-2 protein fragment in PBS buffer pH 9.5. Lane 8 contained *PstI*-EcoR1 NIT-2 fragment in PBS buffer, pH 8.0. Lane 9 had probe only.
Figure 13. Purification of *E. coli*-expressed full length NIT-2 protein. SDS-PAGE analysis of protein samples from purification procedure. Purification under denaturing conditions was carried out as described in Materials and Methods. M, molecular weight marker. Lanes 1-7, fractions eluted with buffer C. Lanes 8 and 9, fractions 1 and 2 eluted with Buffer D. Arrow indicates the purified full length NIT-2 protein.
Figure 14. NIT-2 protein purification by heparin column. NIT-2 protein was expressed in *E. coli* and solubilized, and loaded onto heparin column and eluted as described in materials and methods. Samples from different fractions were then analyzed on a SDS-PAGE gel. M, marker. Lane 1, crude protein extract. Lane 2, flow through of heparin column. Lanes 3-6, fractions 1-4 of heparin column eluate. It is clearly shown that NIT-2 bands were missing in flow through fraction and appeared in eluate fractions. There are multiple bands corresponding to NIT-2 because of premature termination of NIT-2 while being expressed. Arrow indicates the position of NIT-2 protein.
Figure 15. Effect of Chelex on NIT-2 DNA binding ability. NIT-2 protein was incubated with Chelex as described in materials and methods, and then subjected to different renaturing conditions. These samples were then used in EMSA assay. DNA probe used is the BamH1-Rsal DNA fragment from the lao promoter. The NIT-2 truncated protein used was encoded by the BamH1-EcoRI (residues 606-851) fragment. Lanes 1 and 2 contained 0.5 and 1 ug of chelex treated NIT-2 protein plus probe. Lanes 3 and 4 contained 0.5 and 1 ug of untreated NIT-2 protein. Lane 5 and 6 contained 0.5 and 1 ug of chelex treated protein supplied with 2 mM ZnCl2. Lane 7 and 8 contained 0.5 and 1 ug of chelex treated protein supplied with 2 mM ZnCl2 and 2 mM DTT. Lane 9 contained probe only.
Table 1. Equilibrium dialysis of glutamine and NIT-2 protein. The truncated NIT-2 protein and \(^{3}H\)-labelled glutamine were mixed, introduced into a dialysis membrane and dialyzed against buffer. Solutions from both inside and outside the dialysis membrane were tested for their radioactivity. Results from two independent experiments are shown.

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<tr>
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<td>995</td>
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Figure 16. Glutamine interference assay with NIT-2 nuclear extract. EMSA was done with NIT-2 nuclear extract. Different concentrations of glutamine were included in the EMSA buffer. BamHI-RsaI from lao promoter was used as the probe. Lane 8 contained probe only. Lanes 1-7 each contained 2 ug of nuclear extract and lao probe, and 0, 2.5, 5, 10, 25, 50, 100 mM glutamine respectively.
Figure 17 A, B. Chemical crosslinking of NIT-2 fragments. NIT-2 BamH1-EcoR1 (residues 606-851) fragment and BamH1-SstII fragment were subject to glutaraldehyde crosslinking, and analyzed by SDS-PAGE gels. NMR full length protein was used as a control. (A) M, molecular weight marker. Lanes 1-4, 4 ug of BamH1-EcoR1 protein subjected to 0.005% glutaraldehyde for 0, 5, 10, and 30 min. Lanes 5-7, the same NIT-2 protein subjected to 0.01% glutaraldehyde for 5, 10, and 30 min. (B) M, marker. Lanes 1-3, 1, 2, 4 ug of NMR subjected to 0.005% glutaraldehyde for 10 min. Lanes 4-6, 1, 2, 4 ug of NIT-2 BamH1-EcoR1 subject to 0.005% glutaraldehyde for 10 min. Lanes 7-9, 1, 2, 4 ug of NIT-2 BamH1-SstII treated with 0.005% glutaraldehyde for 10 min.
Figure 17
Figure 18 A, B. Identification of NIT-2 from *N. crassa* nuclear extract. (A) mobility-shift experiment with the BamH1-Rsa1 DNA fragment from the lao gene promoter region and *N. crassa* nuclear extract. A DNA probe without NIT-2 binding site is used as control. Lane 4 contained lao probe only. Lane 8 contained control probe only. Lanes 1-3 contained lao promoter plus 2, 4, 8 ug of nuclear extract. Lanes 5-7 contained control probe plus 2, 4, 8 ug of nuclear extract. (B) mobility shift with the same lao promoter and NIT-2/β-gal fusion protein. Lane 1-9 contained lao probe plus 0.00, 0.01, 0.02, 0.04, 0.1, 0.25, 0.5, or 1.0 ug of NIT-2/β-gal fusion protein. One complex was formed between the lao and nuclear extract. No complex was formed between control probe and nuclear extract. A total of three complexes were formed between the lao probe and NIT-2/β-gal fusion protein.
Figure 19 A, B. Mobility shift and western blot assays of nuclear extract from nit-2+ and nit-2− strain. (A) mobility shift assays with nuclear extract. Xba1-Xho1 fragment of nit-3 promoter was used as the probe. Lane 1 and 2 contained DNA probe plus 1.0 and 2.0 ug of nuclear extract from the 300 mM KCl fraction obtained from nit-2+ strain. Lane 3 and 4 contained DNA probe plus 1.0 and 2.0 ug of nuclear extract from the 500 mM KCl fraction from nit-2+ strain. Lane 5 and 6 contained DNA probe plus 1.0 and 2.0 ug of nuclear extract from the 300 mM KCl fraction from nit-2− strain. Lane 7 had the probe only. (B) Western blot assays of nuclear extract. M, molecular weight marker. Lanes 1 and 2 contained nuclear extract samples from NIT-2+ and NIT-2− strain, and analyzed on a SDS-PAGE gel. Lanes 3 and 4 are an exact replica of 1 and 2 and detected with NIT-2 antibody.
Figure 19
Figure 20 A, B. Mobility shift with separate NIT-2 binding sites from lao promoter. BamH1-AvaI of the lao was the smallest fragment used in this experiment, containing one NIT-2 binding site. TaqI-Styl was the second largest probe containing another binding site. BamH1-Styl1 was the largest probe containing both NIT-2 binding sites. (A). Lanes 3, 6, and 9 contained probe only. Lanes 1, 4, 7 contained probe plus 1.0 ug of nuclear extract. Lanes 2, 5, and 8 contained probe plus 2.0 ug of nuclear extract. (B). Lanes f, c, i contained probes only. Lanes d, a, g contained probe plus 0.25 ug of E. coli expressed NIT-2 fragment. Lanes e, b, h contained probe plus 0.5 ug of E. coli expressed NIT-2 fragment.
Figure 21. NIT-2 antibody interference with the NIT-2/DNA interaction. BamH1-Rsal fragment of Lao promoter was used as the DNA probe in EMSA assay. Lane 1 contained DNA probe plus 0.5 ug of NIT2/beta-gal fusion protein. In addition to what lane 1 had, lanes 2-4 contained 1 ul preimmune serum, 1 ul or 2 ul anti-NIT-2 antiserum respectively. Lane 5 contained DNA probe plus 2 ug of nuclear extract. In addition to what lane 5 had, lanes 6-8 contained 1 ul preimmune serum, 1 ul or 2 ul anti-NIT-2 antiserum respectively. Lane 9 contained DNA probe only.
Figure 22. Footprint experiment with lao promoter and N. crassa nuclear extract. BamH1-Styl of lao promoter end-labelled at BamH1 was the probe. Lane 1 contained probe only treated with DNase 1. Lanes 2 and 3 contained probe plus 1.0 and 2.0 ug of NIT-2/beta-gal fusion protein treated with DNase 1. Lanes 4 and 5 contained probe plus 2.0 and 4.0 ug of nuclear extract from wt strain, and treated with DNase 1. Lanes 6 and 7 contained probe plus 2.0 and 4.0 ug of nuclear extract from nit-2 mutant N. crassa strain, and treated with DNase1. The strongest site protected by fusion protein is indicated on lane 2 and 3. Two equally well protected regions by nuclear extract are also indicated on lanes 4 and 5. Bases marked by black dots are those shown to interact with NIT-2 protein by depurination experiment. The third binding site is also shown.
Figure 22
Figure 23 A, B. Affinity of native NIT-2 for double and single GATAs. (A) An XbaI-XhoI DNA fragment from nit-3 promoter region was used as the probe. Site-directed mutagenesis was done to create mutations on GATA sequence. Lanes 1, 3, 5, and 7 contained probes with 2, 1, 1, and 1 GATA sequence respectively. Lane 2, 4, 6, 8 contained probes corresponding to lanes 1, 3, 5, and 7 plus 4.0 ug of nuclear extract. The probe with two GATA was clearly shifted, while mutations within the other probes eliminated the shifted complex. (B) Artificial oligonucleotides were used in mobility shift assay with nuclear extract. Lanes 7, 8, 9 contained the probe with one single GATA plus 2ug, 4ug, and no nuclear extract. Lane 4, 5, 6 contained the probe with another single GATA plus 2ug, 4ug, and no nuclear extract. Lanes 1, 2, 3 contained the probe with both of these GATA sequences plus 2ug, 4ug, and no nuclear extract.
Figure 24 A, B. Localization of NIT-2 protein in vivo. (A) mobility shift with nuclear and cytoplasmic extract. The BamH1-Styl DNA fragment of lao promoter was used as the probe. Lane 1 contained the probe only. Lane 2 and 3 contained the probe plus 4.0 and 8.0 ug of cytoplasmic extract from under repression condition. Lane 4 and 5 contained probe plus 8.0 and 4.0 ug of nuclear extract from cells under repression condition. Lane 6 and 7 contained probe plus 4.0 and 8.0 ug of cytoplasmic extract from cells under induction condition. Lane 8 and 9 contained probe and 8.0 and 4.0 ug of nuclear extract from cells under induction condition. (B). Lanes 1 and 2 are from a coomassie blue stained SDS-PAGE gel, and they contained protein samples also used in lane 7 and 8. Lanes 3 and 4 is the Western blot with NIT-2 antibody corresponding to lane 2 and 1 respectively.
Figure 25 A, B. Step gradient elution of nuclear extract from heparin column and EMSA analysis. A. a 66mer oligonucleotide containing two GATA sequence was used as the probe. Approximately an equal amount of nuclear extract was added in each EMSA assay. Lane 1-4 contained probe plus nuclear extract from 100 mM, 300 mM, 500 mM KCl and flow through fractions. The nuclear extract was from cells grown under nitrogen repressed condition. Lane 5-8 contained probe plus nuclear extract from 100 mM, 300 mM, 500 mM KCl, and flow through fractions. The nuclear extract was from cells grown under induction condition. Lane 9 contained probe plus 0.5 ug of E. coli expressed NIT-2 fragment (BamH1-EcoR1. 220 residues). Lane 10 contained probe only. B. Western blot assay of nuclear extract with NIT-2 antibody. Lane 1 contained nuclear extract sample identical to that in lane 6 in panel A. Lane R contained nuclear extract sample identical to that in lane 2 in panel A.
Table 2. Detection of possible interactions between members of *Neurospora crassa* nitrogen metabolic circuit. None of these constructions yielded a positive filter assay results as one hybrids. "-" represents no color change from filter paper assay of transformants, which resulted from cotransformation using constructions shown in left two columns. "+" represents color change in the filter paper assay. AD, activation-domain; DBD, DNA binding domain.

<table>
<thead>
<tr>
<th>fusion to Gal4 AD</th>
<th>fusion to Gal4 DBD</th>
<th>filter assay results</th>
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</thead>
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<tr>
<td>Nit-3 wl</td>
<td>NMR wl</td>
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</tr>
<tr>
<td>Nit-3 wl</td>
<td>Nit-4 wl</td>
<td>-</td>
</tr>
<tr>
<td>Nit-2 wl</td>
<td>Nit-4 wl</td>
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<tr>
<td>Nit-2 wl</td>
<td>NMR wl</td>
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</tbody>
</table>
Figure 26 A, B. NIT-2/NMR in vitro interaction assay 1. (A) SDS-PAGE gel analysis of protein samples from in vitro NIT-2/NMR interaction assay. M, molecular weight marker. Lane 1 contains eluate from the glutathione-agarose resin incubated with GST-NMR and NIT-2 fragment (residues 522-827). Lane 2 contains eluate from the glutathione-agarose resin incubated with NIT-2 fragment(residues 522-827) only. Lane 3 contains eluate from the glutathione-agarose resin incubated with GST-NMR only. Lane 4 contains eluate from the glutathione agarose resin incubated with GST only. Lane 5 contains eluate from the glutathione agarose resin incubated with GST-NMR and NIT-2 fragment (residues 732-1036). Lane 6 contains eluate from the agarose resin incubated with NIT-2 fragment (residues 732-1036) only. (B) Samples from panel A were transferred to a nitrocellulose filter and analyzed with anti-nit-2 antibody. Lane assignment was exactly the same as in panel A.
Figure 27 A, B, C. NIT-2/NMR in vitro interaction experiment
2. Eluates from the glutathione-agarose resin incubated with
GST or GST-NMR plus NIT-2 proteins were analyzed using anti-
nit-2 antibody. GST only was used in panel A; GST-NMR
(residues 46-488) was used in panel B; GST-NMR (residues 46-
358) was used in panel C. In each panel, NIT-2 (residues 522-
732) was used in experiment represented by lane 1, NIT-
2(residues 732-827) was used in lane 2, NIT-2 (residues 732-
1036) was used in lane 3, and NIT-2(residues 862-1036) was
used in lane 4.
Figure 27
Figure 28. Localization of NIT-2 segments interacting with NMR. Left column represents NIT-2 residues fused to GAL4 activation domain and also expressed in E.coli for in vitro interaction assay. Open bars represent regions of NIT-2 indicated by numbers in left column. Solid boxes represent the NIT-2 zinc finger, and stripped boxes represent two acidic domains of NIT-2. Column under "yeast" represents results from yeast two hybrid system, and column under "GST" represents results from in vitro experiments using GST fusion system. In the column under "yeast", both "++" and "+" represent color change within one hour, "++" is clearly faster than "+". "++", quantitively represents 100+/−30 miller units, and "+" represents 30+/−10 miller units. "−" represents no color change. In the column under "GST", "+" represents detectable interaction in vitro, "−" represents no detectable interaction.
<table>
<thead>
<tr>
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<th>Yeast</th>
<th>GST</th>
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<tbody>
<tr>
<td>1</td>
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<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
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<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>Gal4AD NIT-2 (522-827)</td>
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</tr>
<tr>
<td>4</td>
<td>Gal4AD NIT-2 (732-1036)</td>
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<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Gal4AD NIT-2 (522-732)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Gal4AD NIT-2 (862-1036)</td>
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<td>-</td>
</tr>
<tr>
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<td>Gal4AD NIT-2 (927-1036)</td>
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<td>-</td>
</tr>
<tr>
<td>8</td>
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<tr>
<td>9</td>
<td>Gal4AD NIT-2 (732-827)</td>
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</tr>
<tr>
<td>10</td>
<td>Gal4AD NIT-2 (732-827/1006-1036)</td>
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<td>+</td>
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</table>
Figure 29. Localization of NMR segments interacting with NIT-2. Left column shows the residues of NMR protein fused to GAL4 DNA binding domain. Open bars represent segments of NMR used in interaction study. The right two columns show both the \textit{in vitro} and \textit{in vivo} results of interaction study, with "yeast" representing results from yeast two hybrid system and "GST" representing results from GST-fusion system. A "+" means clear color change within one hour in filter paper assay, a "+/−" means a color change after a 5 hour period, and "−" means no color change.
<table>
<thead>
<tr>
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<tr>
<td>1. NMR wild</td>
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<tr>
<td>2. Gal4DB NMR(46-488)</td>
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<td>+</td>
</tr>
<tr>
<td>3. Gal4DB NMR(46-358)</td>
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<td>-</td>
</tr>
<tr>
<td>4. Gal4DB NMR(46-107)</td>
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<td>-</td>
</tr>
<tr>
<td>5. Gal4DB NMR(46-115/284-488)</td>
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</tr>
<tr>
<td>6. Gal4DB NMR(118-284)</td>
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</tbody>
</table>

Figure 29
Figure 30. Mutations created within NIT-2 zinc finger domain. Mutations created are numbered and shown under the original residues. Four cysteines are shown in bold face, and residues constituting an α-helix are shown in italic form.
CTNCFTQTTPLWRNRPDGQPPLCNAC
  ser  Gly  Gly Gly  AspVal
          5            1            3

GLFLKHLGVRPLSLKTDVIKKRRNR
  Asp Val  Asn Ser
        4            2

Figure 30
Figure 31 A, B. Analysis of NIT-2 mutant proteins for the NIT-2/NMR interaction. (A) Eluates from the glutathione agarose resin incubated with GST-NMR and NIT-2 with different amino acid substitutions were analyzed by Western blot using anti-nit-2 antibody. Lane 5, NIT-2 segment contains residues 732-1036. In lane 6, NIT-2 segment contains residues 757-827 plus residues 1006-1036. Lanes 1-4 contain NIT-2 residues 732-1036 plus 1006-1036 with mutations No. 1, 3, 4, and 5 respectively. (B) Identical experiments as in panel A, but with different NIT-2 segments. Lane 1 contains the same sample as in lane 5 of panel A. Lanes 2-6 contain eluate from glutathione-agarose resin incubated with GST-NMR and NIT-2 segment (residues 757-827) with no mutation and mutations No. 1, 3, 4, and 5 respectively.
Figure 32. NMR interference with the NIT-2/DNA interaction. The BamH1-Styl DNA fragment of the lao promoter was used as the probe. Lane 1 contained probe plus GST-NIT-2 fusion protein. Lanes 2-4 contained the same amount of DNA probe and GST-NIT-2 fusion protein plus increasing amount of GST-NMR protein (moles of GST-NIT-2/moles of GST-NMR= 3.2, 1.6, and 0.8). Lanes 5-7 contained the same amount of DNA probe and GST-NIT-2 protein plus increasing amount of GST protein (moles of GST-NIT-2/moles of GST=1.0, 0.5, and 0.33). Lanes 8 and 9 contained the same amount of DNA probe and GST-NIT-2 plus a truncated NMR fused to histidine-taq (moles of GST-NIT-2/moles of NMR= 1.0, and 0.5). For lane 1, beta scanning showed the shifted probe/unshifted probe=1.0. For Lanes 2-4, the ratios were 0.4, 0.35, 0.30 respectively. For Lanes 5-7, the ratios were 1.1, 1.5, 1.7 respectively. For Lanes 8 and 9, the ratios were 3.8 and 3.1 respectively. A competition between GST-NMR and DNA probe for NIT-2 is clearly indicated.
Figure 32
Table 3. Mutational analysis of activation function of NIT-2 zinc finger. Mutations 1-5, as described in figure 30, were introduced into the one hybrid system as described in materials and methods. Filter paper assay was done for transformants with different mutant constructions. +, color change; -, no color change.

<table>
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<td>+</td>
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LIST OF REFERENCES


