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EXPRESSION AND REGULATION OF CYS3 AND NIT2, THE TWO
POSITIVE REGULATORS FOR SULFUR AND NITROGEN
METABOLISM IN NEUROSPORA CRASSA

DISSERTATION

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

By
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********

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ABSTRACT

In *Neurospora crassa*, sulfur and nitrogen metabolism has been subject to extensive studies for years. The transcription factor CYS3 of *Neurospora crassa* is a positive regulator of the sulfur regulatory circuit which contains many structural genes involved in sulfur metabolism. Expression and degradation of the CYS3 protein is precisely regulated in a sulfur-dependent manner. Cys-3 expression was found to be fully repressed by high concentrations of methionine or inorganic sulfate present in the culture medium, and to be derepressed when these favored sulfur sources were limited. Cys-3 transcripts could be readily detected within two hours after derepression whereas the CYS3 protein was not found until after four hours. CYS3 is stable with a half life greater than 4 hours under low sulfur conditions when it is required for cell growth. However, it is degraded relatively quickly when methionine or inorganic sulfate become available. Upon sulfur repression cys-3 transcripts disappeared within thirty minutes with an estimated half life of 5 minutes whereas CYS3 protein almost entirely disappeared in one hour with a half life of approximately 10 minutes. Site-directed mutagenesis showed that Lys-105 of CYS3 is important for its instability. The change of this single residue from lysine to glutamine resulted in a prolonged half life of CYS3 and impaired responsiveness of CYS3 degradation to sulfur level changes.

As a global transcription factor, NIT2 positively regulates the expression of up to a hundred genes that are related to nitrogen metabolism, and is responsible for lifting of nitrogen catabolite repression when the cellular levels of glutamine or other favored nitrogen sources become limited. Immunoprecipitation of NIT2 protein by antibodies
showed that the NIT2 protein is constitutively expressed, however the expression level can be elevated a few fold when nitrate instead of glutamine is used as the sole nitrogen source. The NIT2 protein is very stable and the stability of NIT2 is not affected by the available nitrogen source. The nit-2 transcripts appear to be very stable and is not regulated by the nitrogen source. The unusually high molecular weight of NIT2 detected in SDS polyacrylamide gel (150 kD) in comparison to the estimated molecular weight (124 kD) suggests a possible post-translational modification. The lack of regulation of cellular levels of nit-2 transcripts and NIT2 protein suggests that post-translational modification of NIT2 and interactions with other proteins, e.g., in the nitrate assimilation pathway, interactions with NIT4 and NMR, may play very important roles in modulating the function of NIT2 in response to environmental stimuli.

The expression of the nit-3 gene (nitrate reductase) is dependent upon nitrogen derepression and nitrate induction and is regulated by two positive-acting transcription factors, NIT2 and NIT4, and a negative regulator, NMR. The presence of a tightly linked cluster of NIT2 and NIT4 binding sites suggested that their close spacing might be required for a synergistic interaction of the NIT2 and NIT4 proteins. However separation of NIT2 and NIT4 binding sites showed no effect on either the expression level or the precise regulation of the nit-3 gene. Studies conducted on the NIT2 site II, which contains only a single GATA element and yet plays a major role in nit-3 gene expression, showed that nucleotides both 5' and 3' of the GATA sequence were important for strong DNA binding in vitro and its activation function in vivo. The nit-3 promoter contains two long AT-rich sequences, one of which is located just upstream of the transcription start sites and is required for optimal promoter function. The nit-3 transcript contains eight TACC repeats in its 5' non-coding region which appear to be involved in mRNA instability. Deletion of these TACC repeats led to a significant increase in the stability of nit-3 mRNA.
To my family
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Chapter 1

Introduction

1.1. Gene regulation in eukaryotes

For almost all living creatures, the genetic information is stored in chromosomes. The passage of this information from chromosomal DNA to RNA to protein is a complex process that can be regulated at many different levels, such as 1) transcription, 2) RNA processing, 3) RNA transport, 4) translation, 5) mRNA degradation, and 6) protein activation and inactivation (Alberts et al., 1994).

As the first step of gene expression, transcriptional control is the best studied and is considered one of the most efficient ways for cells to respond to environmental stimuli. To transcribe or not to transcribe a gene allows cells to maximize the uses of limited resources and therefore to achieve the best output.

RNA processing and RNA transport controls are apparently not as commonly used as transcriptional control by eukaryotes. RNA processing control involves the selective and alternative splicing of hnRNAs to generate mRNA, while RNA transport control involves the selective transport of certain types of mRNA from nucleus to cytoplasm. Control of RNA processing or RNA transport or a combination of both allows cells to achieve the selective expression of certain genes (Alberts et al., 1994).

While eukaryotic protein translation is one of the most conserved biological processes, translational control is not a common phenomenon in gene expression regulation. Instead, overall protein synthesis slows down when cells encounter an
unfavorable growth environment. Phosphorylation of initiation factor eIF-2 is one of the important steps that leads to this slowdown (DeHaro et al., 1996). However, phosphorylated eIF-2 can stimulate the translation of certain special genes, such as yeast transcription factor GCN4, that enable cells to cope with the stressful environment (Alberts et al., 1994; Pain, 1994).

Gene expression can also be controlled by changes in mRNA stability. There are many diverse examples of this regulated event, such as 1) proteins that bind to the 5' leader region of mRNAs, 2) changes in the 3' untranslated region (removal of the 3' UTR or the shortening of the polyA tail), 3) coupling the degradation of mRNA with translation (Alberts et al., 1994; Klaff et al., 1996).

Protein modification is another widespread regulation mechanism. Many systems activate, inactivate or compartmentalize protein molecules after they have been synthesized. Although other types of post-translational modification play important roles in regulating the activation of transcription factors, protein phosphorylation/dephosphorylation is a primary mechanism to coordinate cellular events with external stimuli. Phosphorylation/dephosphorylation may affect the activity of transcription factors at several levels by, 1) sequestering the transcription factors in the cytoplasm and prevent them from gaining access to their target genes, 2) affecting the DNA binding affinity of transcription factors, and 3) modulating the ability of transcription factors to interact with other proteins in the transcriptional machinery. These three different mechanisms are by no means mutually exclusive, and phosphorylation at different or multiple sites can have different effects (Alberts et al., 1994; Calkhoven, 1996).

1.2. RNA polymerase II machinery and class II promoters

In eukaryotes, three distinct forms of nuclear DNA-dependent RNA polymerases are found with different template specificity and nuclear localization. RNA polymerase I is
located in nucleoli and responsible for the transcription of 5.8S, 18S and 28S ribosomal RNA, while RNA polymerase III is located in the nucleoplasm and transcribes 5S ribosomal RNA and all of the transfer RNA molecules. Also located in the nucleoplasm, RNA polymerase II (pol II) transcribes all the messenger RNAs that later can be translated into proteins (Sawadogo, 1990). Due to its role in gene regulation, RNA pol II and the general transcription factors associated with it became the most studied transcription machinery. As a large multisubunit enzyme, RNA polymerase II can not recognize its target promoter directly and form an initiation complex. Accessory factors such as general transcription factors, transcriptional activators and coactivators are needed to initiate the transcription of class II promoters. Originally identified through chromatography, these general transcription factors were named by the fractions that contain them, including TFIIB, TFIID, TFIIIE and TFIIIF. In fraction D, a transcription factor TBP (TATA box binding protein) is able to bind to the TATA box of class II promoters and helps RNA pol II to assemble into a stable complex to initiate transcription (Chang and Jaehning, 1997). TFIIB and TFIIIF are also involved in the initiation stage of transcription while TFIIIE interacts with RNA pol II during the elongation stage. Other transcription factors which bind to the upstream enhancer element of class II promoters like Sp1, also speed up the process to assemble RNA pol II into an initiation complex (Voet and Voet, 1990; Sawadogo, 1990; Alberts et al., 1994).

The class II promoters recognized by the RNA pol II complex, are located in the 5' upstream region of a gene. Binding of RNA pol II machinery opens up the DNA double helix within the promoter for the synthesis of mRNA. Most of the class II promoters share several characteristic elements, including the TATA box, CCAAT box and GC box. The TATA box, which is present in nearly all eukaryotic genes, is located 25 to 30 nucleotides upstream from the transcription start site. The binding of TFIID (TBP) to the TATA box recruits TFIIB, RNA pol II and TFIIIF sequentially to form an initiation complex around the
transcription start site (Buratowski, 1994). The CCAAT box is located at -70 to -80 in class II promoters. Some tissue specific transcription factors such as CTF/NF1, CBP and ACF/NF-Y were shown to be able to bind to this box. The GC box with a consensus GGGCGG sequence is recognized by Sp1 (Alberts et al., 1994).

Besides these common elements that are present in promoters of most of structural genes, there are enhancer elements that are gene-specific. These elements can function from a great distance upstream or downstream of the transcription start site and in either orientation (Maniatis et al., 1987). NIT2, a member of the GATA transcription factor family, binds to DNA sequence with 5' GATA 3' as the core element (Fu and Marzluf, 1989). Several models are proposed for the role of these enhancer elements during transcription. First, these elements may disrupt chromatin due to their low binding affinity for histones, and become accessible to other DNA binding proteins. Second, the special combination of purines and pyrimidines in these elements may alter local DNA structure which gives special access to the RNA pol II machinery. Third, the binding of a gene-specific transcription factor to an enhancer element may loop out the intervening DNA sequence and enable it to interact directly with the RNA pol II machinery and facilitate the initiation of transcription (Voet and Voet, 1990; Lewin, 1994).

1.3. DNA binding domains of transcription factors

Gene expression and regulation is of extreme importance to the growth and development of all organisms, and transcription factors play pivotal roles in these processes by sequence-specific recognition of their binding site(s) and regulating the expression of genes in a timely fashion. Extensive studies of transcription factors has led to a classification of many of these factors into many protein families, each with distinct secondary structures. Zinc finger and leucine zipper families are among the best represented examples.
Zinc finger proteins represent a large family of eukaryotic DNA binding proteins that contain one or more zinc ions as a structural component. First identified in Xenopus, zinc finger proteins often contain tandem repeats of a 30-amino acid region with the conserved sequence Cys-X\textsubscript{2 or 4}-Cys-X\textsubscript{12}-His-X\textsubscript{3-5}-His. Structural studies show that zinc finger region consists of two antiparallel β sheets and an α-helix. The α-helix docks in the major groove of DNA (Alberts et al., 1994; Lewin, 1994). Usually DNA binding proteins with this type of zinc finger contain multiple fingers which strengthen their DNA binding affinity. Some zinc finger proteins such as steroid receptors contain one or more Cys\textsubscript{2}/Cys\textsubscript{2} fingers (Alberts et al., 1994). GATA factors are another example (Marzluf, 1997). Identified in a wide range of organisms as diverse as human, mice, Drosophila, C. elegans and fungi, GATA factors usually have two zinc fingers with a general configuration Cys-X\textsubscript{2}-Cys-X\textsubscript{17}-Cys-X\textsubscript{2}-Cys (except that some fungal GATA factors such as NIT2 from Neurospora and AREA from Aspergillus have only one). The C-terminal finger is required for DNA binding while the N-terminal finger provides stability and specificity (Tsai et al., 1989; Merika and Orkin, 1993). Other proteins with a special Cys\textsubscript{6}/Zn\textsubscript{2} zinc finger have also been identified which in combination with an adjacent basic region functions in DNA binding. The best studied example is yeast transcription factor GAL4. By sharing a pair of cysteine residues, two zinc ions, each held by 4 cysteines, form a binuclear cluster that makes direct contact with DNA (Harrison, 1991; Kraulis et al., 1992; Carey et al., 1989).

Many eukaryotic transcription factors contain a basic-leucine zipper (bZip) motif as their DNA binding domain. Composed of 60 to 80 amino acids, the bZip motif contains two different structural domains. The C-terminal 30 to 40 amino acids form an α-helix that allow dimer formation via a coiled-coil arrangement with hydrophobic interactions. The conserved leucine residues are arranged in heptad repeats and form a coiled-coil structure with a periodicity of 3.5 amino acids, thus every 7 amino acids are in the same structural
environment. The N-terminal 30 to 40 amino acids of each bZip motif contain many basic residues and are responsible for the sequence specific interaction with DNA. With their ability to form homodimers and heterodimers, bZIP proteins can regulate a wide variety of genes using differential DNA binding specificity, or a different combination of activation/repression domains. This mechanism greatly expands the spectrum of the DNA binding specificity of this class of regulatory proteins and serves as an important device to achieve a larger repertoire of gene regulation specificity (Alberts et al., 1994; Lewin, 1994).

1.4. Transactivation domain of transcription factors

Binding to DNA is only half the story of transcription factors. The second half, which requires direct protein-protein interactions with components of the RNA pol II machinery or other transcription factors, is to activate gene transcription. Transactivation domains are responsible for gene activation by the transcription factors. Many different types of transactivation domains have been identified, including acidic-rich (e.g. VP16 and GAL4), glutamine-rich (e.g. Sp1 and CREB), proline-rich (e.g. progesterone receptor), leucine-rich (e.g. NIT4) and isoleucine-rich (e.g. NTF-1) (Cress and Triezenberg, 1991; Ma and Ptashne, 1987; Gill et al., 1994; Ferreri et al., 1994; Feng and Marzluf, 1996; Attardi and Tjian, 1993). The mechanism by which transcription factors accelerate transcription is by promoting the assembly of RNA pol II and general transcription factors at the promoter (Alberts et al., 1994; Lewin, 1994). The negatively charged transactivation domain of GAL4 can facilitate the assembly of TFIIB in RNA pol II machinery which is a limiting step during transcription (Alberts et al., 1994; Lewin, 1994).
1.5. Characteristics of *Neurospora crassa*

A close relative to yeast, the eukaryotic organism *Neurospora crassa* belongs to the fungal *Pyrenomycetes* class, *Ascomycotina* subdivision, *Eumycota* division, and *Mycetas* kingdom (Davis and De Serres, 1970). As a heterotroph, *N. crassa* grows on the cellulosic remains of plants in tropical or subtropical areas. In the laboratory, it can use acetate, glycerol, succinate and a wide variety of monosaccharides and polysaccharides as carbon sources, and ammonium, nitrate, and amino acids as nitrogen sources. A simple medium with a few inorganic salts, trace elements, biotin and a carbon source can support vigorous growth of *N. crassa*. This well defined and simple nutritional requirement enables *N. crassa* to be an excellent laboratory organism.

Encapsulated by a double-layered plasma membrane and a strong cell wall, *N. crassa* contains the typical organelles found in eukaryotes, nuclei, mitochondria, peroxisomes, endoplasmic reticulum, ribosomes and various inclusions. During the vegetative growth stage, it forms mycelia which are multinucleate, branched filaments with septa. As the vegetative culture matures, two types of asexual spores, macroconidia and microconidia, are produced. Averaging 2.5 nuclei each, the bright orange colored macroconidia are formed in large quantity at the tip of the aerial hyphae. At a later stage, microconidia, which are almost exclusively uninucleate, are produced. Microconidia have poor viability in comparison with macroconidia but are useful in characterizing the genotypes. In an environment hostile to vegetative growth, *N. crassa* initiates the sexual reproductive phase which results in sexual spores called ascospores. Both mating type A and α are required in the process. The male gametes and female gametes fuse to form a diploid nucleus, which resolves into eight haploid ascospores by two meiotic and one mitotic divisions. These ascospores are resistant to hostile environments and long-lived (Davis and deSerres, 1970).
Nearly a half century after finding its way into the laboratory, *N. crassa* became one of the genetically well characterized organisms. Thousands of mutants have been isolated. As a haploid organism, it has seven chromosomes referred as linkage groups I to VII. Genes located on different chromosomes follow the Mendelian genetic rules for gene segregation and independent assortment. Crossing over occurs among the genes linked on the same chromosome, with increasing probability as the distance between two genes increases. Gene order and distance can be determined via the calculation of the frequency of cross over and a detailed linkage map has been established for all seven linkage groups (Davis and deSerres, 1970). The genome size of haploid *N. crassa* is $47 \times 10^6$ bp, twice the size of the yeast genome, 93% of which represents unique sequence. Studies of *N. crassa* genes show that they have general features similar to those of vertebrate genes, with nontranslatable leader sequences (including promoters), and often have one or more introns (averaging about 100 bp). Each chromosome has all the typical structures of the eukaryotic chromosome, with nucleosomes, a centromere and telomeres (Mishra, 1991). In the past twenty years, its clear genetic information and well studied life cycle have made *N. crassa* one of the most commonly used model organisms to study gene regulation. Many important genetic concepts have merged from studies with of *N. crassa*, the best example of which is the one gene-one enzyme theory (Beadle and Tatum, 1941).

1.6. Meiotic instability and RIP (repeat induced point mutation)

Meiotic instability of transformed genes in *Neurospora* was first reported in 1973 (Mishra and Tatum). When an int$^+$-transformed strain was crossed with an int$^-$ strain, all the spores produced were int$^-$. Unlike other non-Mendelian genes such as mitochondrial genes that are transmitted uniparentally, transformed genes in *Neurospora* showed biparental nontransmission. Not until 1988, was RIP, the molecular basis of the meiotic instability elucidated (Selker and Garrett, 1988; Selker, 1990). The additional copies of
transformed genes trigger the methylation of cytosine (5-methyl cytosine) within the duplicated sequence. After deamination, 5-methyl cytosine is converted to thymine, which causes GC to AT changes during DNA replication. The frequency of RIP is so high that up to 10% of the duplicated DNA sequences can be altered, which almost always results in loss of function of the gene. The rRNA and 5s rRNA which have multiple copies in Neurospora genome are spared from the action of RIP because of their special location or distribution (Selker, 1990; Perkins et al., 1986; Mishra, 1991).

The understanding of the mechanism of meiotic instability led to the establishment of a powerful new method to generate mutants. Conventional methods using UV or chemicals are relatively inefficient in creating mutants of a particular gene and it is too time consuming to select desired mutants from a pool of random mutants. As a haploid organism, elimination of function of many essential genes often results in lethality in Neurospora. By transforming an additional copy of a wild type gene into Neurospora, then crossing the transformant with the wild type strain, mutants with the target gene damaged can be selected easily from the ascospores produced.

1.7. Gene targeting at the his-3 locus in Neurospora crassa

Unlike E. coli and yeast, no autoreplicating plasmid has been found in N. crassa. Genes stably introduced into N. crassa through transformation insert into the genome, either by homologous recombination or via ectopic insertion (Grant et al., 1984; Paietta and Marzluf, 1985). The frequency of homologous recombination is usually considerably lower than 5%, suggesting that the vast majority of the transformants are the result of random insertion of the transformed gene (Dhawale and Marzluf, 1985). Since the location of its insertion can have a great impact on the expression of a transformed gene, the high percentage of random insertions among the transformants makes it very difficult to make valid comparisons with manipulated genes. A variable copy number of the transformed
gene in different transformants presents another uncertainty. In order to solve this problem, a specific targeting system was developed by Ebbole (1990). A Neurospora crassa strain with a point mutation in the his-3 gene was selected based on its inability to grow on medium without histidine and is used as the host strain. A truncated copy of the his-3 gene which lacks the promoter and first three hundred amino acid residues is added to the plasmid that is destined to be transformed into the his-3 mutant strain. Only through homologous recombination at the his-3 locus can a wild type his-3+ gene be generated, thereby restoring the function of the his-3 gene. By using histidine-independent growth for selection, the random insertion transformants can be efficiently eliminated. The copy number can be determined by genomic Southern analysis of the transformants.

1.8. Sulfur metabolism in N. crassa

For all living creatures, the sulfur-containing amino acids, methionine and cysteine, are very important building blocks for protein synthesis. In mammals, sulfur-containing compounds have diverse functions involving many metabolic processes. Therefore, it is not surprising that a precisely regulated metabolic network controls sulfur metabolism to ensure a steady supply for cell. Deficiency in catabolic enzymes involving sulfur-containing compounds or their regulation causes growth defects in lower organisms, and causes many diseases in higher organisms, including human beings. In the case of rheumatoid arthritis, thiol methyl transferase activity is reduced in patients' red blood cell membranes, and is consistent with a generalized disturbance of sulfur metabolism in rheumatoid disease (Bradley et al., 1991). In Parkinson's disease, reduced S-oxidation capacity is found in a large percentage of patients, suggesting that deficiency in a detoxification pathway involving sulfur metabolites is a contributing factor for the disease (Steventon et al., 1989). S-adenosylmethionine, which is required for the transmethylation reaction, has a wide variety of impacts in the central nerve system, especially on
monoamine neurotransmitter metabolism (Bottiglieri et al., 1994). The biomedical importance of sulfur metabolism is one of the driving forces for a better understanding of the molecular mechanism of sulfur regulation and metabolism.

Due to its simple and well defined nutritional needs, and its well characterized life cycle and genetic background, *N. crassa* is one of the most useful model organisms to study sulfur regulation and has played a leading role in its study for the past thirty years. *N. crassa* is able to use both sulfur-containing amino acids and inorganic sulfate as its sole sulfur source. The sulfate catabolic pathway is shown in Figure 1.1. Upon transfer inside the cell by sulfate permease, inorganic sulfate is first converted to PAPS (3'-phosphoadenosine-5'-phosphosulfate) by ATP-sulfurylase and APS-kinase, then to sulfite by PAPS-reductase. Sulfide is generated finally from sulfite by sulfite reductase. Cysteine is synthesized by transferring the sulfide ion to O-acetylserine by serine transacetylase and cysteine synthetase. Methionine can then be synthesized from cysteine. This sulfur metabolic pathway has also been characterized in yeast and *A. nidulans*. (Marzluf, 1994; Paszeski, 1993).

Many *Neurospora* genes encoding sulfur metabolic enzymes and regulatory proteins have been cloned. Among them, *cys3* was identified as the key positive regulator for the expression of the entire set of sulfur catabolic enzymes. The expression of CYS3 protein is regulated by the availability of the sulfur source. In a sulfur-rich environment, the sulfur containing amino acid, methionine, is a preferred sulfur source for *N. crassa*. When a favored sulfur source is limited, other less readily used sulfur sources like choline-O-sulfate, proteins and aromatic sulfates can be used. Previous studies have shown that in order to use these secondary sulfur-containing compounds, a group of catabolic enzymes which form a tightly regulated sulfur circuit has to be synthesized. These enzymes, including aryl sulfatase, choline sulfate permease, choline sulfatase, external alkaline protease, methionine-specific permease, and sulfate permease I and II, are genetically
unlinked but co-regulated (Marzluf, 1994). Under conditions of sulfur sufficiency, the expression of all the enzymes remains repressed. During sulfur limitation, the repression is released (Marzluf and Metzenberg, 1968). One positive regulator which is responsible for this derepression, named CYS3, was identified first based on genetic analysis (Marzluf, 1994). When switched from high sulfur condition (5 mM methionine or 2 mM sulfate) to low sulfur condition (0.25 mM methionine or 0.1 mM sulfate), *N. crassa* switches on the expression of the CYS3 protein and CYS3 subsequently turns on the expression of all the downstream structural genes (Ketter and Marzluf, 1988; Paietta, 1989). In a mutant strain harboring a point mutation of CYS3 in the basic leucine zipper (bZIP) region that eliminates its DNA-binding ability, the expression of the entire set of sulfur catabolic enzymes is missing even under low sulfur conditions (Burton and Metzenberg, 1972). Two negative regulators for the sulfur circuit, named *scon 1* and *scon 2*, have also been identified. In *N. crassa* strains harboring *scon 1* and *scon 2* mutations, the expression of the sulfur catabolic enzymes becomes constitutive under both sulfur-sufficient and sulfur-limited conditions due to the constitutive expression of CYS3 (Tao and Marzluf, unpublished data; Paietta, 1990).

1.9. Nitrogen metabolism

Nitrogen is another element that can be found in most biomolecules, especially in proteins and nucleic acids, thus the assurance of the supply of nitrogen is of obvious importance to the survival of all living organisms. With many nitrogenous compounds, ammonium is first synthesized, then converted into glutamine and glutamate, which are used to manufacture other metabolites and macromolecules (Mora, 1990). Nitrogen fixation and nitrate assimilation are the two major mechanisms to convert inorganic nitrogen (N₂ and NO₃⁻) to organic nitrogen (NH₄⁺). NO₃⁻ assimilation is much more efficient
(over 100-fold) than N2 fixation (Guerrero et al., 1981). This makes the understanding of the metabolism of nitrate a very interesting and important subject.

In *N. crassa*, the assimilation of nitrate (NO3⁻) to ammonium (NH4⁺) is a well characterized process. Nitrate is first converted to nitrite (NO2⁻) by nitrate reductase via a two-electron transfer reaction, then to ammonium (NH4⁺) via a six-electron transfer reaction catalyzed by nitrite reductase. The utilization of nitrate requires the de novo synthesis of nitrate and nitrite reductases and has to be induced by absence of preferred nitrogen sources including ammonia, glutamine and glutamate, and the presence of nitrate. Nitrate reductase is a large protein with a molecular weight of 228 kDa and functions as a homodimer. A flavin domain at the C-terminus containing FAD, a heme-containing domain in the middle region and a molybdopterin domain at the N-terminus are found in each monomer. Two electrons are transferred stepwise from NADPH to FAD, then to heme, finally to molybdopterin to reduce NO3⁻ to NO2⁻. Nitrite reductase also functions as a homodimer, and has a molecular weight of 290 kDa. FAD, a siroheme prosthetic domain and an iron-sulfur group are used to carry out the six-electron transfer to reduce NO2⁻ to NH4⁺ (Dunn-Coleman et al., 1984).

Two genes, *nit-3* and *nit-6*, encoding nitrate and nitrite reductases respectively, have been identified in *Neurospora*. Studies at the mRNA level indicated that the regulation of these two genes occurs primarily at the transcription level. The *nit-3* mRNA appears quickly after derepression and reaches a steady level within 15 minutes. The turnover of *nit-3* mRNA is also very fast, with a half-life of about 5 minutes (Marzluf, 1981; Okamoto and Marzluf, 1993).

Promoter studies of the two genes showed that two positive regulators, NIT2 and NIT4, are required for the expression of these two genes. NIT2 is a zinc finger protein that belongs to the GATA family of transcription factors. As a global regulator, the NIT2 protein regulates the expression of as many as 100 genes that relate to the metabolism of
nitrogenous compounds. Mutants of nit-2 display a loss of expression of many genes including nitrate and nitrite reductases.

As a pathway-specific regulator, NIT4 mediates the selective activation of the nitrate assimilatory genes. Mutations in nit-4 gene lead to a loss of expression of nit-3 and nit-6. The NIT4 protein is a DNA-binding protein that consists of 1090 amino acid residues. It has a Cys6/Zn2 binuclear zinc cluster as its DNA-binding domain, and a coiled-coil region as its dimerization domain. A palindromic sequence, 5' TCCGc/tGGa/c 3', identified in the nit-3 promoter, is recognized by NIT4 (Evans and Marzluf, unpublished data; Fu et al., 1995).

A negative regulator named NMR (nitrogen metabolic regulation) has also been identified. The expression of many nitrogen-controlled genes in NMR mutants become derepressed in the presence of repressing amounts of ammonia, glutamine or glutamate. The expression of nitrate reductase in nmr mutants, for example, is insensitive to nitrogen catabolite repression but still needs nitrate as inducer to achieve a full scale derepression (Marzluf, 1997). The NMR protein has 488 amino acid residues and contains no distinctive functional domains such as a DNA-binding domain or a protein kinase domain. Studies show that NMR does not exert its repression of nit-3 and nit-6 through protein-DNA interaction at the promoter regions of these two genes, but instead through a protein-protein interaction with NIT2 protein (Xiao et al., 1995).

1.10. Research goals

My research is constituted of three parts. The first part is about transcription factor CYS3, a positive regulator of the sulfur regulatory circuit. My goal was to determine whether expression of this regulatory protein or its mRNA is controlled. I was able to show that the expression and degradation of the CYS3 protein is precisely regulated in a sulfur-dependent manner. Cys-3 transcripts could be readily detected within two hours.
after derepression whereas the CYS3 protein was not found until after four hours. The CYS3 protein is stable with a half life greater than 4 hours under low sulfur conditions. However, it is degraded relatively quickly when methionine or inorganic sulfate become available. Upon sulfur repression, cys-3 transcripts disappeared within thirty minutes, with an estimated half life of 5 minutes, whereas CYS3 protein almost entirely disappeared in one hour, with a half life of approximately 10 minutes.

The second part of my research focused on a study of the nit-3 gene promoter. In Neurospora crassa, the expression of the nit-3 gene (nitrate reductase) is dependent upon nitrogen derepression and nitrate induction and is regulated by two positive-acting transcription factors, NIT2 and NIT4, and a negative regulator, NMR. The presence of a tightly linked cluster of NIT2 and NIT4 binding sites suggested that their close spacing might be required for synergistic interaction of the NIT2 and NIT4 proteins. I was able to show that the NIT2 and NIT4 binding sites can be separated without affecting either the expression level or the precise regulation of the nit-3 gene. Studies conducted on NIT2 site II, which contains only a single GATA element and yet plays a major role in nit-3 gene expression, showed that nucleotides both 5' and 3' of the GATA sequence were important for strong DNA binding in vitro and its activation function in vivo. The nit-3 promoter contains two long AT-rich sequences, and an objective was to determine their functional significance, if any. The nit-3 transcript contains eight TACC repeats in its 5' non-coding region and my results show that they are involved in mRNA instability.

The last part of my research has focused on the expression and regulation of the nit-2 gene and NIT2 protein. In Neurospora, NIT2 is required for lifting of nitrogen catabolite repression when the cellular level of glutamine or other favored nitrogen sources becomes limited. It seemed very important to determine whether expression of the nit-2 gene or synthesis or stability of the NIT2 protein was subject to strict control. Immunoprecipitation of NIT2 protein showed that the NIT2 protein is constitutively expressed, however its
expression level can be elevated a few fold when nitrate instead of glutamine is used as the sole nitrogen source. My results demonstrated that the NIT2 protein is very stable and its stability is not affected by the available nitrogen source. The nit-2 transcripts appear to be very stable as well. Post-translational modification of NIT2 and interactions with other proteins, e.g., in the nitrate assimilation pathway, interactions with NIT4 and NMR, may play very important roles in modulating the function of NIT2 in response to environmental stimuli.
Fig. 1. The sulfur assimilatory pathway. The pathway leading from inorganic sulfate that gives rise to cysteine and methionine is shown, and mutants which affect specific steps in *Neurospora crassa* are identified. Several key enzymes of the pathway are shown in parenthesis.
Chapter 2

Synthesis and differential turnover of the CYS3 protein

2.1. Introduction

Sulfur uptake and assimilation is subject to sophisticated metabolic controls in the filamentous fungus *Neurospora crassa*. Sulfur-containing amino acids and inorganic sulfate are preferred sulfur sources for *N. crassa*. When the favored sulfur sources are missing or limited, other less readily used sulfur compounds such as choline-O-sulfate and aromatic sulfates can be utilized. The utilization of secondary sulfur sources requires the *de novo* synthesis of a set of catabolic enzymes including two sulfate permeases, a methionine-specific permease, aryl sulfatase, choline sulfatase, and an extracellular protease. Several regulatory genes, namely *cys-3*, *scon-1* and *scon-2*, form a hierarchical circuit, with *cys-3* playing the decisive role to ensure *N. crassa* cells have a steady supply of sulfur for growth (Hanson and Marzluf, 1975; Marzluf, 1970; Marzluf, 1994; Metzenberg and Ahlgen, 1966; Paietta, 1987; Paietta et al., 1987; Pall, 1971). Of the three regulatory genes, *cys-3* has been identified as the key positive regulator for the sulfur circuit. Expression of CYS3 protein under sulfur-limited conditions is a prerequisite for the expression of all the structural genes in the sulfur circuit. A *cys-3* null mutant is unable to express any of the sulfur catabolic enzymes and fails to grow on any of the secondary sulfur sources (Marzluf, 1994). *scon-1* and *scon-2* are identified as negative regulators for the sulfur circuit. Mutations in either gene lead to constitutive expression of all the catabolic enzymes regardless of the availability of sulfur sources (Burton and Metzenberg,
The CYS3 protein is expressed in these mutants in repression conditions, although a higher expression level still occurs upon derepression (Tao and Marzluf, unpublished data). Heterokaryon studies showed that the function of scon-1 was intranuclear (Burton and Metzenberg, 1972). It appears that scon-1 prevents CYS3 expression and thus stops expression of the structural genes of the sulfur circuit during repression conditions (Burton and Metzenberg, 1972; Metzenberg and Ahlgen, 1971). The scon-2 gene, which encodes a protein with β-transducin repeats, also acts as a negative regulator for the expression of cys-3 (Ketter et al., 1991; Paietta, 1990). Interestingly, recent studies showed that CYS3 protein acts positively for the expression of scon-2 via CYS3 binding sites located in the scon-2 promoter region (Ketter et al., 1991; Paietta, 1990).

As a basic region-leucine zipper transcription factor, the CYS3 protein shares substantial homology with members of the bZIP protein family, especially JUN, FOS and GCN4, and recognizes a consensus palindromic sequence ATGRYRYCAT (Fu and Marzluf, 1990; Fu et al., 1989; Li and Marzluf, 1996). CYS3 binding sites have been found in the promoter regions of the cloned structural genes of the sulfur circuit, providing molecular evidence that links the expression of sulfur catabolic enzymes to CYS3 (Ketter et al., 1991; Paietta, 1990). Promoter studies suggest that the expression of CYS3 is autoregulated via CYS3 binding sites in the promoter region of cys-3, which enables N. crassa to produce a large pool of CYS3 protein when cells encounter a shortage of sulfur (Fu and Marzluf, 1990). However, whether this CYS3 protein pool is eliminated in a timely fashion when the sulfur supply becomes abundant again is not known.

Studies conducted on cys-3 mutants and the downstream structural genes have suggested that the expression of cys-3 is precisely regulated by the availability of sulfur. Under sulfur-sufficient condition, expression of cys-3 as well as the sulfur circuit is fully repressed. As the sulfur level decreases, derepression of CYS3 synthesis takes place which subsequently leads to expression of the entire set of sulfur catabolic enzymes (Paietta
et al., 1987). However, little is known about the transcriptional or translational regulation of CYS3 expression. In this report I examined regulation of cys-3 transcription and protein synthesis and degradation. Interestingly, I found that the CYS3 protein is subject to differential turnover, depending upon the cellular sulfur supply. Site-directed mutagenesis plus analysis of N. crassa mutant strains have indicated that Lys-105 of CYS3 is important for its instability.

2.2. Materials And Methods

2.2.1. N. crassa and E. coli strains.

The N. crassa wild type strain 74-OR23-1A and mutants cys-3 (allele P22) and cys-3 (NM27t) were obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, Mo. The cys-3 revertant REV21, and cys-3 temperature sensitive revertant REV65 were described before (Marzluf, 1993). Mycelia were cultured in Vogel's liquid medium (Davis and deSerres, 1970) with shaking. The amount of methionine supplement and the culture temperature is indicated for each experiment. E. coli strain DH5α was used for plasmid propagation, CJ236 for single strand DNA template preparation, and BL21(DE3)plys for protein expression.

To study the effect of derepression upon cys-3 expression, N. crassa mycelia were initially grown overnight in 5 mM methionine as the sole sulfur source, then transferred to medium containing 0.25 mM methionine for derepression, and harvested at one hour intervals for 8 hr. For repression studies, mycelia were first cultured in a low sulfur condition (0.25 mM methionine) overnight to allow the expression of CYS3. After increasing methionine to a repression concentration (5 mM), mycelia were harvested at one hour intervals for 5 hr.
2.2.2. Site-directed mutagenesis and CYS3 expression in *E. coli*.

Site-directed mutagenesis was used to generate a new cys-3 mutant K105->Q105. A single strand DNA oligo containing the desired mutation (5'GCC GAG GAA GAC CAG CGA AAG CGC3') was synthesized and annealed to the single strand cys-3 DNA template which was generated using *E. coli* strain CJ236. The daughter strand was synthesized *in vitro* and the double stranded product was used to transform *E. coli*. The mutant was selected and confirmed by DNA sequencing. The construct used for expressing wild type CYS3 protein in *E. coli* was described before (Fu and Marzluf, 1990). The construct carrying cys-3 REV21 was generated by Kristin Coulter (Coulter and Marzluf, 1998). The *E. coli* expression construct carrying the K105Q mutation was made by replacing the corresponding wild type sequence in the coding region of cys-3 with the mutated sequence. Expression and purification of CYS3 was performed as described (Fu and Marzluf, 1990).

2.2.3. Mobility shift assay.

DNA mobility shifts were carried out as described before with minor modifications (Fu and Marzluf, 1990). *E. coli* expressed CYS3 proteins (0.1 μg to 1.0 μg) were used in the mobility shift assays. A 200 bp DNA fragment from the cys-14 promoter containing a strong CYS3 binding site was used as probe. The mixtures of proteins and DNA were incubated at room temperature for 20 min in a total volume of 25 μl binding buffer (12 mM HEPES; 4 mM Tris-HCl, pH 7.9; 50 mM KCl; 1 mM EDTA; 1 mM DTT; 0.3 mg/ml BSA; 10% glycerol) with 3 μg of poly(dI-dC) as nonspecific competitor. Samples were separated on 4% polyacrylamide gels (acrylamide:bisacrylamide=19:1) in 1/4x TBE buffer.
2.2.4. RNA preparation and northern analysis.

Isolation of total RNA from *N. crassa* was done as described by Weaver et al. (1997) with modifications. Mycelia were ground to a fine powder with a mortar and pestle in the presence of liquid nitrogen and suspended in lysis buffer (50 mM NaOAc, pH 5.3; 10 mM EDTA; 1% SDS) at a 1 g/5 ml ratio. An equal volume of acidic phenol/chloroform (equilibrated with the lysis buffer without SDS, pH 5.3, prewarmed at 65°C) was added to the lysate, and the reaction mixture was incubated at 65°C for 30 min with shaking. The aqueous phase was recovered by centrifugation and this process was repeated several times until no protein interface could be seen. Total RNA was precipitated with 0.6 volume of isopropyl alcohol followed by centrifugation.

The RNA samples were fractionated in agarose gels, then transferred to nitrocellulose filters for northern analysis. *cys-3* cDNA and *β-tubulin* cDNA were labeled by the random primer technique using α-32P-dATP and used as probes for northern analysis (GibcoBRL). Hybridization was carried out at 65°C in 1x hybridization buffer (0.5 M NaCl; 0.1 M NaPO4, pH 7.0; 6 mM EDTA; 1% SDS). The membranes were washed at 65°C for 60 min with 1/4x hybridization buffer. Messages of *cys-3* and *β-tubulin* were identified by their size.

2.2.5. Partial purification of CYS3 rabbit polyclonal antibodies

*cys-3* mutant (allele P22) strain was cultured in Vogel's complete media with 5 mM methionine overnight and mycelia were used to prepare whole cell extract. SDS was introduced to the cell lysate to a final concentration of 1%. Cell lysate was denatured at 100°C for 10 min and immobilized on nitrocellulose filter by directly loading on the filter like a regular dot-blot. 2% BSA in TBST was used to block the filter for one hour. The filter was incubated with the CYS3 antibody (serum) for one hour at room temperature. The filter was removed and the procedure was repeated for several times to remove all the
cross-reacting antibodies from the serum. A regular western blot analysis was used to confirm that CYS3 antibody reacted specifically only with CYS3.

2.2.6. Western analysis.

Mycelia were homogenized using a mortar and pestle in the presence of liquid nitrogen and suspended in 2 ml ice-cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.2% sodium azide, 100 μg/ml PMSF, 1% Triton X-100, 2 mM EDTA). Cell debris was removed by centrifugation at 4°C for 5 min using a microfuge at full speed and the supernatant was subjected to Western analysis. The protein concentration of the cell lysate was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, CA). An equal amount of protein for each sample was run on 15% SDS-polyacrylamide gels and transferred to a nitrocellulose filter by electroblotting in glycine buffer (25 mM Tris-HCl, pH 8.3; 192 mM glycine; 20% methanol) as described (Harlow et al., 1986). After blotting, the filter was briefly rinsed with TBST (25 mM Tris-HCl, pH 8.0; 125 mM NaCl; 0.05% Tween 20) and was blocked by TBST containing 4% non-fat milk for 1 hr at room temperature, or at 4°C overnight. The filter was then incubated with rabbit anti-CYS3 antibodies for 1 hr with shaking at room temperature (Kanaan and Marzluf, 1993). The filter was washed with TBST then incubated with horseradish peroxidase-conjugated secondary antibodies (anti-immunoglobulin) (1:7000 dilution, in blocking buffer) for 45 min. After further washing, the antigen-antibody complex on the nitrocellulose filter was detected by the in situ chemiluminescence reaction (ECL system, Amersham) and the result was visualized on X-ray film.

2.2.7. Aryl sulfatase assay.

Mycelia were grown at 30°C in liquid Vogel's minimum medium containing 0.25 mM methionine as the only sulfur source for 16 to 20 hr. Cell lysates were prepared as
described above and protein concentrations were determined by Bio-Rad protein assay. The aryl sulfatase enzyme assay was performed as described before with a few modifications (Metzenberg and Parson, 1966). Cell lysate (50 μl) was mixed with 450 μl of the aryl sulfatase enzyme assay cocktail buffer (6.7 mM p-nitrophenol sulfate, 0.33 M Tris-HCl, pH 8.1). The reaction mixtures were incubated at 30°C for 10 to 60 min depending on the amount of activity, and 1 ml of stop solution (0.5 M NaOH, 90% ethyl alcohol) was added. For the zero-time control, 1 ml of stop solution was added to the mixture without incubation. The reaction mixtures were centrifuged in a microfuge at full speed for 5 min to remove any precipitate. The absorbance at 405 nm of the supernatant was measured with a spectrophotometer.

2.2.8. In vivo labeling of *Neurospora crassa* with $^{32}$P inorganic phosphate and immunoprecipitation

Wild type *N. crassa* 74A conidia were cultured in sulfur-free Vogel's minimum medium supplemented with 5 mM methionine for 20 hours, then transferred to sulfur-free Vogel's minimum medium supplemented with 0.25 mM methionine and cultured for four hours for the derepression of CYS3 protein synthesis. Mycelia were transferred to phosphate-free, sulfate-free Vogel's minimum medium with 0.25 mM methionine and cultured for one half hour to deplete the intracellular phosphate. P-32 labeled inorganic phosphate was added to the medium at 20 μCi/ml and the mycelia were further cultured for another four hours before harvest. The mycelia were homogenized using a mortar and pestle in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.2% sodium azide, 100 μg/ml PMSF, 1% Triton X-100, 2 mM EDTA) in the presence of liquid nitrogen. Cell debris was removed by centrifugation at 4°C for 5 minutes at full speed using a microfuge.

The immunoprecipitation experiment was done as described (Harlow et al., 1986; Lee et al., 1987) with minor modifications. The cell lysate was precleared twice with
formalin-fixed *Staphylococcus aureus* cells (Zymed Lab. Inc.) by incubating at 4°C for 15 min each time with shaking. 10 µl of rabbit polyclonal antibodies against CYS3 protein were added to the cell lysate and the mixture was incubated on ice for one hour. Protein A conjugated Sepharose beads were added to the mixture and incubated at 4°C for one hour with shaking. After the incubation, the Sepharose beads were collected by brief spinning in a microfuge and subjected to an extensive wash using ice cold E1A buffer (250 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.1 mM PMSF, 5 mM EDTA). Bead-bound proteins were examined by SDS polyacrylamide gel electrophoresis and visualized by autoradiography.

2.3. Results

2.3.1. Kinetics of CYS3 expression.

The expression of CYS3 and the catabolic enzymes of the sulfur circuit is precisely regulated by the availability of sulfur. Neither is expressed in the presence of a high concentration of methionine (5 mM) or inorganic sulfate (2 mM). However, when the sulfur level in the medium is limited (0.25 mM methionine or 0.02 mM inorganic sulfate), the expression of CYS3 and subsequently the sulfur catabolic structural genes is derepressed. In order to determine the kinetics of *cys-3* mRNA and protein expression upon derepression, northern and western analyses were carried out. Northern analysis using *cys-3* cDNA as probe showed that upon sulfur derepression the synthesis of *cys-3* mRNA occurs only slowly. No *cys-3* transcripts were detected in RNA from cells grown under repression conditions or within one hour after derepression (Fig 2.1, lanes 1 and 2). *cys-3* mRNA first appeared at a low level two hours after derepression and increased for two more hours before leveling off (Fig. 2.1, lanes 3, 4, and 5). The bottom panel of figure 1A shows the same filter probed with β-tubulin gene as an internal control,
indicating approximately equal loading of RNA in each lane. The expression of CYS3 protein is an even slower process. Western analysis using rabbit anti-CYS3 antibodies showed no detectable CYS3 protein accumulation within the first three hours after derepression. CYS3 protein first appeared at four hours and kept increasing in the next four hours before it reached a plateau (Fig. 2.2). The CYS3 protein actually appears as two distinct bands (Fig. 2.2), suggesting the possibility of post-translational modification. Aryl sulfatase, a downstream structural gene regulated by CYS3, was chosen as an indicator for expression of the sulfur circuit. The enzyme activity was detected about five hours after derepression (Fig. 2.3).

2.3.2. Kinetics of cys-3 mRNA and protein turnover.

When cells growing under sulfur-limited conditions encounter an abundance of sulfur, the multiple sulfur-regulated permeases and enzymes are no longer needed. It was of interest to determine whether the sulfur circuit was shut down in response to repression conditions. I found that upon establishment of sulfur repression, cys-3 mRNA and protein turn over in a rapid fashion. Northern analysis showed that the disappearance of cys-3 mRNA was very quick, and the transcript was undetectable after 20 min. The estimated half life of mRNA at this transition stage was approximately 5 min (Fig. 2.4). Western analysis indicated that the turnover of CYS3 protein is also a rapid process with an estimated half life of about 10 min (Fig. 2.5). CYS3 nearly completely disappeared within one hour after sulfur repression.

The stability of CYS3 protein during sulfur derepression conditions was also determined. Cycloheximide was added to the culture medium at 2x10^{-4} M to block de novo protein synthesis. Western analysis suggested that CYS3 was very stable under low sulfur conditions, with an estimated half life of about 4 hours (Fig. 2.6). A control experiment using the same concentration of cycloheximide to block the induction of
*Neurospora* nitrate reductase was conducted at the same time. Nitrate reductase enzyme assay indicated that protein synthesis was completely inhibited under these conditions (Fig 2.7).

The 4-hour half life of CYS3 protein under the low sulfur condition was confirmed by using a *cys-3* temperature sensitive mutant REV65, which behaves as the wild type at 27°C but as a *cys-3* null mutant at 37°C when it fails to synthesize CYS3 as well as all the downstream sulfur catabolic enzymes. By shifting the culture temperature from 27°C to 37°C, CYS3 protein synthesis is halted without altering the normal cell growth conditions. Western analysis showed that the pre-existing CYS3 protein was very stable under the low sulfur condition (Fig. 2.8). These results strongly suggest that differential turnover of CYS3 occurs under different growth conditions.

Since cycloheximide is known to selectively stabilize some highly unstable proteins, apparently due to a block of the synthesis of proteases, it was important to determine whether cycloheximide would stabilize CYS3 in cells transferred from low to high sulfur medium. When high methionine medium and cycloheximide (2x10^-4 M) were added to a culture at the same time, we found that cycloheximide did stabilize CYS3 somewhat, giving a longer half life of about 30 minutes (Fig. 2.9); however, the turnover of CYS3 was still much faster than under sulfur-limited conditions. The difference in the turnover rate of CYS3 under different growth conditions suggests that a specific sulfur-dependent signal leads to CYS3 elimination when it is no longer needed.

### 2.3.3. Lysine 105 of CYS3 is required for rapid sulfur-regulated degradation.

REV21, which was isolated as a revertant of the *cys-3* null mutant, carries three changed amino acid residues, K105Q, R106Q and F116Y (Fu et al., 1989; Coulter and Marzluf, 1998). Analysis of expression of the downstream structural gene, aryl sulfatase,
revealed that in REV21 the mutated CYS3 had an impaired transactivation function and retained about 10% activity compared to the wild type protein. However, western analysis indicated that the CYS3 protein level was significantly elevated in REV21 compared to the wild type strain under the derepression condition. Since the expression of CYS3 protein is autoregulated, the CYS3 level in REV21 would be expected to be lower than in wild type. Mobility shift analysis suggested that the reduction of transactivation activity was primarily due to a lower affinity of DNA binding, as shown below. One possible explanation for the elevated CYS3 protein level is that the stability of CYS3 protein in REV21 is increased. To examine this possibility, the stability of the mutant CYS3 protein was examined at the low sulfur to high sulfur transition stage. Indeed, the mutant CYS3 protein of REV21 was found to have a prolonged half life of about 40 minutes compared with 10 minutes for wild type, and was still present after 2 hours (Fig. 2.10). Thus the amino acid substitutions appear to alter the turnover of the CYS3 REV21 protein during sulfur repression, perhaps by partially hindering a sulfur-triggered degradation of CYS3.

Since lysine is often modified for ubiquitin-controlled proteolysis, a new mutant K105Q was constructed which has only one amino acid substitution, lysine 105, changed to glutamine. The K105Q mutant was transformed into the N. crassa cys-3 null mutant. Transformants carrying K105Q showed proper response to sulfur regulation, with about 20% transactivation activity comparing to wild type CYS3 protein. The sulfur-regulated degradation of CYS3 K105Q was examined at the low sulfur to high sulfur transition stage. Western analysis revealed that, like REV21, CYS3 K105Q had a prolonged half life of about 40 minutes (Fig. 2.11).

The DNA binding ability of K105Q, REV21, and wild type CYS3 proteins was examined. These three forms of CYS3 were expressed in E. coli and were partially purified (Fig. 2.12). An identical amount of each CYS3 protein was used in a mobility shift analysis. As shown in figure 2.13, analyses with two concentrations of protein
revealed that WT CYS3 protein has strong DNA binding ability while the two mutant forms of CYS3 were significantly weaker in DNA binding. In order to measure the percentage shift of the probe, the density of each band was quantified with a phosphoimager and the percentage shift of the probe in each reaction is indicated at the bottom of figure 2.13. The K105Q mutant protein possesses about 20% of wild type binding affinity while REV21 possesses only about 10%. This explains why these mutant proteins only poorly turn on the structural genes, despite their presence in amounts greater than the wild type level.

2.3.4. CYS3 is a phosphoprotein.

The first hint that CYS3 might be subject to post-translational modification came from its unusual appearance on SDS polyacrylamide gel. It runs as a doublet at 35 kDa, 9 kDa larger than the expected size. The second hint came from the comparison of *E. coli* expressed CYS3 which runs at 31 kDa, 4 kDa smaller than the *N. crassa* expressed CYS3 (Fig 2.12). A third line of evidence came from a mutagenesis study of CYS3. Alanine substitution for serine and threonine residues in the N-terminal region of CYS3 resulted in a hyperactive protein, further suggesting that phosphorylation may modulate the CYS3 protein (Kannan and Marzluf, 1993). In order to test this hypothesis, I directly examined the possible phosphorylation of CYS3 expressed in *N. crassa*. In vivo labeling using 32-P inorganic phosphate was carried out as described in Material and Methods. CYS3 protein was immunoprecipitated as described using the rabbit anti-CYS3 antibodies and following electrophoresis. The results were visualized by autoradiography. My results suggested that CYS3 indeed is a phosphoprotein under physiological conditions (Fig. 2.14, lane 2). To test whether or not the phosphorylation state of CYS3 changed in response to the changing of the sulfur concentration, another sample was labeled in low sulfur medium and then incubated in high sulfur medium for 10 min before harvest. CYS3 protein was immunoprecipitated and analyzed on an SDS polyacrylamide gel (Fig. 2.14, lane 3).
Compared to the sample in lane 2 which was labeled strictly in low sulfur medium, one expect a 50% decrease in the radioactive signal in lane 3 simply due to the turn over of CYS3 protein at the low sulfur to high sulfur transition if the phosphorylation state of CYS3 remained the same at both culture conditions. Interestingly, I saw no decrease but instead a slight increase in signal in lane 3 suggesting that there is an increase in the phosphorylation of CYS3 due to sulfur repression conditions.

To address a possible biological function of the CYS3 phosphorylation, I examined the two hyperactive cys-3 mutants described before. There are two Ser/Thr rich regions in CYS3, one located at the N-terminal and the other at the C-terminal end, which may contain the potential phosphorylation site(s). Deletion studies showed that the C-terminus Ser/Thr rich region has little affect on CYS3 function (Kannan and Marzluf, 1993). CYS3MK40 and CYS3MK30-50 are two point mutants that change different serine and threonine residues into alanines, namely MK40 (T13A, T14A) and MK30-50 (T12A, T13A, T14A, Y15A, T16A, S17A, T19A), mimicking presumably the hypophosphorylated stage of CYS3 (Kannan and Marzluf, 1993). In N. crassa transformants expressing CYS3MK40 and CYS3MK30-50, the protein expression is increased about three and five fold respectively, compared to wild type while the activity of aryl sulfatases increased only about two fold. This result suggested that the changes in the N terminal amino acids may have more dramatic effect on protein stability than on the transactivation activity of CYS3. In order to test this hypothesis, the stability of CYS3 MK40 and CYS3MK30-50 was examined at the low sulfur to high sulfur transition, which is characterized by the fast turnover rate of CYS3 with an estimated half life about 10 minutes. Both CYS3MK40 and CYS3MK30-50 showed prolonged half life compared to the wild type CYS3, estimated to be about 30 minutes and 60 minutes, respectively (Fig. 2.15, Fig. 2.16). This result showed that the changes in the amino acid residues indeed increase CYS3 protein stability.
and explains the elevated CYS3 levels in the transformants. It is not possible to rule out the possibility that these changes also affect the transactivation activity of CYS3.

2.4. Discussion

The CYS3 protein plays a central role in controlling the expression of an entire family of structural genes which encode enzymes for acquisition of sulfur. CYS3 is composed of 236 amino acids and has a bZIP domain that confers sequence-specific DNA binding to elements with a consensus sequence ATG-RYRYCAT. The cys-3 gene is itself highly regulated by the negative-acting scon genes and is not expressed, or only very weakly so, during sulfur repression conditions. Thus, when cells growing with repressing levels of sulfur (i.e. 5 mM methionine) experience derepression conditions (i.e. 0.25 mM methionine), expression of cys-3 must occur before the various structural genes can be activated. Synthesis of aryl sulfatase occurs only approximately five hours after wild-type cells are transferred from high sulfur to low sulfur conditions. It was of interest to determine the time course of cys-3 expression in cells undergoing the transition from sulfur repression to derepression conditions. I found that this is a very slow process, requiring approximately two hours before cys-3 mRNA can be detected and two to three additional hours to reach a maximum level. It required at least four hours following derepression to detect the CYS3 protein, which apparently explains the long delay in appearance of aryl sulfatase activity after cells are shifted from high to low levels of sulfur. Further reduction of the methionine concentration to 0.025 mM or use of a sulfur-free medium only slightly shortened the time period (to about three hours) before the CYS3 protein could be detected by western analysis. The slow response of the sulfur regulatory circuit to the shift from sulfur repression to derepression conditions almost certainly is due to the presence of a substantial internal pool of sulfur compounds, which must be utilized before the cells actually experience sulfur limitation. The ability to accumulate stored forms of sulfur, e.g.,
choline-o-sulfate, insures that *Neurospora* has sufficient intracellular sulfur for prolonged growth even in sulfur-poor environments before expression of the entire family of sulfur catabolic enzymes becomes necessary.

Another significant change in environmental conditions occurs when cells growing under sulfur starvation conditions, with the sulfur circuit fully activated, suddenly encounter an abundance of sulfur. Under the new conditions of excess sulfur, the sulfur catabolic enzymes would no longer be required. I found that upon establishment of sulfur repression, synthesis of *cys-3* mRNA apparently stopped immediately and the pre-existing mRNA turned over rapidly and was completely gone within twenty minutes. Moreover, the CYS3 protein also turned over quickly, with a half life of approximately ten minutes. The rapid loss of *cys-3* mRNA and protein insures that the expression of the sulfur structural genes is turned off quickly upon sulfur repression.

A differential rate of turnover of the CYS3 protein was observed depending upon sulfur availability. The CYS3 protein was stable, with a half life of about four hours, in sulfur-derepressed cells. In contrast, during sulfur repression CYS3 turned over considerably more rapidly, with a half life estimated to be approximately ten minutes. The finding that cycloheximide partially stabilized the CYS3 protein suggested that its sulfur-dependent rapid turn over required de novo protein synthesis, possibly of a proteolytic enzyme. The CYS3 protein with 236 amino acids, when expressed in *E. coli*, runs in SDS-polyacrylamide gels as a single species with a size of 31 kDa, whereas in *Neurospora* cells, CYS3 appears as a doublet of approximately 35 kDa, suggesting that it may be subject to post-translational modification. The faster migrating form of the doublet of CYS3 from *Neurospora* cells is first observed when cells are derepressed disappeared first when cells are subject to sulfur repression. One interesting possibility is that CYS3 is controlled by phosphorylation and dephosphorylation. An experiment to determine whether CYS3 is phosphorylated revealed a doublet of 32P-labeled protein bands around
35 kDa which was immunoprecipitated by anti-CYS3 antibody from cells grown under the sulfur derepression condition but not from cells grown under the sulfur repression condition, although a considerable background was present. It appeared that there is a slight increase in the label intensity from the sample that with the ten-minute high sulfur treatment (Fig. 2.14, lane 3), which should otherwise show a decrease in intensity due to the loss of CYS3 protein. Although not conclusive, I speculate that CYS3 is a phosphoprotein and a high sulfur signal may trigger further phosphorylation of CYS3. The attempt to determine a biological function of the phosphorylation proved to be even less conclusive. There are two Ser/Thr rich regions in CYS3, one at the N-terminus, another at the C-terminus. Deletion and mutagenesis studies suggested that only the Ser/Thr region in the N-terminus is essential for CYS3 function (Kannan and Marzluf, 1993). Changing of these serine and threonine residues to alanine, which presumably mimics the hypophosphorylated stage, generated CYS3 proteins with longer half lives and better transactivation activities.

In an attempt to address the mechanism involved in the selective degradation of the CYS3 protein, I found that amino acid residue lysine 105 is important in determining CYS3 instability. Both cys-3 REV21 and cys-3 K105Q mutant proteins, each with lysine 105 replaced with glutamine, showed an elevated cellular accumulation and prolonged half life. It is well known that the ubiquitin-dependent proteolytic pathway, which involves the covalent attachment of ubiquitin to specific lysine residues of target proteins, is responsible for the selective degradation of proteins in eukaryotes. The bZIP protein, c-JUN, which shares certain features with CYS3, is selectively degraded by the ubiquitin-dependent pathway (Treier et al., 1994). My results implicate lysine 105 as a determinant of CYS3 stability, suggesting that the ubiquitin pathway could be involved in CYS3 turnover.
Fig. 2.1 Derepression of *cys-3* mRNA.

The derepression process was achieved by shifting wild type *N. crassa* mycelia from medium with a repressing level of methionine (5 mM) to medium with a derepressing level of methionine (0.25 mM). Mycelia were harvested and total RNA was prepared. RNA blot analysis revealed the *cys-3* transcript of 1.6 kb. β-tubulin (2.0 kb) was used as an internal control. The harvest time of each sample is indicated at the top of each panel.
Figure 2.1
Fig. 2.2 Derepression of CYS3 protein.

Wild type *N. crassa* mycelia were grown under the sulfur repression condition (5 mM methionine) overnight and shifted to medium with derepressing levels of methionine (0.25 mM). Mycelia were harvested and whole cell extracts were prepared. Western blot analysis of CYS3 protein was carried out using rabbit anti-CYS3 antibodies. The harvest time of each sample is indicated at the top of each panel.
Fig. 2.3 Time course of aryl sulfatase activity.

Wild type *N. crassa* mycelia were grown under derepression conditions (5 mM methionine) then shifted to medium with 0.25 mM methionine. At different time points, mycelia were harvested and whole cell lysates were prepared for enzyme assay. OD$_{405}$, optical density at 405 nm.
Figure 2.3
Fig. 2.4 Turnover of cys-3 mRNA.

The turnover kinetics of cys-3 mRNA were examined at the transition stage when the methionine level in a culture was increased from low (0.25 mM) to high (5 mM). 74A mycelia were harvested at different times after addition of methionine and were used to prepare total RNA. RNA blot analysis was used to detect the cys-3 transcript. The half life of cys-3 mRNA was estimated using the SigmaGel program.
Figure 2.4
Fig. 2.5 Turnover of CYS3 protein.

The turnover kinetics of CYS3 protein were also examined at the transition stage from sulfur derepression to repression by raising the methionine concentration in the culture medium. Samples were taken at various times, 0, 0.5, 1, 2, 3 hours following the addition of methionine. Whole cell extracts were used for immunoblot analysis of CYS3 protein. The half life of CYS3 protein was estimated by the SigmaGel program.
Figure 2.5
Fig. 2.6 Differential elimination of CYS3 protein.

The cellular level of CYS3 protein was examined after introducing cycloheximide \((2 \times 10^{-4} \text{M})\) to a wild type culture in medium containing a derepressing amount of methionine \((0.25 \text{ mM})\). Samples were harvested at different times after the addition of cycloheximide. In lane 6, a control sample grown without cycloheximide was harvested at the same time as the sample in lane 5, which was grown in cycloheximide for 4 hours.
Fig. 2.7 Nitrate reductase assay.

A block of the synthesis of nitrate reductase using cycloheximide (2x10^{-4}M) was used as the control experiment for the differential elimination of CYS3 protein. 74A mycelia were grown in Vogel's medium supplemented with 20 mM glutamine, then transferred to medium supplemented with 40 mM KNO₃ for the induction of synthesis of nitrate reductase. Cycloheximide was introduced to half of the cultured samples during the transfer to monitor the block of protein synthesis. Samples were harvested at various times and were tested for the enzyme activity of nitrate reductase.
Figure 2.7

Graph showing nitrate reductase activity over time with and without cycloheximide.
Fig. 2.8 Turnover of mutant CYS3 protein under derepressing condition.

Temperature sensitive mutant REV65 was grown first at the permissive temperature (27°C), then shifted to the non-permissive temperature (37°C) to block further production of CYS3 protein. The turnover of pre-existing CYS3 during the period 0 to 3 hours was analyzed by western analysis.
Fig. 2.9  Turnover of CYS3 under S-repressing conditions in the presence of cycloheximide.

The cellular level of CYS3 protein was examined after shifting *N. crassa* mycelia from medium of derepression condition (0.25 mM methionine) to the repression condition (5 mM methionine) and introducing cycloheximide to 2x10^{-4} M.
Fig. 2.10 Turnover of REV21 CYS3 mutant protein upon sulfur repression.

The turnover of REV21 CYS3 protein was monitored after shifting *N. crassa* mycelia from low-sulfur medium to high-sulfur medium. The harvest time of each mycelia sample is indicated at the top of each panel. CYS3 protein was detected by Western analysis.
Fig. 2.11 Turnover of K105Q CYS3 protein upon sulfur repression.

The turnover of this mutant CYS3 protein was monitored after shifting *N. crassa* mycelia from low-sulfur medium to high-sulfur medium. The harvest time of each mycelia sample is indicated at the top of each panel. CYS3 protein was detected by Western analysis.
Figure 2.11
Fig. 2.12  *E. coli* expressed CYS3 proteins.

Wild type and mutant CYS3 proteins were expressed in *E. coli* and were partially purified.
Coomassie Blue stained SDS-PAGE was used to confirm the presence of CYS3 proteins.
Figure 2.12
Fig. 2.13 DNA mobility shift analysis of expressed CYS3 proteins.

0.2 μg protein was used in lanes 2, 3 and 4; 0.6 μg protein was used in lanes 5, 6 and 7. A 200 bp DNA fragment taken from the cys-I4 promoter containing a strong CYS3 binding site was end labeled by $^{32}$P and was used for the mobility shift assay. Free DNA fragment and CYS3-DNA complex are indicated by arrows. The percentage shift of the probe in each reaction as determined by a phosphoimager is indicated at the bottom of the picture.
Fig. 2.14 Phosphorylation of *N. crassa* CYS3 protein.

Cells were grown in high sulfur medium overnight, then transferred to low sulfur medium for 3 hr for the derepression of CYS3 synthesis. Cells were labeled with inorganic $^{32}$P (20 μCi/ml) for three hours. One culture sample received 5 mM methionine for 10 min before harvest. Whole cell lysates were prepared and CYS3 protein was immunoprecipitated.
High sulfur
Low Sulfur
Low to High

Figure 2.14

CYS3
Fig. 2.15 Turnover of CYS3 MK40 mutant protein.

The repression kinetics were examined at the transition stage when the methionine level in culture was increased from low (0.25 mM) to high (5 mM). Mycelia were harvested at different times after the addition of methionine to the medium and were used to prepare protein extracts. Western blot analysis was used to detect the CYS3 protein.
Figure 2.15

The figure shows a gel electrophoresis with molecular weight markers in kDa on the left side and time points at the bottom: 0 hr, 0.5 hr, 1 hr, 2 hr, and 3 hr. The protein CYS3 is indicated by an arrow and is present at 29 kDa at 1 hr and 3 hr.
Fig. 2.16 Turnover of CYS3 MK30-50 mutant protein.

The repression kinetics were examined at the transition stage when the methionine level in the culture was increased from low (0.25 mM) to high (5 mM). Mycelia were harvested at different times after the addition of methionine to the medium and were used to prepare protein extract. Western blot analysis was used to detect the CYS3 protein.
Figure 2.16
Chapter 3

Analysis of a distal cluster of binding elements and other unusual features of the promoter of the highly regulated nit-3 gene of Neurospora crassa

3.1 Introduction

In Neurospora crassa, nit-3 (nitrate reductase) is one of the well characterized genes in the nitrogen regulation circuit. The expression of nitrate reductase is precisely regulated by the availability of inducer nitrate and the absence of primary nitrogen sources such as glutamine, glutamate and ammonia (Fu and Marluf, 1987). When a primary nitrogen source is abundant, the expression of nit-3 is fully repressed. Limitation of the favored nitrogenous compounds represents the signal for derepression of many structural genes in the nitrogen regulation circuit via a global transcription factor, NIT2. However, in order to utilize secondary nitrogen sources, such as purines, amides, nitrate, nitrite, most amino acids and proteins, a second pathway-specific positive regulator is usually also required for the full scale derepression of the genes that encode the catabolic enzymes of a particular pathway. One well documented example of a pathway-specific regulator is the NIT4 protein. In the presence of nitrate, NIT4 acts in conjunction with NIT2 to turn on the expression of nit-3 and nit-6 (nitrite reductase) genes (Marzluf, 1997).

Since the nit-2 gene was identified (Stewart and Vollmer, 1986), its regulation and function have been subject to extensive study. nit-2 is constitutively expressed in Neurospora, however the nit-2 mRNA level increases three- to four-fold during nitrogen
limitation (Fu and Marzluf, 1990). The nit-2 gene encodes a 116 kDa protein with a single Cys-X_2-Cys-X_17-Cys-X_2-Cys zinc finger motif and a basic region that serves as the DNA binding domain (Fu and Marzluf, 1990). The Aspergillus nidulans homolog, AREA protein and NIT2 share a high degree of homology with the carboxyl finger of the vertebrate GATA transcription factor family (Tsai et al., 1989; Wall et al., 1988; Kudla et al., 1990). In common with other members of this transcription factor family, NIT2 shows high binding affinity to the consensus DNA sequence T/A(GATA)A/G (Fu and Marzluf, 1990; Orkin, 1992). NIT2 binding sites have been found in the promoters of nit-3, nit-6 and several other structural genes within the nitrogen regulation circuit, e.g. alc (allantoicase) and lao (L-amino acid oxidase), and even in the nit-2 gene, suggesting a possible autogenous regulation (Chiang and Marzluf, 1994).

The pathway specific transcription factor NIT4 is a protein with an amino terminal Zn(II)_2Cys_6 type of zinc finger that constitutes its DNA binding domain (Yuan et al., 1991). Northern analysis showed that the nit-4 gene is constitutively expressed at an extremely low level (Fu et al., 1989). Mobility-shift and DNA-footprinting experiments showed that a symmetrical sequence TCCGCGGA and a closely related sequence are recognized by NIT4. Binding sites for NIT4 were found in the promoter of the nit-3 gene (Fu et al., 1995) and potential NIT4 sites can be identified in the nit-6 gene via sequence analysis.

Besides the two positive regulators, a negative-acting regulatory gene, nmr (nitrogen metabolic regulation), is also involved in regulation of the nitrogen circuit. Nitrate reductase and other nitrogen-related enzymes become constitutively expressed in nmr mutants (Premakumar et al., 1980; Fu et al., 1988). The NMR protein does not have DNA binding activity but functions as a negative regulator through a protein-protein interaction with NIT2, interfering with NIT2-DNA binding (Xiao et al. 1995; Pan et al., 1997).
Three NIT2 and two NIT4 binding sites have been identified in the nit-3 promoter (Fu and Marzluf, 1990; Fu et al., 1995). Removal of either all the NIT2 sites or of the two NIT4 sites resulted in complete elimination of nit-3 promoter activity, indicating that both NIT2 and NIT4 are required for expression (Chiang and Marzluf, 1995). Inspection of the nit-3 promoter revealed that it possesses some intriguing features, including the fact that a cluster of NIT2 and NIT4 binding sites is located at a considerable distance, approximately 1 kb, upstream of the gene; the promoter also contains two long AT-rich regions. Moreover, eight repeats of the sequence TACC occur in the transcribed region of the gene but prior to the initiation codon for synthesis of the nitrate reductase protein. In this chapter, I report a detailed study to examine the functional significance, if any, of these unusual features of the nit-3 promoter.

3.2. Materials and Methods

3.2.1 N. crassa and E. coli strains.

The N. crassa wild type strain 74-OR23-1A was obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, Mo. The RIP15 (nit-3 his3) mutant has been described (Chiang and Marzluf, 1995). Mycelia were cultured in Vogel's liquid medium (Davis and deSerres, 1970) with shaking. The amount of glutamine, KNO3 and uric acid supplement is indicated for each experiment. E. coli strain DH5α was used for plasmid propagation, and CJ236 for single stranded DNA template preparation.

3.2.2. Site-directed mutagenesis

Site-directed mutagenesis was used to generate all the nit-3 mutant constructs. DNA oligos containing the desired mutation were synthesized and annealed to the single
stranded nit-3 DNA template which was generated using E. coli strain CI236. The daughter strand was synthesized in vitro and the double stranded product was used to transform E. coli. Each mutant was selected and confirmed by DNA sequencing. All the mutants were then cloned into vector pDE1 (FGSC).

3.2.3 Mobility shift assay.

DNA mobility shifts were carried out as described before with minor modifications (Fu and Marzluf, 1990). E. coli expressed NIT2 proteins (0.1 μg to 1.0 μg) were used in the mobility shift assays. A 34 bp DNA fragment containing the NIT2 binding site II was used as probe. The mixtures of proteins and DNA were incubated at room temperature for 20 min in a total volume of 25 μl in binding buffer (12 mM HEPES; 4 mM Tris-HCl, pH 7.9; 50 mM KCl; 1 mM EDTA; 1 mM DTT; 0.3 mg/ml BSA; 10% glycerol) with 3 μg of poly(dI-dC) as nonspecific competitor. Samples were separated on 4% polyacrylamide gels (acrylamide:bisacrylamide=19:1) in 1/4x TBE buffer.

3.2.4 RNA preparation and Northern analysis.

Total RNA from N. crassa was isolated as described by Weaver et al. (1997) with modifications. Mycelia were ground to a fine powder with a mortar and pestle in liquid nitrogen and suspended in lysis buffer (50 mM NaOAc, pH 5.3; 10 mM EDTA; 1% SDS) at a 1 g/5 ml ratio. An equal volume of acidic phenol/chloroform (equilibrated with the lysis buffer without SDS, pH 5.3, prewarmed at 65°C) was added to the lysate, and the reaction mixture was incubated at 65°C for 30 min with shaking. The aqueous phase was recovered by centrifugation, and this process was repeated several times until no protein interface could be seen. Total RNA was precipitated with 0.6 volume of isopropyl alcohol followed by centrifugation. The RNA samples were fractionated in agarose gels, and transferred to nitrocellulose filters.
nit-3 cDNA and β-tubulin cDNA were labeled with random primers using $^{32}$P-dATP for use as probes for northern analysis (GibcoBRL). The hybridization was carried out at 65°C in 1x hybridization buffer (0.5 M NaCl; 0.1 M NaPO$_4$, pH 7.0; 6 mM EDTA; 1% SDS). The membranes were washed at 65°C for 60 min with 1/4x hybridization buffer. The nit-3 and β-tubulin mRNAs were identified by their sizes.

3.2.5 Nitrate reductase assay.

Mycelia were grown at 30°C in liquid Vogel's minimum medium containing 20 mM glutamine as the only nitrogen source for 12 to 16 hr, then transferred to medium containing 40 mM KNO$_3$ for 3 to 4 hr to allow expression of the nit-3 gene. Mycelia were harvested by filtering through miracloth and were lysed in lysate buffer (0.1 M KPO$_4$, pH 6.8; 1 mM β-mercaptoethanol; 0.5 mM EDTA; 1% NaCl) using a Mini-beadbeater BX-4 (Biospec Products). Protein concentrations were determined with the Bio-Rad protein assay. Nitrate reductase enzyme assays were performed as described before with a few modifications (Garrett and Cove, 1967). Cell lysate (100 µl) was mixed with 400 µl of buffer (22 mM KPO$_4$, pH 7.75; 22 mM NaNO$_3$; 5.5 mM Na$_2$SO$_3$; 0.11 mM FAD) and incubated at 30°C for 30 min. 50 µl NADPH (0.2 mM) was added to initiate the reaction and the mixture was incubated at 30°C for 15 to 30 min depending on the amount of activity. 0.1 ml of stop solution (25% barium acetate) was added and the precipitate was removed by centrifuging in a microfuge at full speed for 1 min. For the zero-time control, the stop solution was added to the mixture without incubation. 0.5 ml of the supernatant of each reaction mixture was mixed with 2.5 ml 0.2% sulfanilamide (diluted from 1% stock) in 20% v/v HCl, and 0.026% wt/vol naphthylthylene-diamine-dihydrochloride. The absorbance of these samples was measured at 540 nm with a spectrophotometer.
3.3. Results

3.3.1. Potential communication between NIT2 and NIT4 sites

Of the binding sites found in the *nit-3* promoter, two NIT2 and two NIT4 binding sites are clustered together about 1 kb upstream of the transcription start sites (Fig. 3.1). A third NIT2 site is located very close to the transcription start sites. Site-directed mutagenesis was used to study the biological function of the individual NIT2 and NIT4 sites. Surprisingly, the proximal NIT2 site III, which consists of four GATA elements and has strong in vitro binding affinity for the NIT2 protein, is the least important one for *nit-3* promoter activity. Loss of the most upstream NIT2 site I, which also contains multiple GATA elements, reduced promoter activity by 50%. Mutation of NIT2 site II with a single GATA element showed the most dramatic effect, with a 90% loss of promoter activity. The two NIT4 sites contribute equally to promoter activity in that mutation of either one results in a 50% reduction and loss of both leads to complete loss of *nit-3* expression (Fig. 3.1).

In an attempt to further dissect the *nit-3* promoter, it was important to determine whether there were additional unknown elements located distal to the transcription start sites. A deletion mutant, dl-BP, was generated by removing 600 bp of *nit-3* promoter between the NIT2-NIT4 cluster and the proximal NIT2 binding site. This construct was targeted to the *his-3* locus of a *nit-3* RIP mutant host (RIP15). Analysis of multiple transformants showed that the mutant promoter gave approximately the same activity as the wild type promoter (Fig 3.2). This result suggested that there is no obvious functional reason for the large distance between the cluster of NIT2-NIT4 elements and the transcription start sites, and also showed that the intervening 600 bp of the promoter region does not appear to contain any important regulatory element(s).
The next question addressed was whether it is necessary that the NIT2 and NIT4 sites of the upstream cluster be close together to maintain proper function, possibly to allow a direct protein-protein interaction between NIT2 and NIT4 factors. In order to answer the question, a series of mutants was generated. First a unique restriction enzyme site (Sma I) was engineered between the NIT2 and NIT4 sites, then DNA fragments ranging from 22 bp to 200 bp were inserted at this Sma I site. The constructs (SmaI(-8), 22, 34, 56, 68, 124, 200) were named after the length of DNA fragment inserted. These insertion mutant constructs as well as one carrying the wild type promoter were targeted to the his-3 locus and multiple transformants of each were examined. The promoter activity showed a slight decrease for constructs Sma I(-8), 22 and 34 and was at the wild type or a somewhat greater level for constructs 56, 68, 124 and 200 (Fig. 3.2). Nitrogen regulation was normal in the transformants carrying each of these mutant constructs. The presence of glutamine fully repressed the expression of the nit-3 gene, while nitrate was required for induction. The presence of both glutamine and nitrate led to a low level of expression (approximately 10% of fully derepressed value), while other poor nitrogen sources like allantoin or uric acid did not induce nit-3 at all (less than 5% of induced level) (Fig. 3.3). This study demonstrated that it is not critical that NIT2 and NIT4 elements of the cluster be close to one another for proper promoter function and for normal nitrogen regulation.

3.3.2. Anatomy of a single GATA site

Nearly all strong NIT2 binding sites have two or more closely spaced GATA elements, with the exception of site II in the nit-3 promoter. In vitro mobility shift studies demonstrated that this site, which contains only a single GATA element, strongly binds the NIT2 protein. Deletion of this site results in up to 90% loss of the in vivo activity of the promoter. Deletion of all three NIT2 sites (in construct dl-34) resulted in essentially a complete loss of nit-3 expression, and dl-34 transformants could not grow in medium
using nitrate as the only nitrogen source. In order to further analyze the function of the single GATA site, a series of mutants were generated. By using dl-34 as the recipient, a short double-stranded fragment containing the GATA core element of site II and its flanking sequence (12 bp on each side) was inserted, either at the original location (named dl-343), or at a different location about 180 bp upstream of the transcription start sites (named dl-344). Analysis of transformants that received these constructs showed that returning the GATA element back to its original location restored about 60% of the wild type promoter activity, suggesting that this single GATA plays a unique role in regulating the promoter. Transformants with the single GATA element inserted just before the transcription start sites had about 40% of the wild type promoter activity. By comparison to construct dl-3, in which the N1T2 cluster (sites I and II) was removed, this single GATA element is stronger at activation (3 fold greater) than the N1T2 site III which contains multiple GATA copies (Fig 3.4).

In an attempt to determine why the NIT2 site II when inserted near the transcription start site was somewhat less effective in restoring the promoter activity than when located far upstream, a deletion mutant dl-5 which reduced the distance between NIT4 and NIT2 sites from 600 bp to about 100 bp was constructed from dl-344. In dl-5, the two NIT4 sites are located about 100 bp upstream of the inserted N1T2 site II. The dl-5 promoter functioned at the same level as dl-344. This result suggested that the relative orientation of NIT4 and NIT2 sites may have some effect on the promoter activity (Fig. 3.4).

Previous results had suggested that substitution of single nucleotides 5' or 3' of a GATA element had marginal affects upon NIT2 binding affinity (Chiang and Marzluf, 1994). To further analyze why the single GATA element of site II was so highly active, site-direct mutagenesis was used to change its flanking sequences. The 5' flanking sequence was changed from AGTA to CTAC; the 3' flanking sequence was changed from CGTA to GTAG (Fig. 3.5 A). The effect, if any, of these changes was analyzed both in
vivo and in vitro. The in vitro mobility shift analysis with a synthetic oligo containing this single GATA with its wild type flanking sequences showed very good NIT2 protein binding. In contrast, identical oligonucleotides with base substitutions on the 5' or on the 3' side of the GATA core element showed a significantly reduced binding affinity for NIT2, 15-fold and 7-fold lower, respectively (Fig. 3.5 B). The results of in vivo expression analysis with these altered promoters correlated very well with the mobility shift analysis. The engineered nir-3 promoters containing either the wild type copy of NIT2 site II or the mutant forms were transformed into the Neurospora nit-3 RIP15 mutant. Transformants carrying the two mutant promoters with altered flanking sequences showed a dramatic reduction in promoter activity compared to the wild type copy (Fig. 3.6). This result indicated that both the 5' and 3' flanking nucleotides and the GATA core sequence contribute to the NIT2 protein-DNA interaction in vivo, as reflected in nir-3 gene expression.

3.3.3. AT-rich sequences

Besides the major transcription factor binding sites, the nir-3 promoter has several other interesting features. Two AT-rich sequences are found in the nir-3 promoter, one located about 1 kb upstream of the transcription start sites (AT I), the other located just in front of the transcription start sites (AT II). Deletion of AT I (construct dl-1) increased nit-3 expression slightly, while deletion of AT II (construct dl-2) resulted in more than 70% loss of nit-3 gene expression (Fig. 3.7). Deletion of both AT sequences and the 1 kb promoter region between them (construct dl-12) resulted in the same loss of promoter activity as did deletion of just the AT II sequence. Insertion of the AT II sequence 200 bp upstream of the transcription start sites into construct dl-2 (yielding dl-2+ATII) partially restored activity (60% of the wild type promoter). Insertion of an additional copy of the AT II sequence to the wild type promoter (construct WT+ATII) resulted in only a slight
increase of the promoter activity. These results suggested that the proximal AT-rich sequence II is essential for full transcription of the \textit{nir-3} gene.

### 3.3.4. Study of TACC repeated sequence

Eight repeats of the sequence TACC are situated about 30 bp down stream of the transcription start sites and thus will be present in the \textit{nit-3} mRNA. Deletion of this repeated sequence resulted in an increase of nitrate reductase activity. As shown in Figure 3.8 A, the TACC deletion mutant had about twice the activity as did transformants carrying the wild type construct. It was suspected that this increase in enzyme activity was due to a change in the stability of the \textit{nit-3} transcript. In order to test this hypothesis, the \textit{nit-3} mRNA half-life was determined at the induction-repression transition. Mycelia were initially grown in medium with 40 mM KNO$_3$ as the sole nitrogen source, and then transferred to medium with 20 mM glutamine. This change in the nitrogen source prevented any further synthesis of \textit{nit-3} mRNA, thus allowing me to analyze the turnover rate of the pre-existing \textit{nit-3} transcripts. Cells were harvested at 0, 5, 10, and 20 min after the shift and total RNA was prepared. Northern analysis revealed that the mRNA stability of the TACC-deletion mutant is increased, with an estimated half life of 10 min (Fig. 3.8 B) in comparison to the wild type mRNA which has a half life of 5 min (Okamoto et al., 1991). This result suggested that an increase in the half life of \textit{nit-3} transcript is responsible for the increase in enzyme activity in transformants containing the \textit{nit-3} mutant lacking the TACC repeated sequences.

### 3.4. Discussion

In \textit{Neurospora crassa}, environmental signals determine the expression pattern of nitrate and nitrite reductase. Limitation for a primary nitrogen source and the presence of inducer nitrate, metabolic signals integrated via NIT2 and NIT4 proteins respectively, are
both required for the expression of these enzymes. In the 5' promoter region of the \textit{nit-3} gene, multiple NIT2 and NIT4 binding sites have been identified. The arrangement of these binding sites is rather intriguing, with only a single NIT2 site located at -180 bp, and a cluster of two NIT2 and two NIT4 sites situated at about 1 \textit{kb} upstream of the transcription start sites. In the mutagenesis studies, I found that the far upstream cluster has the major effect for the \textit{nit-3} promoter activity, while the proximal NIT2 site plays only a limited role despite its having multiple GATA core elements and showing strong in vitro binding to NIT2 protein. Removal of up to 600 bp of the promoter sequence between the cluster of NIT2 and NIT4 elements and the proximal NIT2 site showed no significant effect on the promoter activity, implying that there are no important elements in this entire stretch of 600 bp that function to modulate the level of promoter activity or to regulate its function. This result also indicates that there is no obvious requirement that the cluster of regulatory elements be so far (at least 1 \textit{kb}) upstream of the transcription start sites.

A similar phenomenon has been observed in \textit{Aspergillus nidulans} as well (Punt et al., 1995). The niaD and niiA genes, which specify nitrate and nitrite reductase respectively, are transcribed divergently from a common intergenic control region, within which multiple NIRA (NIT4 homolog) and AREA (NIT2 homolog) binding sites were identified scattered throughout the entire region. Mutagenesis results showed that of the 10 AREA binding sites, only four located in the central region appear to be biologically important for nitrogen repression/derepression. Loss of those AREA sites located near the transcription start sites of the niaD and niiA genes showed no detectable effect.

Like many \textit{Neurospora} genes, \textit{nit-3} lacks a TATAA box and deletion of a TATA-like sequence located near the start sites has no demonstrable effect on transcription (Tao and Marzluf, unpublished result). Another unusual feature of the \textit{nit-3} promoter is the presence of two long AT-rich sequences, one located far upstream, the other immediately before the transcription start sites. The upstream AT-rich sequence is immediately
downstream of the cluster of NIT2 and NIT4 binding elements, which suggested the possibility that it might function to keep these sites in an open conformation for interaction with the regulatory factors, for example by excluding nucleosomes. However, our results reveal that this AT-rich sequence does not appear to play a significant role in modulating either the regulation or the expression level of the nit-3 gene. In contrast, the AT-rich sequence just upstream of the transcription start sites is required for the optimal expression of nit-3 and its loss led to a significant decline (about 70% loss) of promoter activity. Studies in yeast, plants and mammals have suggested that AT-rich sequences in promoter regions may have divergent effects on transcription. DNA binding proteins have been identified in these diverse organisms which can bind specifically to AT-rich sequences and act as either an activator or as a repressor (Zhou et al., 1993; Tjaden and Coruzzi, 1994; Winter and Varshavsky, 1989).

Many organisms possess multiple GATA factors, each of which recognizes binding sites with the same core GATA sequence. Mice and humans contain at least six different GATA factors, each of which activates a particular set of target genes. The expression of these GATA factors is developmentally regulated to give a distinctive but overlapping pattern of tissue-specific distribution, which is at least one major determinant for their specificity in controlling downstream genes (Orkin, 1992; Merika and Orkin, 1993).

*Neurospora crassa* contains at least five distinct GATA factors, NIT2, WC1, WC2, SRE and NGF1, all of which appear to be constitutively expressed within the same vegetative cells despite their diverse functions (Ballario et al., 1996; Zhou and Marzluf, 1998; Feng and Marzluf, unpublished results). The coexistence of these multiple GATA factors, with similar DNA binding specificity, raises a question as to how each controls its own unique set of target genes. The studies presented here highlight two features which may be central to the specificity exerted by NIT2 in controlling nitrogen regulated structural genes. First, activation of the nit-3 gene is completely dependent upon the presence of
both NIT2 and NIT4 which bind at a cluster of elements in the promoter (Fu and Marzluf, 1987b). Secondly, it was shown that both the 5' and 3' flanking nucleotides of the single GATA core element in NIT2 binding site II are of critical importance for its strong NIT2 binding affinity in vitro and its physiological function in vivo. The NIT2 binding sites I and III, each with multiple GATA sequences, bind the NIT2 protein in vitro with a greater affinity than does the single GATA element in site II. Yet site II was found to be the most important for in vivo function, revealing that DNA binding examined in vitro cannot be relied upon to indicate function in vivo.

Comparison of the flanking nucleotides in all eight of the GATA elements present in the nit-3 promoter revealed an expanded consensus NIT2 binding site, 5' GATGATAAG (Table 1). The single GATA element in site II, the one most important for nit-3 gene expression, is the only GATA sequence in the promoter with a perfect match to this expanded binding sequence. Moreover, changing the nucleotide sequence on either side of this special GATA element led to a drastic reduction in both the strength of NIT2 binding in vitro and nit-3 gene activation in vivo. These findings show that the context of the single GATA element of site II is important and that a proper extended sequence is required for optimal function. This feature may help explain the weak level of derepression observed with two other genes of the nitrogen control circuit, lao and alc, which possess less perfect NIT2 binding sites.

In many cases, the regulation of gene expression is achieved at different levels. Within the Neurospora nitrate assimilation pathway, the expression of nitrate reductase appears to be regulated mainly at the transcriptional level by NIT2 and NIT4. However, our mutagenesis study suggested that the stability of nit-3 messenger RNA might also affect nit-3 gene expression. Removal of eight TACC repeats from the 5' untranslated region doubles the half life of nit-3 mRNA as determined at the transition stage from nitrogen-derepression to nitrogen-repression conditions. Differential turnover of nitrate
reductase mRNA has also been observed in *Chlorella vulgaris* under nitrogen-repressed conditions (Cannons and Pendleton, 1994).
Table 3.1 Summary of nucleotides that occupy 5' and 3' flanking regions of the GATA element of the NIT2 binding sites
Fig. 3.1 In vivo analysis of nit-3 promoter.

Schematic drawing of nit-3 promoter and several mutant constructs. The first two NIT2 binding sites were mutated individually in constructs A1 and A2. The two NIT4 sites were changed individually in A3 and A4. All the constructs were built into pDE1 vector and targeted to the his3 locus using a nit-3 his-3 double mutant (RIP15A) as the host. Nitrate reductase activity was assayed in cells under nitrate derepression conditions. Nitrate reductase activity for the construct with the wild type promoter is set at 100. I, II, and III, NIT2 binding sites; A and B, NIT4 binding sites. The sites which were mutated in each construct are indicated by removing the corresponding box.
Figure 3.1
Fig. 3.2. In vivo nit-3 promoter analysis.

The insertion mutants were generated by inserting DNA oligos with different lengths between the two upstream NIT2 sites and the two NIT4 sites. The two DNA oligos used in cloning are 34-mer 5' GACATGTACCCCTACGACGTGCCCGACTACTCCC 3' and 22-mer 5' GGGAATAAGAATTCTTATTCCC 3'. Sma I has the indicated restriction sites inserted between the NIT2 and NIT4 sites. The insertion mutants are designated by the number of base pairs inserted between NIT2 and NIT4 sites. RIP15 was used as the host strain to analyze the promoter activity of each construct via nitrate reductase expression levels. Transformants harboring the wild type promoter were assigned an activity of 100.
Figure 3.2
Figure 3.3 In vivo analysis of nitrogen regulation of the nit-3 promoter mutants. Mycelia were initially grown in Vogel’s minimum medium with 20 mM glutamate as nitrogen source for 12 to 16 hr, then transferred to media containing 40 mM KNO$_3$, 40 mM KNO$_3$ and 20 mM glutamine, or 20 mM uric acid as nitrogen sources and grown for 3 hr. Nitrate reductase was analyzed and the activity (OD$_{540}$x10$^{-2}$/mg.min) of each sample is shown.
Figure 3.3
Fig. 3.4 In vivo nit-3 promoter analysis.

Diagram of nit-3 promoters showing combinations of deletions and insertions of NIT2 binding sites. All constructs were built into pDE1 and targeted to the his3 locus in RIP15A, and transformants were assayed for nitrate reductase activity. Relative activities are indicated with activity of the wild type construct set equal to 100. I, II, and III, NIT2 sites; A and B NIT4 sites.
Figure 3.4
Fig. 3.5 In vitro mobility shift assay using the NIT2 site II (5' AATTCTATGGTACGTAGATAAGTACCACCCGTG 3') and related mutant oligos and *E. coli* expressed NIT2 protein (DNA binding region).

A. The sequence of NIT2 site II and the two mutants with altered 5' and 3' flanking region.

B. DNA mobility shift experiment. 34 bp DNA oligos labeled with $^{32}$P were quantified and equal amounts of each was tested for NIT2 binding as described in Material and Methods. NIT2-DNA complex and free oligo are indicated. The percentage shift of the probe in each reaction as determined by a phosphoimager is indicated at the bottom of each lane.
A  NIT2 site II:

5' GGTACGTAAGTACTACCAC 3'

B  oligos  wild type  AGTA->CTAC  CGTA->GTAG

<table>
<thead>
<tr>
<th>NIT2</th>
<th>0</th>
<th>0.2ug</th>
<th>0.5ug</th>
<th>0</th>
<th>0.2ug</th>
<th>0.5ug</th>
<th>0</th>
<th>0.2ug</th>
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<tr>
<td>NIT2-DNA complex</td>
<td></td>
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<td>Free oligo</td>
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31% 56%  2% 4% 4% 12%

Figure 3.5
Fig. 3.6 In vivo nit-3 promoter analysis.

dl343 contains only the NIT2 site II while CTAC and GTAG contain the NIT2 site II with altered flanking sequences. Transformants for each were assayed for nitrate reductase activity with that for dl343 set at 100. Open boxes represent NIT4 binding sites; the filled box represents NIT2 site II.
Figure 3.6
Fig. 3.7 In vivo analysis of manipulated nit-3 promoters.

The two AT-rich regions are indicated in the diagram. Schematic drawings show the deletion and insertion of AT-rich regions in the nit-3 promoter in different constructs. Each construct was transformed into the nit-3 his-3 host strain and assayed for nitrate reductase activity. Activity for the transformants with wild type construct was set at 100. dl= deleted segment.
Figure 3.7
Fig. 3.8 Functional analysis of TACC repeats.

A. In vivo analysis of TACC repeated sequence. The TACC deletion mutant was generated and nitrate reductase of multiple transformants was examined. The relative enzyme activity was calculated using wild type construct as 100.

B. RNA blot analysis of nit-3 transcript (3.1 kb) (wild type and TACC mutant) and β-tubulin (2.0 kb) control was carried out as described in Materials and Methods.
Figure 3.8

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Chapter 4

The expression and the stability of *nit*-2 mRNA and protein

4.1 Introduction

In *Neurospora crassa*, transcription factor NIT2 regulates the expression of many genes that are related to nitrogen metabolism. Besides nitrate and nitrite reductases, its targets include L-amino acid oxidase, phenylalanine-ammonia lyase, general amino acid transporters, purine transporter and catabolic enzymes, and even an extracellular protease (Marzluf, 1997). Together with a negative regulator, NIT2 mediates the repression of its target genes when the primary nitrogen sources, i.e. glutamine, glutamate and ammonia, are available. Overriding nitrogen repression requires not only the presence of an inducer or substrate of a certain pathway but also the absence of primary nitrogen sources, suggesting that NIT2 acts in a higher hierarchical level of the nitrogen regulation network.

NIT2 is a DNA binding protein and contains a Cys$_2$/Cys$_2$ zinc finger with a 17-amino acid central loop and an adjacent basic region as its DNA binding domain (Fu and Marzluf, 1987). It belongs to the GATA transcription factor family which is named after its trademark binding sequence GATA. The ever increasing number of GATA family members found in vertebrates, Drosophila, plants and fungi and their diverse functions suggests an important role of this family in global transcriptional regulation. Besides NIT2, 4 additional GATA factors have been cloned in *N. crassa*, including WC1 and WC2 which are involved in light regulation, SRE which is involved in iron regulation, and NGF-
1 with an unknown function (Ballario et al., 1996; Feng and Marzluf, unpublished data; Zhou et al., 1998).

Analysis of the nit-2 transcript indicates that the nit-2 gene is constitutively expressed in N. crassa using either primary or secondary nitrogen sources, however at a higher level when only secondary nitrogen sources are provided. This indicates that the expression of nit-2 mRNA may be subject to nitrogen control (Fu and Marzluf, 1987). No information of the protein expression level of NIT2 is available.

In Aspergillus nidulans, the nit-2 homologue, areA, has also been isolated. It encodes a protein of 876 amino acid residues with an overall 42% homology to the NIT2 protein. Within the 50-amino acid DNA binding region, the homology between NIT2 and AREA is almost 100%. Not surprisingly, the Neurospora nit-2 gene has been shown to complement Aspergillus areA mutants (Davis and Hynes, 1987). Like its counterpart, AREA is also a global positive regulator that mediates the expression of many genes that encode nitrogen catabolic enzymes (Crawford and Arst, 1993). The absence of primary nitrogen sources (ammonium and L-glutamine) and the presence of secondary nitrogen sources are both needed to lift the nitrogen repression modulated by AREA and NMR-1. Selective expression upon demand of these enzymes gives A. nidulans the versatility to use a wide variety of nitrogen sources. Like other members of the GATA family, AREA recognizes GATA core elements in DNA. GATA sequence have been found in the promoter region of many AREA-controlled genes (Wilson and Arst, 1998). 10 copies of the GATA element are found in the intergenic control region between niiA gene (nitrite reductase) and nia D gene (nitrate reductase), which are coregulated by AREA. Mutation of the four GATA elements that are located in the middle of the control region results in loss of the repression/derepression control (Punt et al., 1995).
The expression of *areA* gene has also been studied. Under nitrogen-starved conditions, 3 *areA* transcripts with sizes of 3.9 kb, 3.6 kb, and 3.2 kb are found. When ammonium is provided, the level of all 3 species is greatly reduced (Langdon et al., 1995). 13 copies of the GATA element are found in the promoter region of *areA* gene. A point mutation changing the GATA nearest to the transcription start site for the 3.2 kb transcript eliminates that transcript completely, providing evidence for a potential autoregulation of the *areA* gene (Langdon et al., 1995). The stability of *areA* transcripts is also regulated. At the 3' untranslated region, 2 copies of a 28-nucleotide direct repeat sequence with 6-nucleotide overlap were found to be important for the half life of *areA* mRNA. Deletion of the 2 repeat sequences led to a derepression phenotype for many nitrogen related genes. Further studies showed that the half life of this mutant *areA* mRNA was altered significantly under different growth conditions. Under nitrogen repression and nitrogen-starved conditions, the half life of the mutant *areA* mRNA is 25 min and 22 min, while the wild type *areA* mRNA is 7 min and 40 min, respectively. The prolonged half life of mutant *areA* mRNA under nitrogen repression conditions appears to be the basis for the derepressed phenotype (Platt et al., 1996).

Due to the close relation of the two fungal species and the high homology between NIT2 and AREA, the nitrogen regulation systems in *N. crassa* and in *A. nidulans* have been compared extensively. It is of considerable interest to know whether or not *nit-2* and *areA* themselves share similar regulatory mechanisms to modulate their expression. In this report, I show that despite all the sequence and functional similarities found between the two genes, the expression and regulation of the *nit-2* gene is quite different from that of the *areA* gene.
4.2 Materials and Methods

4.2.1 *N. crassa* and *E. coli* strains

The *N. crassa* wild type strain 74-OR23-1A and the Ssu-1 strain were obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, Mo. The *nit-2* nonsense mutant (allele KGP0220) and the suppressed nonsense mutant Ssu-1 *nit-2* (allele KGP0220) were isolated in this laboratory (Perrine and Marzluf, 1986). Mycelia were cultured in Vogel's liquid medium (Davis and deSerres, 1970) with shaking. The amount of glutamine and KNO₃ supplement is indicated for each experiment.

4.2.2 RNA preparation and Northern analysis

Isolation of total RNA from *N. crassa* was done as described by Weaver et al. (1997) with modifications. Mycelia were ground to a fine powder with a mortar and pestle in the presence of liquid nitrogen and suspended in lysis buffer (50 mM NaOAc, pH 5.3; 10 mM EDTA; 1% SDS) at a 1 g/5 ml ratio. An equal volume of acidic phenol/chloroform (equilibrated with the lysis buffer without SDS, pH 5.3, prewarmed at 65°C) was added to the lysate, and the reaction mixture was incubated at 65°C for 30 min with shaking. The aqueous phase was recovered by centrifugation and this process was repeated several times until no protein interface could be seen. Total RNA was precipitated with 0.6 volume of isopropyl alcohol followed by centrifugation. The RNA samples were fractionated in agarose gels and transferred to nitrocellulose filters.

*nit-2* cDNA and *β-tubulin* cDNA were labeled by random primer labeling using ³²P-labeled dATP as probes for Northern analysis (GIBCOBRL). Hybridization was carried out at 65°C in 1x hybridization buffer (0.5 M NaCl; 0.1 M NaPO₄, pH 7.0; 6 mM
EDTA; 1% SDS). The membranes were washed at 65°C for 60 min with 1/4x hybridization buffer. Messages of nit-2 and β-tubulin were identified by their size.

4.2.3 Partial purification of rabbit anti-NIT2 polyclonal antibodies

Antiserum from rabbits that had been immunized with a truncated NIT2 protein was available in the laboratory. However, it gave high background and appeared to react with many different Neurospora proteins. In order to reduce the background, Neurospora cell lysate lacking NIT2 protein was used to adsorb the crossreacting species from the antiserum. nit-2 RIP mutant strain was cultured in Vogel's complete medium over night and mycelia were used to prepare a whole cell extract. SDS was introduced to the cell lysate to a final concentration of 1%, and the cell lysate was denatured at 100°C for 10 min. The denatured cell lysate was immobilized on a nitrocellulose filter by directly loading it on the filter. BSA (2%) in TBST was used to block the filter for one hour. The filter was incubated with the NIT2 antibody for one hour at room temperature, then removed. The procedure was repeated for several times with fresh filters. A Western blot analysis was used to test the quality of the treated NIT2 antibody.

4.2.4 Western analysis

Mycelia were homogenized using a mortar and pestle in the presence of liquid nitrogen and suspended in 2 ml ice-cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.2% sodium azide, 100 μg/ml PMSF, 1% Triton X-100, 2 mM EDTA). Cell debris was removed by centrifugation at 4°C for 5 min at full speed using a microfuge and the supernatant was subjected to Western analysis. The protein concentration of the cell lysate was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, CA). An equal amount of protein for each sample was run on 10% SDS-polyacrylamide gels and
transferred to a nitrocellulose filter by electroblotting in glycine buffer (25 mM Tris-HCl, pH 8.3; 192 mM glycine; 20% methanol) as described (Harlow et al., 1986). After blotting, the filter was briefly rinsed with TBST (25 mM Tris-HCl, pH 8.0; 125 mM NaCl; 0.05% Tween 20) and was blocked by TBST containing 4% non-fat milk for 1 hr at room temperature, or at 4°C over night. The filter was then incubated with rabbit anti-NIT2 antibodies for 1 hr with shaking at room temperature. The filter was washed with TBST then incubated with horseradish peroxidase-conjugated secondary antibodies (anti-immunoglobulin) (1:7000 dilution, in blocking buffer) for 45 min. After further washing, the antigen-antibody complex on the nitrocellulose filter was detected by the \textit{in situ} chemiluminescence reaction (ECL system, Amersham) and the result was visualized on X-ray film.

4.2.5 Immunoprecipitation

The immunoprecipitation experiment was done as described (Harlow et al., 1986; Lee et al., 1987) with minor modifications. Cell lysate was precleared twice with formalin-fixed \textit{Staphylococcus aureus} cells (Zymed Lab. Inc.) by incubating at 4°C for 15 min each time with shaking. 10 μl of rabbit polyclonal antibodies against the NIT2 protein was added to the cell lysate and the mixture was incubated on ice for one hour. Protein A conjugated Sepharose beads were added to the mixture and incubated at 4°C for one hour with shaking. After the incubation, the Sepharose beads were collected by brief centrifugation in a microfuge and subject to an extensive wash using ice cold buffer (250 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.1 mM PMSF, 5 mM EDTA). Bead-bound proteins were examined by SDS polyacrylamide gel and visualized by autoradiography.
4.2.6 Nitrate reductase assay

Mycelia were grown at 30°C in liquid Vogel's minimum medium containing 20 mM glutamine as the only nitrogen source for 12 to 16 hr, then transferred to medium containing 40 mM KNO₃ for 3 to 4 hr to allow expression of nit-3 gene. Mycelia were harvested by filtering through miracloth and were lysed in lysate buffer (0.1 M KPO₄ pH 6.8; 1 mM β-mercaptoethanol; 0.5 mM EDTA; 1% NaCl) using a Mini-beadbeater BX-4 (Biospec Products). Protein concentrations were determined with the Bio-Rad protein assay. Nitrate reductase enzyme assays were performed as described before with a few modifications (Garrett and Cove, 1967). Cell lysate (100 μl) was mixed with 400 μl of buffer (22 mM KPO₄, pH 7.75; 22 mM NaNO₃; 5.5 mM Na₂SO₃; 0.11 mM FAD) and incubated at 30°C for 30 min. 50 μl NADPH (0.2 mM) was added to initiate the reaction and the mixture was incubated at 30°C for 15 to 30 min depending on the amount of activity. 0.1 ml of stop solution (25% Barium acetate) was added and the precipitate was removed by centrifuging in a microfuge at full speed for 1 min. For the zero-time control, the stop solution was added to the mixture without incubation. 0.5 ml of the supernatant of each reaction mixture was mixed with 2.5 ml 0.2% sulfanilamide (diluted from 1% stock) in 20% v/v HCl; and 0.026% wt/vol naphthylthylene-diamine-dihydrochloride. The absorbance of these samples was measured at 540 nm with a spectrophotometer.

4.3 Results

4.3.1 Analysis of the stability of nit-2 transcript under different growth conditions

Many regulatory genes are themselves subject to regulation by other factors and at many different levels. The nit-2 transcript was found to be present under both nitrogen repression and derepression conditions, but at a higher level in the later situations (Fu and
Marzluf, 1987). In order to study the stability of the nit-2 transcript using different nitrogen sources, proflavin was used as the transcription blocker (Cybis and Weglenski, 1972). The efficacy of proflavin was examined using nitrate reductase as the reporter gene. 74A mycelia were grown using glutamine (20 mM) as the sole nitrogen source, then shifted to medium with only KNO3 (40 mM) to achieve the full scale induction. Different amounts of proflavin were added to parallel cultures at 0 hr during the nitrate reductase induction test, and mycelia samples were harvested at different time points to examine the enzyme activity. As shown in Figure 4.1, at 30 μg/ml, proflavin did not block the production of nitrate reductase, and at a concentration of 60 μg/ml, the production of nitrate reductase was reduced by 70%. At a concentration of 75 μg/ml or greater, a 100% block of the enzyme production was achieved. For the following experiments, 100 μg/ml of proflavin was used to inhibit transcription.

The stability of nit-2 transcript was examined under conditions using either glutamine (Nitrogen repression) or KNO3 (N-derepression). 74A mycelia were grown in Vogel's minimum medium supplemented with either 20 mM glutamine or 40 mM KNO3 overnight to allow the amount of nit-2 mRNA to reach a steady level. Proflavin was introduced to the cultures to block any further transcription of the nit-2 gene. Mycelia were harvested at different time points and total RNA was prepared as described in Methods and Materials. Northern analysis using nit-2 DNA as probe detected two species of nit-2 transcripts with the size of 3.3 kb and 3.5 kb, suggesting that there may be two active transcription start sites. The nit-2 transcript is very stable under both nitrogen derepression and nitrogen repression conditions. During the 90 min test period, the nit-2 transcript level showed almost no change at all, suggesting that the nit-2 transcripts are stable and its half life is not affected by the nitrogen sources used (Fig. 4.2).
4.3.2 Detection of NIT2 protein in *Neurospora crassa*

Although it has been established that *nit-2* is constitutively transcribed under both nitrogen-limited and nitrogen-sufficient conditions, it is not known whether or not NIT2 protein is present in both situations. By using a specific immuno-absorption method, rabbit polyclonal antibodies were partially purified to increase their specificity to detect the NIT2 protein, as described in Materials and Methods. Wild type (74A) mycelia were grown in nitrogen-free medium supplemented with 20 mM glutamine or 40 mM KNO₃. The *nit-2 RIP* mutant was grown in Vogel's complete medium as a negative control. Whole cell lysates were prepared from these cultures and the NIT2 protein was immunoprecipitated as described in Materials and Methods. After gel electrophoresis of the resuspended precipitates, the same rabbit polyclonal antibodies were used in western analysis to detect NIT2. As shown in Figure 4.3, the NIT2 protein is not expressed in *nit-2 RIP* mutant as anticipated (lane 1); NIT2 is present in wild type *Neurospora* cells using both primary (glutamine) and secondary nitrogen (KNO₃) sources (lanes 2 and 3). Correlated with the elevated *nit-2* mRNA level in cells using KNO₃, the NIT2 protein was also accumulated to a higher level under this derepression condition. The size of the NIT2 protein is noticeably larger (about 150 kDa) than its calculated molecular mass (124 kDa), suggesting a possible post-translational modification.

The expression of NIT2 protein in a *nit-2* nonsense mutant (allele KGP0220) and the suppressed-nonsense revertant Ssu-1 *nit-2* (KGP0220) was also examined. No full length NIT2 protein is detected in the *nit-2* nonsense mutant that can not utilize the secondary nitrogen sources (Figure 4.3, lane 4), although the possible presence of a truncated NIT2 protein can not be ruled out. More interestingly, the full-length NIT2 protein was detected in the suppressed mutant with restored NIT2 activity (lane 5), and a higher level of expression is achieved when a secondary nitrogen source is used (lane 6).
The complete lack of this protein band in the nonsense mutant and its restoration in the suppressed mutant strongly confirms its identity as the NIT2 protein.

4.3.3 Stability of NIT2 protein under different growth conditions

In order to obtain a complete picture of the regulation involved in the expression of NIT2 protein, it is essential to know any possible regulation at the protein level. I started to address this question by analysis of the stability of the NIT2 protein. Cycloheximide (2x10^{-4} M) was used to inhibit protein synthesis and nitrate reductase was employed as the control to monitor its efficacy. At 2x10^{-4} M, cycloheximide achieves a complete block of the synthesis of nitrate reductase (data not shown). Wild type mycelia were grown overnight in nitrogen-free Vogel's minimum medium supplemented with either glutamine (20 mM) or KNO_3 (40 mM) to allow the NIT2 protein to accumulate to a steady level. Then cycloheximide was introduced to stop protein synthesis. During the 3 hr test period, mycelia samples were harvested at different time points and were used to prepare whole cell lysates as described in Materials and Methods. NIT2 protein was examined by western analysis using the cleaned rabbit anti-NIT2 polyclonal antibodies. As anticipated, the NIT2 protein appears to be very stable under nitrogen derepression conditions, the situation where it functions to turn on downstream genes, and no change in the NIT2 protein level was detected during the three hour period examined (Figure 4.4A). Significantly, the NIT2 protein also appears to be very stable under N-repressed conditions (Figure 4.4B). These results indicate that the NIT2 protein has a very long half life in vivo and that its stability is not affected by different nitrogen sources, including those that lead to N-repression or N-derepression.
4.4 Discussion

Nitrogen metabolism is a highly regulated process in fungi like *Neurospora crassa* and *Aspergillus nidulans*. In *Neurospora*, one of the best-studied pathways is the nitrate assimilation pathway, which includes structural genes *nit-3* (nitrate reductase) and *nit-6* (nitrite reductase). Two positive regulators, NIT2 and NIT4, and one negative regulator, NMR (nitrogen metabolite repression), are required to modulate the proper regulation of this pathway. Under nitrogen repression conditions, NMR is the key regulator that prevents the expression of both *nit-3* and *nit-6*. NMR has no DNA-binding ability and appears to act by directly binding to NIT2 and inhibiting the latter's positive action (Xiao et al., 1995). Glutamine or its derivative appear to be important as the cellular nitrogen-repression signal (Marzluf, 1997). NMR and/or NIT2, or other unknown factor(s) may bind to the signaling molecule and exert repression by inhibiting the positive action of NIT2. Recent studies showed that NIT2 directly interacts with NMR through two different regions, the zinc finger and the 12 amino acid residues at the C-terminal end (Xiao et al., 1995). Mutations that abolish the NIT2-NMR interaction lead to the nitrogen derepression phenotype (Pan et al., 1997). This result convincingly demonstrates that both NIT2 and NMR are involved in nitrogen metabolite repression and that an interaction of these two proteins plays a central role in establishing repression.

Under derepression conditions, NIT2 and NIT4 are both needed to turn on *nit-3* and *nit-6* genes. Mutants of either *nit-2* or *nit-4* completely lack nitrate and nitrite reductases and are unable to use NO$_3^-$ as nitrogen source (Marzluf, 1997). Very recent studies showed that NIT2 directly interacts with the pathway-specific regulator NIT4 and that the two synergistically activate the transcription of target genes (Feng and Marzluf, 1998). The critical function of NIT2 in the nitrogen metabolite derepression process suggests that NIT2 protein may be present in cells constantly, regardless of the nitrogen
sources provided. Another line of evidence indicating that NIT2 is constitutively expressed is the quick response of synthesis of nitrate reductase to inducer NO₃⁻. Nitrate reductase mRNA can be detected as rapidly as a few minutes after induction and reaches a steady level in 15 minutes (Okamoto et al., 1993). The enzyme can be detected 10 min after induction (Tao and Marzluf, unpublished data). This suggests that the NIT2 protein is present at considerable levels even in cells supplied with abundant favored nitrogen sources, and thus is able to function immediately upon N-derepression.

Studies conducted in Aspergillus nidulans gave somewhat different results. AREA mutants with a truncated C-terminus (the very last 12 amino acid residues) also showed a derepressed phenotype for the nitrate assimilation pathway, suggesting that the AREA protein, the NIT2 counterpart, works in a similar fashion during nitrogen metabolite repression as does NIT2 (Platt et al., 1996). It is well documented that both AREA and NIRA (the counterpart of NIT4 in A. nidulans) proteins are required for derepression of nitrate assimilation genes (Punt et al., 1995). The induction of the nitrate reductase in Aspergillus nidulans is also a very rapid process, with only 7 to 8 min lag before enzyme begins to appear, suggesting the de novo synthesis of the AREA protein is not needed prior to the synthesis of nitrate reductase (Cove, 1966). These similarities suggest that the AREA protein should also be present constitutively to exert its control over the nitrogen repression/derepression process, as its counterpart NIT2 does in Neurospora.

Intriguingly, the areA gene was shown to be expressed at an extremely low level under nitrogen repression conditions (10 mM ammonium) (Langdon et al., 1995). The stability of the areA transcript is affected by the availability of nitrogen sources. In the presence of 40 mM ammonium, the areA transcripts have a half life as short as 7 min. The instability of areA mRNA is partly due to the 2 copies of a repeated sequence located in its 3' untranslated region. Removal of the two repeats leads to a prolonged half life (25 min) and a parallel derepressed phenotype (Platt et al., 1996). These results suggest that areA
transcripts are absent or at extremely low levels during nitrogen repression, due to the combined regulatory mechanisms that act at the transcriptional and post-transcriptional level. So far no information is available about the cellular AREA protein levels. It will be most interesting to see how the AREA protein behaves under both repression and derepression situations.

My results about *nit-2* expression and its regulation are consistent with previous observations made in *Neurospora*. I found that the *nit-2* gene is constitutively expressed. Both *nit-2* mRNA and NIT2 protein are present at a relatively high level even under nitrogen repression conditions, and are further increased several fold under nitrogen derepression conditions. The *nit-2* mRNA lacks the repeated sequence at its 3' untranslated region as found in the *areA* mRNA. A *nit-2* gene deleted for almost the entire 3' utr maintains nitrogen regulation as found in the wild type strain (Feng and Marzluf, unpublished data). Both *nit-2* mRNA and NIT2 protein are very stable under both nitrogen repression and nitrogen derepression situations. Their half life is not affected by the nitrogen sources. The lack of substantial regulation at both transcriptional and post-transcriptional levels suggests that direct protein-protein binding, such as the NIT2-NMR and NIT2-NIT4 interactions, may play a pivotal role in regulating NIT2 function in response to an ever changing environment.
Figure 4.1 Efficacy of proflavin.

The efficacy of proflavin to block transcription was examined using nitrate reductase as a reporter. Different concentrations of proflavin (0 to 105 µg/ml, as indicated on the right hand side of the graph) were tested during a one hour period.
Figure 4.1
Figure 4.2  RNA blot analysis of nit-2 transcripts under different culture conditions.

The stability of nit-2 transcript was analyzed in cells grown in Vogel's minimum medium supplemented with either 20 mM glutamine or 40 mM KNO₃. Proflavin (100 μg/ml) was used to block RNA synthesis and mycelia samples were harvested at different times after the introduction of the drug. Two nit-2 mRNAs with estimated sizes of 3.5 kb and 3.3 kb were detected, as indicated by two arrows. β-tubulin transcript was used as the internal control.
Figure 4.3 Immunoprecipitation of NIT2 protein.

The nit-2 RIP mutant (NIT2') which is used as a negative control, and nit-2 nonsense mutant (allele KGP0220) were grown in Vogel's complete medium. Wild type strain 74A and the suppressed nonsense mutant (Ssu-1 nit-2 (KGP0220)) were grown in Vogel's minimum medium with different nitrogen sources, as indicated. After immunoprecipitation, each sample was resuspended, subjected to gel electrophoresis, and examined by a western blot as described in Materials and Methods. Lane 1, the nit-2 RIP mutant; lane 2, 74A cultured with 20 mM glutamine; lane 3, 74A cultured with 40 mM KNO3; lane 4, nit-2 nonsense mutant (allele KGP0220); lane 5, the suppressed nonsense mutant (Ssu-1 nit-2 (KGP0220)) cultured with 20 mM glutamine; lane 6, the suppressed nonsense mutant (Ssu-1 nit-2 (KGP0220)) cultured with 40 mM KNO3.
Figure 4.3
Figure 4.4 Analysis of the stability of NIT2 protein under different culture conditions.

The presence of NIT2 protein was followed after introducing $2 \times 10^{-4}$ M cycloheximide to cells in medium containing either 20 mM glutamine (A) or 40 mM KNO$_3$ (B) as nitrogen sources. Samples were harvested at different time points after the addition of cycloheximide. The NIT2 protein was detected by western analysis.
Figure 4.4

A

B

97 kD  200 kD

I 2 3 4 5 6

0 hr 0.5 hr 1 hr 1.5 hr 2 hr 3 hr

97 kD  200 kD

I 2 3 4 5 6

0 hr 0.5 hr 1 hr 1.5 hr 2 hr 3 hr
Chapter 5

General Discussion

As the key regulator for the sulfur regulatory circuit, the CYS3 protein governs the expression of an entire set of sulfur catabolic enzymes. Under sulfur-rich conditions, the absence of CYS3 protein leads to the silencing of the genes which encode the enzymes in the sulfur circuit. Upon sulfur depletion, CYS3 needs to be synthesized prior to the expression of the circuit. My research showed that this is a relatively slow process. The detection of cys-3 transcripts takes about two hours after Neurospora is subject to sulfur derepression and the appearance of the CYS3 protein takes even longer, about three to four hours. The appearance of products of the downstream structural genes has a longer delay of about five to six hours, as represented by arylsulfatase. The lag period between sulfur derepression and the appearance of cys-3 transcripts is presumably due to a large internal pool of sulfur compounds, which must be depleted before derepression actually occurs. The CYS3 protein is very stable under derepression conditions and has a half life about 4 hr. When the sulfur supply becomes abundant again, both the CYS3 protein and the sulfur catabolic enzymes are no longer needed. The rapid elimination of CYS3 ensures the shut down of the entire sulfur circuit. In comparison to the slow induction process, my work showed that establishment of sulfur repression is a relatively rapid process. The cys-3 transcripts are gone in 20 min with an estimated half life of about 5 min, while the CYS3 protein disappears within one hour with a half life of 10 min. The dramatic change of
CYS3 stability suggests that CYS3 is actively degraded during sulfur repression conditions. The regulated synthesis and degradation of the CYS3 protein makes it possible to fine tune the regulation of sulfur circuit and to quickly respond to the ever changing environment.

By changing one lysine residue (Lys105) to glutamine, the half life of CYS3 is prolonged to 40 min, as observed in a revertant of a cys-3 mutant (Rev 27). This result suggests that ubiquitin-mediated protein degradation with Lys105 as a potential target site may be involved in the CYS3 elimination process. Independent research done by Kumar and Paietta (1998) provides strong evidence supporting this hypothesis from a very different angle. One of the negative regulators of the sulfur circuit, scon-2 (sulfur controller 2), has been cloned. The expression of scon-2 is positively regulated by CYS3 and is limited to low sulfur conditions (Kumar and Paietta, 1995). Its lack of a DNA-binding domain and the parallel expression pattern suggest that SCON2 may exert its regulatory function on the sulfur circuit through a direct interaction with CYS3. Just such a pattern has been shown in yeast Saccharomyces cerevisiae, in which Met4p and Met30p, the yeast homologues of CYS3 and SCON2, interact directly with each other (Thomas and Surdin-Kerjan, 1995). An F-box, a novel protein motif involved in ubiquitin-mediated proteolysis, has been identified in the SCON2 protein, which provides a helpful hint for the possible function of SCON2. Point mutations changing some of the conserved amino acids in the F-box of SCON2 lead to a sulfur auxotroph phenotype, i.e. when transformed into a scon-2 mutant, transformants have a phenotype similar to cys-3 null mutants and are unable to grow without methionine supplement. Northern analysis revealed that like the cys-3 null mutant, no cys-3 transcript was detected in these scon-2 point mutants. A model was proposed based on these observations, featuring SCON2 as a potential E3 of the ubiquitin-protein ligase complex that degrades CYS3 protein. While wild type SCON2 protein may negatively regulate the sulfur circuit via the physical elimination of CYS3.
under high sulfur conditions, it does not target CYS3 for degradation under low sulfur conditions. In contrast, those scon-2 point mutants described above may have a deregulated activity and eliminate CYS3 with or without a high sulfur signal, which results in a phenotype similar to a cys-3 null mutant (Kumar and Paietta, 1998).

The precisely regulated expression of the sulfur circuit in response to the environmental signals provides a good example for a mechanism of operation of a complex genetic network that contains multiple regulatory and structural genes. The expression and elimination of CYS3 as regulated by sulfur signals, illustrates how regulation at both transcriptional and post-transcriptional levels can be coordinated to allow cells to control an entire area of metabolism.

Like the sulfur control circuit, the nitrogen regulatory circuit involves another intricate network with multiple regulatory genes and many structural genes that encode nitrogen catabolic enzymes of different nitrogen metabolic pathways. In this circuit, the nitrate assimilation pathway which contains nitrate reductase (nit-3) and nitrite reductase (nit-6), is excellent example for the coordinated regulation of gene expression. NIT2, a global positive regulator that controls up to 100 genes related to the nitrogen metabolism, NIT4, a pathway specific positive regulator restricted to the nitrate assimilation pathway, and NMR, a global negative regulator, are all involved in modulating the expression of nitrate assimilation pathway.

Since NIT2 plays such an important role in nitrogen regulation, much effort has been devoted to address its function. As a member of the GATA transcription factor family, NIT2 binds to DNA elements with a consensus GATA core sequence. One concern for an enhancer with such a short core element is that statistically the GATA sequence can appear approximately every 256 bp throughout the entire genome. How NIT2 and other GATA factors select and bind to the right binding site(s) from such a large pool of GATA sequences is certainly an interesting question. Moreover, a further
complexity is the fact that multiple GATA factors are found in many organisms, ranging from fungi to vertebrates, that all recognize the GATA sequence (Orkin, 1992). At least six GATA factors are found in vertebrates. The expression pattern of these proteins overlaps, although they do have a different tissue specificity and developmental profile (Merika and Orkin, 1993). In *Neurospora*, besides NIT2, four other GATA factors have been cloned, and apparently all coexist in the vegetative cells. Of these four genes, WC1 and WC2 (white color 1 and 2) are involved in blue light regulation, SRE (siderophore regulator) is a negative regulator for iron intake and metabolism, and the function of NGF-1 (new GATA factor 1) is yet unknown (Ballario et al., 1996; Zhou and Marzluf, 1998; Feng and Marzluf, unpublished data). How each of the 5 *Neurospora* GATA factors distinguishes itself from the others in recognition of the binding sites of its target genes is a challenging question. A very recent study that demonstrates a NIT2-NIT4 protein-protein interaction synergistically activates the transcription of the *nit-3* gene provides one mechanism that can partially answer the question. Of the five known *Neurospora* GATA factors, only NIT2 can interact with the NIT4 protein, suggesting that the pathway specific regulator may play an important role in positioning the correct GATA factor (NIT2) at its target genes via a direct protein-protein interaction (Feng and Marzluf, 1998). My own studies of the interaction of the NIT2 protein and its binding sites in *nit-3* gene also provide evidence for another mechanism of GATA factor specificity. Both the in vivo *nit-3* promoter analysis and the in vitro mobility shift analysis showed that besides the GATA core element, both its 5' and 3' flanking sequences are very important for optimizing the protein-DNA interaction. In the *nit-3* promoter, the second NIT2 binding site (NIT2 site II) with only one copy of GATA sequence is by far the most influential one. Changing the flanking sequence on either side (four nucleotides at a time) of this NIT2 site greatly reduced the strength of its interaction with NIT2, which translated into a 90% loss of the *nit-3* promoter activity. This result suggests that although the core GATA element is essential for DNA
binding by all the GATA factors, the flanking sequences may provide added specificity to distinguish between them. One experiment to test this hypothesis would be to introduce the NIT2 site II into a different promoter, such as the promoter of allantoase, which only weakly responds to NIT2 regulation. Based on this hypothesis, the expression of the modified allantoase gene should respond strongly to NIT2.

The regulation of \textit{nir}-2 itself at both transcriptional and post-transcriptional levels is another interesting topic. In \textit{Aspergillus nidulans}, the \textit{areA} gene, which is the counterpart of \textit{nir}-2, is found to be regulated at both levels by the nitrogen sources present in the medium. Growth on a preferred nitrogen source such as ammonia leads to an extremely low level of transcription of \textit{areA} gene as well as a reduced half life of \textit{areA} transcripts (Langdon et al., 1995; Platt et al., 1996). Although no information is available about the \textit{ARE} protein, it is very likely that the \textit{ARE} protein is not constantly present when ammonia is used due to the lack of the \textit{areA} mRNA. Despite their high protein sequence homology and functional similarities, my work showed that the expression and regulation of \textit{nir}-2 is very different from \textit{areA}. It is known that \textit{nir}-2 is constitutively expressed and that its expression level can be elevated several fold when using a secondary nitrogen source (Fu and Marzluf, 1987). My work further showed that the \textit{nir}-2 mRNA is very stable and its stability is not affected by the status of the nitrogen sources used. Two \textit{nir}-2 transcripts with estimated size of 3.5 kb and 3.3 kb were detected while using both primary and secondary nitrogen sources, suggesting there may be two active transcription start sites. It seems that the smaller transcript is present at a slightly higher level than the larger one when glutamine was used as the nitrogen source. However when NO$_3^-$ was used, both transcripts are present at equal abundance and display a similar stability. In accordance with the constitutive transcription, the NIT2 protein is present constantly at a fairly high level regardless of the nitrogen sources provided. The NIT2 protein level is also elevated accordingly as the \textit{nir}-2 mRNA level increases when only a secondary nitrogen
source is available. The NIT2 protein is very stable and its stability is not affected by the nitrogen sources used. These results are consistent with the observations I made about the production of nitrate reductase during the NO$_3^-$ induction. The rapid appearance of nitrate reductase during induction with only about 10 min lag between the introduction of NO$_3^-$ and the appearance of nitrate reductase suggested that no NIT2 synthesis is needed prior to the production of nitrate reductase.

The constant presence of NIT2 even under the nitrogen repression conditions presents a challenging situation since a mechanism is needed to keep the NIT2 inactive during nitrogen repression. There are several possible mechanisms that could achieve this goal. First, NIT2 transactivation activity may be modulated by a post-translational modification, such as phosphorylation/dephosphorylation, which is widely used in higher organisms to achieve a fast and accurate regulation. The molecular weight of NIT2 appears significantly larger than the calculated one, suggesting the possibility that NIT2 is modified, e.g. by phosphorylation. The phosphorylation status of NIT2 could be rapidly changed according to the nitrogen repression/derepression signal, thereby modulating NIT2 activity. To test this hypothesis, in vivo protein labeling using 32-P inorganic phosphate followed by immunoprecipitation using anti-NIT2 antibody can directly test whether NIT2 protein indeed is a phosphoprotein. By examining the phosphorylation status of NIT2 under nitrogen repression and nitrogen derepression conditions, the biological function of possible NIT2 phosphorylation can be addressed.

Secondly, NIT2 can be inactivated by a direct protein-protein interaction with the negative regulator NMR. No DNA binding domain has been found in NMR, therefore it is possible that NMR exerts its negative regulatory function to its target genes via a direct interaction with NIT2. A direct NIT2-NMR interaction was first demonstrated by Xiao et al. (1995). The interaction domain on NIT2 maps to two regions, one is a short α-helical motif within the NIT2 DNA binding domain, the other is at the C-terminus. A mutagenesis
study showed that removing the last 12 amino acid residues of NIT2 abolishes the NIT2-NMR interaction as well as the nitrogen repression (Pan et al., 1997). This result provides compelling evidence portraying the NIT2-NMR interaction as central to nitrogen repression. Both NMR and NIT2 are required for the process. The negative effect of the NIT2-NMR interaction may be due to the masking the NIT2 transactivation domain by NMR while the NIT2-NMR complex remains on the promoter. Alternatively, NMR may prevent NIT2 from binding its target promoters and thereby block the transactivation function of NIT2.

Thirdly, glutamine or its derivatives, which serve as the signal compounds for nitrogen repression, may associate directly with NIT2, NMR, a NIT2-NMR complex or other protein(s) and cause a conformation change in NIT2. This altered conformation may change the NIT2 activation domain, or weaken its DNA binding ability, or both, and thus inactivate the NIT2 protein for turning on of downstream genes.

To test these hypotheses, a new technique, ligation mediated PCR in vivo footprinting, can be used to obtain a clear view about how NIT2 functions under physiological conditions when different nitrogen sources are provided. By using DMS (dimethyl sulfate), an alkylating agent which can freely penetrate cell membranes, nuclear DNA can be methylated at guanines in intact cells. Regions occupied by DNA binding proteins often have an altered accessibility to DMS, which yields a different guanine methylation pattern. After the in vivo DMS treatment, nuclear DNA can be purified and cleaved at the methylated guanine residues with piperidine. These cleavage products then are PCR-amplified using a sequence-specific primer at one end and a double-strand common linker that is ligated to the other end of double-strand PCR product. A 32-P labeled primer is employed after the PCR amplification process, and the radioactive signal is detected by radiography after gel electrophoresis. By comparing the in vivo DNA
footprints obtained in wild type and appropriate mutants, the binding sites of a particular regulatory protein can be visualized (Mueller and Wold, 1989).

Using the nit-3 promoter region which has two NIT2 binding sites and two NIT4 binding sites clustered together as the candidate for in vivo footprinting, a great deal of information can be learned about how NIT2 associates with DNA under nitrogen repression and nitrogen derepression conditions. Under nitrogen derepression conditions, NIT2 associates with NIT4, which may give an enlarged protected region covering both NIT2 and NIT4 binding sites. Under nitrogen repression conditions, NMR instead of NIT4 joins with NIT2. If the NIT2-NMR complex remains on DNA, the NIT2 binding sites would still be protected, in which case the NIT2-NMR interaction is likely to mask the NIT2 transactivation domain. Alternatively, if the NIT2-NMR complex can no longer bind to DNA, the NIT2 binding sites will not be protected, which would imply that the NIT2-NMR interaction causes the disassociation of NIT2 from its binding sites.

By performing this experiment in different mutant Neurospora strains instead of wild type, a great deal more can be learned about the nitrogen regulation. For example, by studying the nit-2 transformants with mutations lacking the last 12 amino acids at its C-terminus, information about how nitrogen repression mediated by NMR is established can be learned. Since NIT2 interacts with NMR in two different regions, mutation of just the C-terminus site may or may not disrupt the in vivo NIT2-NMR interaction. If the in vivo NIT2-NMR interaction is disrupted, indicated by a change in the footprinting profile, it would be convincing that the 12 C-terminus amino acids are crucial for establishing NMR-mediated nitrogen repression. This 12 amino acid domain could be added onto other transcription factors that are not involved in nitrogen regulation to see if NMR can regulate their action. If the in vivo NIT2-NMR interaction is not disrupted, indicated by the footprinting profile, the last 12 amino acids of NIT2 may allow NMR to interfere with NIT2 transactivation domain.
The presence of two different nit-2 transcripts may present another interesting twist to the regulation of nit-2 gene. Although only limited regulation is found at the transcription level of nit-2 gene, it will be important to know if the transcription level or the stability of two transcripts are differentially regulated by different nitrogen sources. In Aspergillus nidulans, there exists three areA transcripts with sizes of 3.9 kb, 3.6 kb and 3.2 kb. The smallest one showed autoregulation by the AREA protein via a GATA site near its transcription start site (Langdon et al., 1995). Several GATA sites have been found in nit-2 promoter, and it will be interesting to determine if any of them may have a dominant effect on the synthesis of either transcript.

In summary, much remains to be learned about nitrogen regulation. As a part of this never ending story, my work has shown that although both are global-acting positive regulators, the expression and regulation of CYS3 and NIT2 are very different. CYS3 is regulated heavily at both transcriptional and post-transcriptional levels, while NIT2 appears to be constantly present and its activity can rapidly be switched "on" and "off". The commonalities and differences between CYS3 and NIT2 illustrate the versatility and creativity of Mother Nature.
Bibliography


