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ISOLATION AND CHARACTERIZATION OF A NEW GENE, *SRE*, WHICH
ENCODES A GATA-TYPE REGULATORY PROTEIN THAT CONTROLS IRON
TRANSPORT 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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1998

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Developmental Biology Graduate Program

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ABSTRACT

Multiple GATA factors, regulatory proteins with consensus zinc finger motifs that bind to DNA elements containing a GATA core sequence, exist in the filamentous fungus, *Neurospora crassa*. One GATA factor, NIT2, controls nitrogen metabolism, whereas two others, WC-1 and WC-2, regulate genes responsive to blue light induction. A gene encoding a new GATA factor, named SRE, was isolated from *Neurospora* using a PCR-mediated method. Sequence analysis of the new GATA factor gene revealed an open reading frame specifying 587 amino acids, which is interrupted by two small introns. Unlike all previously known *Neurospora* GATA factors which possess a single zinc finger DNA binding motif, SRE contains two GATA-type zinc fingers. The deduced amino acid sequence of SRE shows significant similarity with URBS1 of *Ustilago* and SREP of *Penicillium*.

A loss of function mutation was created by the rip procedure. Analysis of *sre*⁺ and *sre*⁻ strains revealed that SRE acts as a negative regulator of iron uptake in *Neurospora* by controlling the synthesis of siderophores. Siderophore biosynthesis is repressed by high iron concentrations in the wild-type strain but not in *sre*⁻ mutant cells. The *sre*

promoter contains a number of GATA sequences; however, expression of *sre* mRNA occurs in a constitutive fashion and is not regulated by the concentration of iron available to the cells.

SRE was expressed in *E. coli* as a GST fusion protein. *In vitro* gel mobility shift assays of SRE GST fusion proteins revealed that SRE binds specifically to DNA fragments which contain a GATA sequence. Both zinc fingers of SRE contribute to the DNA binding. *In vivo* tests revealed that the *sre* gene mutated in either of the zinc fingers fails to function in negative regulation in the iron metabolic pathway in *Neurospora*.

To my mother, for her support without knowing
what I have been doing.

To my father, for his stubbornness but trust.

To my advisor, a real gentleman, the best educator
during my 23 years' learning experience.

To all the wonderful people I have met.

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2. Zhou, LW, Haas, H, and Marzluf, GA (1998) Isolation and characterization of a new gene, *sre*, which encodes a GATA-type regulatory protein that controls iron transport in *Neurospora crassa*. *Molecular General Genetics*. 259, 532-540.

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CHAPTER 1

INTRODUCTION

1.1. The biology of *Neurospora crassa*

Neurospora crassa, a eukaryotic organism and a member of the fungal class ascomycetes, can be found growing on plant remains in tropical areas. It also has a common name as the pink bread mold because of its contamination in bakeries. This heterotroph's growth only requires a simple carbon source, such as acetate, succinate, glycerol or glucose, a nitrogen source, such as nitrate, nitrite, ammonium or amino acids, a few simple salts, trace elements, and a single vitamin, biotin (Davis and DeSerres, 1970).

The structure and organization of the cells of *Neurospora crassa* are similar to those of other eukaryotes, e.g. the presence of the organelles, mitochondria, nuclei, and cell membranes. Different types of cells form during the *Neurospora* life cycle, including hyphal cells, aerial hyphae, microconidia, macroconidia, and ascospores. *Neurospora* normally grows as a haploid, forming multinucleate, branched filaments, or hyphae, the so-called vegetative system. The hyphae are segmented by incomplete cross-walls, which have pores that allow the cellular contents including nuclei,

organelles, and cytoplasm to migrate in the direction of growth. The hyphae are multi-nucleated. A hyphal system is also called a mycelium. Hyphal anastomosis, the fusion of hyphae, can occur within or between strains (Davis and DeSerres, 1970). The multinucleate character of *Neurospora* makes heterokaryon tests possible because nuclei with different genotypes can exist in the same cell.

As a *Neurospora* culture matures, it can produce three different types of spores, depending on the growth conditions. These different kinds of spore can be used for different purposes in genetic studies. Macroconidia are produced during the *Neurospora* asexual life cycle and have an orange color, the basis for the common name "pink bread mold". These conidia are used to inoculate *Neurospora* vegetative cultures or for sexual crossing (Davis and DeSerres, 1970). Macroconidia have also been used to make *Neurospora* competent cells, the spheroplasts, which are used for *Neurospora* transformation. Macroconidia are multinucleate, averaging 2.5 nuclei per cell. Normally, foreign DNA only transforms one of the nuclei during transformation. This phenomenon sometimes makes further analysis of the phenotype of the transformant difficult because of the presence of different types of nuclei.

The other type of asexual spore, microconidia, are formed late in growth by a process quite different from macroconidia formation, and can also be generated in the

laboratory by controlling the growth medium. Microconidia are smaller in size and have poor viability. Microconidia are almost always uninucleate, so that vegetative cultures derived from microconidia will have a single genotype (Davis and DeSerres, 1970).

When *Neurospora* grows on medium with poor nutrition either naturally or under laboratory controlled conditions, it will enter the sexual cycle. The ascospores formed from this process are dormant and can survive without germination for long periods of time, allowing *Neurospora* to tolerate severe growth conditions. The sexual cycle can also be called a cross because of the requirement for two different mating types, A and a. *Neurospora* are haploid and morphologically hermaphroditic, and a normal strain of either mating type can produce male "gametes" and female "gametes". The sexual cycle starts with gamete fusion, i.e., the male "gametes" (macroconidia or microconidia) with protoperithecia of the opposite mating type. The protoperithecium is a protective sheath, which contains female gametes--ascogonia. After several division of the parental nuclei, nuclear fusion occurs. The generated diploid nucleus quickly undergoes two rounds of meiotic divisions and one mitotic division. The resulting ascus has eight ascospores. Each of them is haploid and can give rise to a vegetative culture after heat (60°C) activation (Davis and DeSerres, 1970). The ratio and the order of the genotypes of the ascospores can be used to

calculate gene recombination and linkage, which is very useful in genetic analysis.

Two techniques which are closely dependent on the *Neurospora* life cycle have been well developed and widely used in *Neurospora* molecular biology studies: transformation and rip mutation. *Neurospora* transformation with foreign DNA is accomplished by using spheroplasts, which are made by using enzymes to partially digest the cell wall of germinated conidia. Further treatment with CaCl_2 makes the cell competent to take up foreign DNA (Case et al., 1979; Akins and Lambowitz, 1985). Direct electroporation of conidia can also achieve transformation, but the transformation efficiency will be somewhat lower (Chakraborty and Kapoor, 1990).

Like most eukaryotes, the transformed DNA is ectopically integrated into the *Neurospora* genome at random locations. Homologous recombination only occurs at a very low frequency, less than 5% of total transformants (Dhawale and Marzluf, 1985; Paiette and Marzluf, 1985). Usually, multiple copies of the DNA are integrated into the genome when the concentration of DNA used for the transformation is high. This, together with the fact that foreign DNA is randomly integrated, puts the expression of introduced genes DNA under the influence of different genetic backgrounds. Thus, results concerning the expression level of transformed genes must be interpreted with caution.

A widely used method, which places all of the foreign genes into the same genetic background, is targeted transformation. This method uses a special *N.crassa his-3* mutant strain, and a 5'-truncated *his-3* gene in the transformation vector, and with selection for *his*⁺. A single copy of the truncated *his-3* gene, together with the target gene on the same vector, is integrated into the *his-3* locus of the genome by homologous recombination. Both random transformation and targeted transformation have been used successfully in *Neurospora* molecular biology studies.

Rip mutation (Repeat induced point mutation) is another technique that has been very valuable in *Neurospora* molecular biology studies. This phenomenon occurs in the sexual life cycle of *Neurospora*, triggered by duplicated DNA sequences. The duplicated regions can be linked or unlinked. The result of this mutation is multiple G-C to A-T point mutations along the duplicated region of both copies, which are also heavily methylated. The point mutations and methylation are independent events (Selker and Ganet, 1988; Cambareri et al., 1989). Both rip and methylation can result in the inactivation of both copies of genes residing on the duplicated DNA segments. This method has been used to disrupt endogenous genes.

The *Neurospora* haploid nucleus has seven chromosomes, linkage groups I-VII, about 27Mb total DNA (Perkins and Barry, 1977; Krumlauf and Marzluf, 1979), with an estimated

number of 10,000 genes in the genome. Introns exist in *Neurospora* but both the number and length of introns are lower than in higher eukaryotes. Presently, the *Neurospora* Genome Project is underway at the University of New Mexico.

Until the whole genomic sequence is determined, researchers in *Neurospora* molecular biology use a method called sib selection to clone *Neurospora* genes. This method was first described by Akins and Lambowitz (1985). This method uses pools of plasmids carrying *Neurospora crassa* genomic DNA to transform mutant *Neurospora* spheroplasts. By complementation of the mutant phenotype, the plasmid which carries the wild type gene that corrects the mutant phenotype is identified. This method has been further modified by using cosmid libraries to introduce larger genomic DNA fragments and a dominant selection marker, the benomyl resistance gene (Vollmer and Yanofsky, 1986).

There are several important research fields in *Neurospora* molecular biology. Circadian rhythm is an exciting topic and of all the model systems that scientists are using, the *Neurospora* system is the one that has given the clearest picture so far. Several genes involved in circadian rhythm, *frq*, *white collar1* and *white collar2*, have been isolated and characterized. The white collar genes encode GATA type transcription factors. A model describing how white collar gene products regulate transcription of *frq*, plus negative autoregulation of the *frq* gene itself, was proposed to

explain the expression of FRQ in a time-of-day dependent manner (Aronson et al., 1994; Dunlap et al., 1996; Crosthwaite et al., 1997; Garceau et al., 1997; Liu et al., 1997).

Metabolism might appear to be an old research topic since Beadle and Tatum won the Nobel prize for their work in *Neurospora* forty years ago (Beadle and Tatum, 1941). But modern molecular biology, which studies the genes and their regulation of various metabolic pathways, such as nitrogen regulation and sulfur regulation in *Neurospora*, brings new life to this topic (Marzluf, 1997a; Marzluf, 1997b).

The discovery of three different forms of a subunit of G proteins and three ras genes from *Neurospora* opened research on signal transduction in *Neurospora* (Turner and Borkovich, 1993; Ito et al., 1997). Several unique topics of *Neurospora* biology such as rip mutation attract considerable attention. Insights into this unique phenomenon in *Neurospora* can give a clue to the general phenomena of gene methylation and gene silencing. Recently, a new related process called "quelling" was discovered in *Neurospora* that has raised even more interest in this research field. Quelling is a form of gene inactivation that occurs without mutation and thus is reversible (Romano and Macino, 1992; Cogoni et al., 1996; Cogoni and Macino, 1997).

1.2. Eukaryotic gene regulation and GATA factors

Eukaryotic gene regulation can occur at several different levels. Starting in the nuclei, after gene transcription, the transcript is processed and exported. Most transcripts serve as template for translation. The translated protein can undergo modification, such as phosphorylation, and can be transported to different cellular compartments or even secreted out of the cell (Lewin, 1994).

Gene transcription takes place in the cell nucleus and requires RNA polymerase and different types of transcription factors. Regulation can happen at both transcription initiation and elongation, although initiation is the more important of the two. After transcription, mRNA splicing removes the introns from the hnRNA, and a 5' cap and 3' poly(A) tail are added to the mRNA before it is exported through nuclear pores to the cytoplasm. Different types of regulatory machinery control all these processes, including mRNA transport. The mRNA has several fates after being exported to the cytoplasm. It can undergo immediate translation, but mRNAs can also be stored in the cytoplasm as a hnRNP for translation when they are needed. Moreover, mRNA localization also contributes to regulated spatial expression of some particular gene products. The processes of translation initiation, elongation and mRNA degradation are all subject to regulation (Lewin, 1994).

Among the many levels of gene regulation in eukaryotes, transcription initiation is the most important step. Control of initiation is achieved by the basal transcriptional apparatus and factors that act at upstream activation sites (Buratowski, 1994; Tjian and Maniatis, 1994). Almost all of the mRNAs in eukaryotes are transcribed by RNA polymerase II. The transcription initiation complex consists of RNA polymerase II plus basal transcriptional factors, such as TFIIA, B, C, D, E, F, H, J. They help in recruiting and phosphorylation of polymerase II, resulting in the activation of transcription (Peterson and Tjian, 1990). However, the basal transcription level will be very low without the help of upstream transcriptional factors. An optimal levels of regulated transcription require the presence of a core promoter, the basal transcription factors, upstream enhancers or silencers, the binding sites of transcriptional factors, and the presence of transcriptional factors. Moreover, other factors such as the chromosomal location of the gene, chromatin structure, and extent of DNA methylation can also influence the transcription level (Lewin, 1994).

Transcription factors, which contribute to the activation or suppression of polymerase II transcription, include several large families of DNA binding proteins, such as the zinc finger family, the leucine zipper family, and the helix-loop-helix family. Among them, the zinc finger family contributes the largest group of transcription factors.

According to the type and number of the amino acids within the fingers, transcription factors in the zinc finger family can be further divided into several subfamilies, such as Cys₂/His₂, Cys₆, and Cys₄ (Table 1). Many of these transcription factors have a DNA binding domain and one or more activation motifs, which interact with proteins of the basal transcription apparatus to activate transcription (Pabo, 1992).

GATA factors (GATA element binding proteins) refer to a family of transcription factors that recognize a consensus GATA DNA element. Members of this family share high identity within their DNA binding motif, a Cys₂/Cys₂ type zinc finger with a central loop of 17-18 amino acids (Figure 1) (Omichinski et al., 1993; Omichinski et al., 1993). DNA binding proteins of the GATA family occur in widely different organisms including mammals (Evans and Felsenfeld, 1989; Tsai et al., 1994; Trainor et al., 1990; Fujiwara et al., 1996), birds (Laverriere et al., 1994), *Xenopus* (Xu et al., 1997), plants (Chang et al., 1996), *Drosophila* (Romain et al. 1993), *C.elegans* (Speith et al., 1991), and fungi (Fu and Marzluf, 1990; Voisard et al., 1993). These GATA factors all have either one or two consensus Cys₂/Cys₂ type zinc fingers which determine their DNA binding ability.

In vertebrates, six GATA factors (GATA1 to GATA6) have been identified. Each of them contributes to different tissue specific gene expression or organ development (Tsai et

al. 1994; Ting et al., 1996; Kuo et al., 1997; Trainor et al., 1990; Fujiwara et al., 1996; Laverriere et al., 1994; Xu et al., 1997). Multiple GATA factor encoding genes have also been isolated from various fungi. Three GATA-factors, NIT2 (Fu and Marzluf, 1990), WC-1 (Ballario et al., 1996) and WC-2 (Linden and Macino, 1997), have been characterized in *N. crassa*. In *A. nidulans*, two GATA factors, AREA (Kudla et al., 1990) and NSDD (Chae et al. 1995), and in *P. chrysogenum*, three GATA factors, NRE (Haas and Marzluf, 1995), SREP (Haas et al., 1997a) and NREB (Haas et al. 1997b), have been identified. At least six GATA factor encoding genes- *GLN3* (Minehart and Magasanik, 1991), *DAL80* (Cunningham and Cooper, 1991), *NIL1* (Stanbrough et al. 1995), *GZF3* (Soussi-Boudekou et al. 1997), and *ASH1* (Sil and Herskowitz, 1996)-exist in the yeast *Saccharomyces cerevisiae*. On one hand, different GATA factors appear to control distinct areas of cellular function. NIT2 is the master regulator of nitrogen metabolism (Fu and Marzluf, 1990), while WC-1 and WC-2 determine blue light responses and circadian rhythms of *N. crassa* (Crosthwaite et al., 1997). On the other hand, different GATA factors can also be involved in the same area of cell metabolism, e.g. Gln3p, Dal80p, Nil1p, and Gzf3p are all involved in the control of the nitrogen circuit in *S. cerevisiae* (Minehart and Magasanik, 1991; Cunningham and Cooper, 1991; Stanbrough et al., 1995; Soussi-Boudekou et al., 1997).

Numerous studies have been conducted to investigate the mechanism that GATA factors use to regulate downstream gene transcription. The expression of the vertebrate GATA factors, which have two Cys₂/Cys₂ fingers, is tissue specific or developmental stage specific. The GATA-1 gene is expressed in erythropoietic cells and multipotential progenitor cells. This gene is critical for hematopoietic gene expression (Orkin, 1992). The GATA-1 protein alone is not enough to turn on its specifically regulated genes; rather it acts synergistically with other trans-acting regulatory factors to control downstream regulated genes. The carboxyl terminal finger is primarily responsible for DNA binding, while the amino terminal zinc finger of GATA-1 is involved in DNA binding stability and specificity, dimerization, and protein-protein interactions with SpI and EKLF (Crossley et al., 1995; Merika and Orkin, 1995). This amino-terminal finger also interacts with FOG, a multitype zinc finger protein, which acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryotic differentiation (Tsang et al., 1997). Similar protein-protein interactions also occur between the N-terminal zinc finger of GATA-2 and general transcription factors, JUN and FOS (Kawana et al., 1995). It was also shown that the N-terminal finger of human GATA-3, along with its flanking region, is involved in nuclear localization, but is not required for binding to the promoter of its regulated gene (Yang et al., 1994).

Research on the two zinc fingers of fungal GATA factor URBS1 indicates that the C-terminal finger of URBS1 is required for its DNA binding to the promoter region of its downstream regulated gene *sid1*, and also for the iron mediated regulation of siderophore production in *Ustilago maydis*. Mutation in the N-terminal zinc finger, which can reduce URBS1 DNA binding ability, had no obvious effect on its biological function in *sid1* gene expression (An et al., 1997). The solution NMR structure of the chicken GATA-1 zinc finger domain was determined (Omichinski et al., 1993) (Figure 1). A small single "finger" peptide from the GATA-1 protein binds specifically to DNA as a zinc or iron complex (Omichinski et al., 1993).

Quite different from the vertebrate GATA factors, all the GATA factors found in fungi have only one zinc finger, which is similar to the vertebrate C-terminal zinc finger. This zinc finger determines the DNA binding ability of these GATA factors (Fu and Marzluf, 1990b; Ballario et al., 1996; Kudla et al., 1990; Chae et al., 1995; Haas and Marzluf, 1995). Research also indicates that the zinc finger of NIT2, which is a GATA type positive regulator of nitrogen metabolism of *Neurospora crassa*, is also involved in protein-protein interaction of NIT2 with NIT4, a pathway specific regulator (Feng and Marzluf, 1998), and of NIT2 with NMR, a global negative regulator (Xiao et al., 1995; Pan et al., 1997). This suggests that some of the roles played by the

single zinc finger of fungal GATA factors may have been taken over by the N-terminal zinc finger of vertebrate GATA factors during the long history of protein evolution.

All the GATA factors, whether from vertebrates or from fungi, can recognize a GATA sequence. Different GATA factors do have some preference for bases at the binding site flanking the GATA sequence (Merika and Orkin, 1993). This could be one mechanism used to determine the regulation specificity of GATA factors. However, for the *Neurospora* GATA factors, NIT2, WC-1, and WC-2, *in vitro* gel mobility shift assays revealed that the differences in the flanking region of the GATA sequence is not enough to explain their individual regulatory specificity. A specific protein-protein interaction between NIT2 and the pathway-specific regulator NIT4 is required for synergistic activation of the regulated *nit3* gene (Feng and Marzluf, 1998). *In vivo*, both mechanisms could contribute to the regulatory specificity of GATA factors.

GATA factors can be involved in different cellular control functions, but can also be involved in the same metabolic pathway. Functional overlap has been found for vertebrate GATA factors, GATA-1 and GATA-2. They both play a positive role in their regulated processes (Xu et al., 1997). Positive-acting GATA factors and negative-acting GATA factors could act on the same pathway. An example is GLN3 (positive) and DAL80 (negative) in the nitrogen circuit in *Saccharomyces*

cerevisiae. It has been speculated that competition between DAL80 and GLN3 for the same binding site could allow these opposite-acting regulators to control the same process (Cunningham et al., 1994).

One of the well studied metabolic pathways which is regulated by a GATA factor in *Neurospora* is the nitrogen regulation circuit. *Neurospora* uses favored nitrogen sources, such as ammonia, glutamine, and glutamate preferentially. But it can also utilize various secondary nitrogen sources, such as nitrate, purines, or amino acids. This requires the expression of a large group of genes that encodes enzymes to use these substrates (Marzluf, 1996, 1997). The expression of these genes requires the absence of a favored nitrogen source. This whole system is controlled by the global positive regulator NIT2, a GATA type transcription factor, and NMR, which is a global negative regulator. The expression of a specific structural gene also requires a pathway specific regulator. *nit3*, the gene which encodes nitrate reductase, which is needed for the utilization of nitrate, is regulated positively by both NIT2, the global regulator and by NIT4, a pathway specific regulator (Marzluf, 1996, 1997). NIT2 contains a single Cys-X₂-Cys-X₁₇-Cys-X₂-Cys zinc finger DNA binding domain and two acidic and basic regions, which might be relevant for activation (Fu and Marzluf, 1990a). Both the zinc finger motif and the immediate downstream basic region together constitute a DNA binding domain (Fu and

Marzluf, 1990b). NIT4 possesses a single GAL4 like Cys₆/Zn₂ binuclear type zinc finger DNA binding motif near the amino terminus (Fu et al., 1989; Yuan et al., 1991). Three NIT2 binding sites and two NIT4 binding sites have been identified in the *nit3* gene promoter. *In vivo* evidence suggests that a protein-protein interaction between NIT2 and NIT4 determines the regulatory specificity of the global regulator NIT2. Specific protein-protein interactions may determine the regulatory specificity for all of the other GATA factors in *Neurospora*, although all of these GATA factors overlap in their DNA binding capability (Feng and Marzluf, 1998).

NMR, a global negative regulator, controls the nitrogen circuit genes in a different manner. This gene encodes a small protein of 488 amino acid residues. No DNA binding domain has been identified in this protein. NMR can interact with the NIT2 zinc finger and a small carboxyl terminal tail region *in vitro*. And *in vivo*, mutated NIT2 proteins, which are mutated or deleted for these two regions but still can function in gene activation, no longer mediate nitrogen repression. These results imply that the NIT2-NMR interactions are required to establish nitrogen catabolic repression, believed to reflect the cellular content of glutamine (Pan et al., 1997).

A prerequisite to decipher the mechanisms that allow specific regulation of gene expression by related transcription factors is the identification of all the

factors present. It seemed possible that additional GATA factors existed in *Neurospora* besides NIT2, WC-1 and WC-2. In the study reported here, a PCR-mediated approach was used to isolate a new GATA factor-encoding gene from *Neurospora*. The DNA sequence and deduced amino acid sequence of this new protein revealed that it contains two zinc fingers, and thus it differs from the other GATA factors from *Neurospora*, which have only a single finger motif. Additionally, the putative amino acid sequence displays a high degree of homology with URBS1 from *Ustilago* (Voisard et al., 1993) and SREP from *Penicillium* (Haas et al., 1996). Functional studies show that this new GATA factor, named SRE, is a negative regulator of siderophore biosynthesis and iron homeostasis in *Neurospora*.

1.3. Iron metabolism in fungi and *Neurospora*

Iron, an essential nutrient element, is an important component for many enzymes in all cells. But iron is not readily available in aquatic or terrestrial environments or in animal hosts. Iron exists in nature as ferric hydroxide, with Fe^{+++} concentration of only 10^{-18}M at pH7. This Fe^{+++} concentration is not high enough to be used directly by microbes, including bacteria and fungi. On the other hand, an excess amount of iron in the cell is toxic to the cells (Neiland, 1995).

Microbes have developed various strategies for acquiring iron while at the same time protecting themselves from potential iron toxic effects. The major strategy used by bacteria and fungi to acquire iron includes production of siderophores during conditions of iron limitation. Siderophores, cyclic hexapeptides, can solubilize ferric hydroxides and extract iron from other organisms. The siderophores are secreted into the environment and sequester iron to allow its uptake and storage within the microbial cells (Neilands, 1995; Huschka et al., 1985; Matzanke et al., 1987). It has been suggested that siderophores are an important means for pathogenic microorganisms to acquire iron from their hosts (Neilands, 1995; Neilands and Leong, 1986). Several siderophores or iron chelating analogs are utilized to treat iron toxicity in humans (Neilands, 1995). Most microorganisms synthesize several kinds of siderophores

(Neilands, 1995; Guerinot, 1994). *Neurospora* elaborates four different siderophores, coprogen, coprogen-B, ferrichrome, and ferricrocin (Figure 14). Their synthesis is highly regulated by iron availability within the environment (Huschka et al., 1985).

Various microbes have developed regulatory mechanisms to facilitate iron uptake when it is needed and to prohibit iron uptake to avoid toxic effects when the cells have an excess amount of iron. These dual features are acquired by controlling the cellular synthesis of siderophores. Siderophores have been correlated with the virulence of both microbial animal and plant pathogens. An acquired iron supply for many pathogenic species is critical for their virulence. For pathogens that infect humans, the high affinity iron salvaging system of the microbes enables them to compete with host iron binding mechanisms to obtain the iron they require for growth and metabolism (Finkelstein et al., 1983; Weinberg, 1990).

Repression of biosynthesis of extracellular siderophores occurs at 10^{-5} M iron for *Ustilago maydis*. Through controlling the synthesis of siderophores, microbes can control the uptake of iron into the cells. *sid1*, the gene which encodes the first enzyme that leads to siderophore synthesis, and *urbs1*, the negative regulator of the siderophore synthesis pathway, have been isolated and characterized from *Ustilago maydis* (Mei et al., 1993; Voisard et al., 1993). L-ornithine-

N⁵-oxygenase, the enzyme encoded by *sid1*, converts ornithine into N-hydroxyornithine through hydroxylation, and then N-hydroxyornithine is acetylated to form δ -N-acetyl- δ -N-hydroxyornithine. After several additional steps, which are still not clear, siderophores are produced. This pathway is highly conserved among all the fungi (Figure 13) (Mei et al., 1993). URBS1, a GATA factor, negatively controls the siderophore synthesis pathway in *Ustilago*. There are several GATA factor binding sites in the promoter region of *sid1*, which allow URBS1 to bind to its promoter and regulate its expression (An et al., 1997a). It was also found that only the C-terminal zinc finger of URBS1 is involved in the DNA-binding mediated regulation (An et al., 1997b).

The results reported here describe the isolation and characterization of SRE, a negative regulatory gene for siderophore biosynthesis in *Neurospora*. These studies have provided a better understanding of the regulatory circuit that governs iron metabolism in fungi.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial and Fungal Strains:

Neurospora crassa wild type strain 74OR23, a *his-3* mutant strain Y234M723, an *arg-12* mutant strain CD3, and the triple mutant strain *arg-5,ota,aga* were obtained from the Fungal Genetics Stock Center (University of Kansas Medical Center). The *nit2* rip mutant was made in this lab. The *sre* rip mutant was created during this study. The wild type strain, *nit2* rip mutant, and *sre* rip mutant were grown on Vogel's minimum medium. Y234M723 was grown on Vogel's minimum medium plus histidine, *arg-12* was grown on Vogel's minimum medium plus arginine, and the triple mutant on minimum medium supplemented with 1mM ornithine or 1mM arginine and 1mM spermidine. *Escherichia coli* XL1Blue (*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac⁻ F'[proAB⁺ lacI^q lacZ M15 Tn10(tet^r)*]) was used as host strain for DNA manipulation.

2.2 Medium, Chemical and Reagents:

Low-iron (LI) medium was normal Vogel's minimum medium with iron omitted from the trace elements. High-iron (HI) medium was supplemented with 1mM FeSO₄ for solid cultures and

10uM FeSO₄ for liquid cultures. Crossing medium was 1X Westguards plus 0.1% sucrose and 4% agar noble (Singer et al., 1995; Davis and DeSerres, 1970). Ornithine, arginine and spermidine (from Sigma) were each used at a final concentration of 1mM for liquid cultures.

Restriction enzymes, M-MLV reverse transcriptase, and a random primer labeling kit were from BRL (Bethesda Research Laboratories). Taq polymerase was from Boehringer and the Thermo sequencing kit was from Amersham.

2.3 Transformation of *E. coli*

E. coli competent cells were prepared following the standard procedure of Sambrook et al. (1989). Transformation was done by incubating DNA and the competent cell mixture on ice for 30 min, and then the mixture was heat shocked at 42°C for 2 min. After incubation at 37°C for another 30 min, the cells were spread on LB plates with ampicilin selection.

2.4. Isolation of plasmid and cosmid DNA from *E. coli*

Isolation of plasmid DNA was done by the alkaline method as described by Sambrook et al. (1989) with an additional RNaseA digestion of the DNA sample before precipitation with ethanol.

Because of the low copy number of cosmid DNA in *E. coli* cells, isolation of cosmid DNA was done with cultures that were grown in LB medium with the addition of chloramphenicol

to a final concentration of 170 ug/ml to amplify the cosmid DNA. Isolation of cosmid DNA was done following the same procedure used to isolate plasmid DNA.

2.5. Isolation of DNA fragments

DNA fragments for probes or subcloning were isolated from agarose gels with 0.5 ug/ml ethidium bromide. The concentration of the gel was determined by the size of the DNA fragment to be isolated. The agarose gel was run in TAE buffer following the procedure described by Sambrook et al. (1989). Two methods were used to purify DNA fragments from gels depending on the size of a particular DNA fragment. The Gene Clean kit (from Biol01, Inc., La Jolla, California) was used to purify DNA fragments around 1,000bp. Larger DNA fragments were purified with Quantum Prep™ Freeze'N Squeeze Spin Columns (from Bio-Rad), which causes less damage to larger DNA fragments than does the Gene Clean kit.

2.6. Radiolabeling of DNA fragments.

DNA probes used for Southern blots and cosmid library screening were prepared with a random primer labeling kit (from Bethesda Research Laboratories) to label DNA fragments isolated with the above-described procedures.

DNA fragments used for gel mobility shift assays were radioactively labeled by filling in using DNA polymerase I

(Klenow fragment) or using T4 DNA polymerase if the DNA had a 5' overhang at the end.

After labeling, each reaction mixture was passed through a G-50 Sephadex spin column to purify the DNA probe by removing the unincorporated [α - 32 P]dATP.

2.7. Isolation of single-stranded DNA and site-directed mutagenesis.

Single-stranded DNA was isolated by following the method described in the Bio-Rad Muta-Gene™ Phagmid in vitro Mutagenesis Kit Instruction Manual (Bio-Rad, Richmond, CA). Small scale single-stranded DNA was isolated by a rapid preparation protocol (Katayama, 1990).

The DNA plasmid, which was the template of the single strand DNA, was transformed into CJ236 competent cells. A single colony was inoculated into 5ml 2xYT medium with 100ug/ml ampicillin together with 10ul helper phage (VCSM13 10^7 - 10^8 pfu/ml) and grown at 37°C for 2 hours. Kanamycin was added to a final concentration of 70 ug/ml to select the *E. coli* cells, which were infected with helper phage. The cell culture was continuously incubated at 37°C for 16 to 24 hours. Then, 1 ml of the cell culture was centrifuged at 14,000 rpm to remove the *E. coli* cells. The supernatant was taken and treated with RNaseA for 30 min at 37°C. Then, 150ul 20% PEG/2.5M NaCl was added to the sample and incubated on ice for 15min, when it was centrifuged at 14,000rpm to

precipitate phage particles. The phage were suspended in 400ul 0.3M NaOAc pH6.0/1mM EDTA, and then extracted with PCA. The single-stranded DNA was precipitated by addition of ethanol.

Site-directed mutagenesis and further *E. coli* transformations were done by following the instruction manual from the Bio-Rad Muta-Gene™ Phagmid *in vitro* Mutagenesis Kit.

2.8. DNA sequencing

DNA sequencing was carried out by the dideoxynucleotide chain termination method (Sanger et al., 1977) with Thermo Sequenase from Amersham LIFE SCIENCE. With this thermo sequencing method, the radioactive label is incorporated into the DNA sequencing reaction product by the use of four [α -³²P] dideoxynucleotide (ddNTP) terminators (G, C, A, T). This method largely prevents the labeling of prematurely terminated molecules and compression of bands on the sequencing gel.

Plasmid DNA was used as template. Most primers used had a length around 15 nucleotides. So instead of using the standard thermo cycle condition from the instruction manual (dGTP: 95°C, 30sec; 55°C, 30sec; 72°C, 1min and dITP: 95°C, 30sec; 50°C, 30sec; 60°C 5-10min), which is suitable for primer sizes around 25 nucleotides, the reaction conditions were changed (dGTP: 95°C, 30sec; 50°C, 30sec; 63°C, 2min and dITP: 95°C, 30sec; 45°C, 30sec; 55°C, 10min). Some adjustment was

also made according to the GC content of the primer being used.

Preparation of the glycerol-tolerant gel and gel treatment after electrophoresis followed the procedures described in the sequencing kit instruction manual.

2.9. Cosmid DNA library screening

The ordered cosmid DNA library was stored in about 32 96-well plates. Cosmid DNA isolated from each of the 96 individual clones of a plate was pooled in a microfuge tube; each such tube contains cosmid DNA representing 96 clones of a particular plate.

First, the dot-blot technique was applied to each of the pooled cosmid DNA samples. One μ l of each DNA sample was transferred to a piece of nitrocellulose paper and dried. Denaturing of the DNA, neutralization, and hybridization of the filter followed the standard procedure described in "Molecular Cloning" (Sambrook et al., 1989).

When a positive signal with the dot-blot was obtained, the corresponding 96 clones were replicated to an LB plate with ampicillin. After incubation of the plate at 37°C overnight, the colonies were transferred to a nitrocellulose filter following standard procedures (Sambrook et al., 1989). The filters were UV-cross linked, and then prehybridization and hybridization followed the standard procedure from "Molecular Cloning" (Sambrook et al., 1989). The PCR product

(see below) was used as the template to make the DNA probe for both dot-blot and cosmid DNA library screening.

The filter was finally exposed to X-ray film. A colony, which gave a strong signal on the X-ray film was chosen for further analysis.

2.10. *Neurospora* genomic DNA isolation

The genomic DNA used in degenerate PCR and Southern blot analysis was obtained by following the short protocol described by Leach et al. (1986). Mycelia from 16-30 hour cultures were harvested and rinsed with water. The mycelia were mechanically diced and put into a small test tube, to which 0.7ml LETS buffer (0.1M LiCl, 10mM EDTA, 10mM Tris pH8.0 and 0.5%SDS) and glass beads were added. After severe vortexing for about 2min, the mixture was extracted with PCA (phenol : chloroform : isoamyl alcohol = 25 : 24 : 1) and the upper aqueous phase was taken. DNA in the aqueous phase was precipitated with ethanol, dried, and resuspended in 40 ul TE (10mM Tris, 1mM EDTA pH8.0).

A small amount of this DNA could be used for PCR reactions. However, for restriction enzyme digestion, diluted DNA had to be used because the DNA sample prepared by this method is impure. Normally, restriction digestion was done in a volume of 400ul, in which 10ul of the DNA sample and 20u of enzyme were mixed and incubated at 37°C for at least 5 hours (or overnight). After digestion, the reaction mixture was

ethanol precipitated, redissolved, and RNaseA was added to degrade any RNA before the sample was loaded on agarose gels for electrophoresis, which was followed by Southern blots.

2.11. Southern blot analysis

Either cosmid DNA or digested genomic DNA was loaded on agarose gels and after electrophoresis following standard procedures (Sambrook et al., 1989), the DNA in the gel was denatured in 1.5M NaCl/0.5M NaOH for 30min with gentle shaking. Then the gel was neutralized with 1.5M NaCl/1.0M Tris-HCl, pH8.0 with one change of buffer.

Transferring DNA from the gel followed the standard procedure with 10xSSC as the transfer buffer. The derived nitrocellulose filter was UV cross-linked and treated with prehybridization buffer (50% deionized formamide, 6xSSC, 0.25% dehydrated milk) for 2-5 hours. In the meantime, a radiolabeled DNA probe was prepared as described above. Hybridization was at 42°C overnight after adding the radiolabeled DNA probe. The blot was rinsed twice in 2xSSC (with optional 0.1% SDS) for 10min at room temperature with gentle shaking. The blot was then washed with 0.1x SSC and 0.1% SDS at 65°C until the background radioactivity of the filter was very low, as checked with a Geiger counter. The filter was wrapped with Saran wrap and exposed to X-ray film overnight, or longer, depending on the strength of the signal on the filter.

2.12. Isolation of mRNA and northern blot analysis

Whole cell RNA from *N. crassa* was isolated as follows: Frozen (-70°C) mycelia were pulverized in a pre-chilled mortar and pestle, and then suspended in 5ml of UNSET (8M Urea, 150mM NaCl, 100mM Tris pH7.5, 1.0mM EDTA, 2% SDS). After extracting five times with Phenol/Chloroform, one-tenth volume of 3M NaAc pH5.2 and two volumes of cold ethanol were added to precipitate RNA at -70°C. The pellet was washed with 70% ethanol, then resuspended in TE or water. The RNA derived from this method was used in northern blots and RT-PCR.

Northern blots were performed by loading whole cell RNA samples (50ug RNA in 20mM MOPS pH7.0, 8mM sodium acetate, 1mM EDTA, 2.2M formaldehyde, 50% formamide, 50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF, ethidium bromide) on 1% formaldehyde gels (1% agarose, 0.02 MOPS pH7.0, 8mM sodium acetate, 1mM EDTA pH8.0, 2.2M formaldehyde). After electrophoresis, the RNA was blotted to a nitrocellulose filter and UV cross-linked. The filter was put in prehybridization solution (50% formamide, 5XSSPE, 2X Denhardt's, 0.1% SDS) and prehybridized for 2 hours at 42°C. Then, the ³²P labeled DNA probe, derived from the EcoRI fragment of the *sre* gene, was added to the prehybridization solution. After hybridization overnight, the filter was washed with 2XSSC, 0.1% SDS and then 0.1XSSC and 0.1%SDS until the background was very low, and then exposed to X-ray film (from Kodak) at -70°C.

2.13. Degenerate PCR and RT-PCR

Two degenerate primers that cover the coding region for the conservative zinc finger region of GATA factors were used: 5'ACACCATTTGTGGCGA3' and 5'AGACCGCAAGCGTTGCA3' (Figure 2). The conditions used for PCR were 33 cycles of 1 min at 94°C, 1 min at 40°C, and 1 min at 72°C.

RT-PCR was done by adding 50ug whole cell RNA to the reverse transcription system, which contains 50mM Tris pH 8.3, 75mM KCl, 10mM DTT, 3mM MgCl₂, 0.5mM each dGTP, dTTP, dCTP, dATP, 10ug/ml oligo(dT)₁₂₋₁₈, and 200 units M-MLV reverse transcriptase. The total volume was adjusted to 50ul, then the sample was incubated at 37°C for 60 minutes. The derived nucleic acid was used as template to amplify cDNA of the *sre* gene. The reaction mixture (5ul cDNA, 5ul 150ug/ml of each primer, 4ul 5mM dNTP mixture, 10ul 10X PCR amplification buffer in a total volume of 100ul) was heated 2 min at 94°C and centrifuged one second to collect condensate. Then 0.5ul (2.5u) Taq DNA polymerase (from Boehringer) was added to the mixture. Following PCR (39 cycles at 1 min 94°C, 2 min 55°C, and 2 min 72°C and a final round 2 min 55°C and 7 min 72°C), the products were used for cloning or for direct sequencing using an internal primer.

2.14. *Neurospora* transformation

Neurospora crassa spheroplast preparation and transformation were done as described by Akins and Lambowitz

(1985) with the following modification. Following the Novozyme digestion and washing with several changes of sorbitol and STC (1M Sorbitol, 50mM Tris pH8.0, 50mM CaCl₂), the spheroplasts were used for transformation. Heparin (5ul of 5mg/ml in STC, made fresh) was mixed with 1 ug DNA and incubated on ice for 5 minutes, when 100 ul of spheroplasts was added. The mixture was put on ice for 30 min, and then, 1ml PTC was added, and the mixture was incubated at room temperature for another 20 min. The spheroplasts were added to 10 ml top agar (1X Vogel's, 2.8% agar noble, 18.2% sorbitol, 2% sorbose, 0.05% fructose, 0.05% glucose, 0.5% biotin), which was immediately poured onto 25 ml bottom agar (1X Vogel, 1.5% agar, 2% sorbose, 0.05% fructose, 0.05% glucose, 0.5% biotin). Transformants were picked after two days incubation at 30°C.

2.15. Rip mutation

A DNA fragment carrying the *sre* gene linked to a truncated *his3* gene in pDEI vector was targeted to the *his3* gene locus of the *his3*⁻ mutant strain. The *his*⁺ transformants were selected on minimum medium on which the host strain can not grow. Genomic DNA was isolated from the putative transformants and Southern blots were used to check the copy number of the introduced DNA fragment in each transformant. One transformant, which had one extra copy of the target DNA fragment, was crossed with a wild type strain of the opposite

mating type. Ascospores were heat treated at 68°C for 30 minutes and spread on Vogel's minimum medium. Single colonies were picked and tested for growth on different media to determine their phenotype (Singer *et al.*, 1995; Davis and DeSerres, 1970).

2.16. Heterokaryon test

Heterokaryon test was done by mixing mycelia of two different mutant strains of the same mating type on Vogel's minimum medium. The mutant strains used should require some supplement in the minimum medium for them to grow (Davis and DeSerres, 1970).

Thus, the *sre* mutant strain was crossed with a *his3* mutant strain to get a double mutant, which required histidine to grow and also showed *sre* mutant phenotype. The other mutant strain chosen was *arg-12*, which requires arginine for growth. Both mutant strains alone were tested and could not grow on Vogel's minimum medium without any supplement. Then, their mycelia were mixed and inoculated into minimum medium. After several day's incubation at 30°C, hyphal anastomosis resulted in a heterokaryon, which had both types of nuclei and grew vigorously on minimal medium. The heterokaryon was saved for further phenotype test.

2.17. Siderophore tests

Three methods were employed to detect the presence of siderophores: colony color, a ferric perchlorate assay, and thin-layer chromatography.

Wild type and *sre* mutant strains were grown on low-iron solid medium supplemented with 1mM FeSO_4 . *sre* mutant cells will keep secreting siderophores, which form a complex with free iron, giving the mutant colonies an orange color. In contrast, wild type colonies remain white because siderophore synthesis was suppressed at the high iron condition (Fig.15.) (Voisard et al., 1993).

A ferric perchlorate assay was done by growing *Neurospora* for 48 hours in liquid media (low-iron or high-iron). The culture was centrifuged, the supernatant was collected, and an equal volume of perchlorate assay solution (5mM $\text{Fe}(\text{ClO}_4)_3$, 0.1M HClO_4) was added. The absorbance at 495nm was determined using fresh medium as the control (Atkin et al., 1970)

The same cultures were also used for thin-layer chromatography. Siderophores were extracted from the cell-free liquid growth medium as described by Garibaldi and Neilands (1955). A small volume of each siderophore-iron extract was applied to silica gel plates (from Sigma). Thin layer chromatography was then carried out using methanol:water (9:1, v/v) as the solvent system (Budde and Leong, 1989).

2.18. Enzyme assay

L-ornithine N⁵-oxygenase enzyme activity was determined as previously described (Mei et al. 1993) with minor changes. *N. crassa* cells grown on low-iron or high iron medium were collected and suspended in potassium phosphate buffer (pH8.0). The cells were disrupted with a Mini-bead-beater (from Biospec Products) using two periods of 3 minutes. The homogenate was centrifuged at 12,000g for 15 minutes, and the supernatant was saved to measure enzyme activity. Plattner's method (Plattner et al. 1989) with minor changes was used to determine the enzyme activity. The reaction mixture (200ul 0.5mM potassium phosphate, pH8.0, 50ul 10mM NADPH, 10ul 0.5mM FAD, 400ul cell extract, 150ul 10mM L-ornithine, 200ul H₂O) was incubated at 30°C for 2 hours before 0.5ml 0.2M perchloric acid was added to stop the reaction. The same amount of perchloric acid was added to one sample before incubation as a control. Then, the samples were centrifuged and 1ml supernatants were taken for the subsequent iodine oxidation test (Tomlinson et al., 1971). The supernatants were mixed with 4ml 6% NaAc, 0.5ml 1% sulfanilic acid (in 25% acetic acid), and 0.2ml 1.3% iodine (in glacial acetic acid). The mixture was kept at room temp for about 5-7 min before 0.2ml 0.1N sodium thiosulfate and 0.1ml 0.6% a-naphthylamine (in 30% HAC) was added. The reaction mixture was further incubated at room temp for another 30min. After brief centrifugation to remove any denatured protein, the

absorbance at 520nm was determined. The protein concentration was determined by adding 1ul protein sample to 1:5 diluted protein assay solution (Bio-Rad) and determining the absorbance at 595nm. The derived concentration was used to determine specific activity of the enzyme.

2.19. Protein expression and purification

The pGEX-2T vector was chosen to express the full length SRE protein and different mutant forms of the SRE protein. Appropriate restriction sites were created in the coding region of the *sre* gene to facilitate in frame cloning of the corresponding *sre* gene fragment. Then the fragment to be expressed was subcloned into pGEX-2T so that it formed a chimeric protein with GST tag. The GST tag was at the N-terminus of the fusion protein.

The constructed plasmid was transformed into *BL21(DE3)pLysS*. A single colony was picked and inoculated into 5ml LB medium containing ampicillin. After growth overnight, 500ml of fresh medium was inoculated with the overnight culture. The culture was kept growing at 37°C until A_{600nm} reached 0.6. IPTG was added to a final concentration of 0.1 mM. The culture was further grown for 3-7 hours, then the cells were pelleted and suspended in 1/50-1/100 culture volume of MTPBS (150mM NaCl, 16mM Na_2HPO_4 , 4mM NaH_2PO_4 , pH7.3). Sonication was used to disrupt the cells. The whole cell lysate was centrifuged at 15,000 rpm for 20 min at 4°C.

To the supernatant, 800 μ l of glutathione-agarose-beads were added. The fusion protein was allowed to bind to the beads for 30 min at room temperature with gentle shaking. Then the glutathione agarose beads were washed with 10 ml MTPBS for 5 times. Finally, 200 μ l freshly prepared 50 mM Tris HCl (pH7.5) containing 5 mM reduced glutathione was added to the bead-protein mixture to elute the fusion protein. The purified protein was examined by gel electrophoresis and its concentration was determined with the Bio-Rad protein assay solution.

2.20. Gel electrophoresis mobility shift assay

Radiolabeled DNA probes and purified GST-SRE fusion proteins were prepared as described above. The 32 P radiolabeled DNA probes were incubated with various amounts of GST-SRE fusion proteins for 30 min at 25°C in a buffer containing 12mM Hepes, 4mM Tris HCl pH7.9, 110mM KCl, 1mM EDTA, 3mM MgCl₂, 1mM DTT, 10% glycerol, and 0.3mg/ml BSA. The reaction mixture was loaded onto 4% native polyacrylamide gels and electrophoresed in 0.5xTBE (0.0445M Tris-borate, pH 8.2, 0.001M EDTA) for about 2 hours at 200 volts until the bromophenol blue reach the bottom of the gel. The gel was dried and exposed to X-ray film. The X-ray film was scanned by densitometer to determine the ratio of free and shifted probes.

2.21. Assay of nitrate reductase

Mycelia from a 30ml overnight growth culture were harvested and rinsed with distilled water. Then the mycelia were divided into two equal amounts and was transferred to both nitrogen repression and derepression/induction media and incubated for another 4 hours at 30°C with gentle shaking.

The mycelia were again harvested and suspended in extraction buffer (100mM phosphate, pH6.8, 1mM β -mercaptoethanol, 0.5mM EDTA, and 1% NaCl). The cells were disrupted with a Mini-bead-beater (from Biospec Products) using two periods of 3 minutes. The homogenate was centrifuged at 12,000g for 15 minutes, and the supernatant was saved to measure enzyme activity as described (Garrett and Cove, 1976). Activity was calculated from measurements taken at 25 min reaction time. The protein concentration was determined by protein assay solution from Bio-Rad, and the derived concentration was used to determine specific enzyme activity.

CHAPTER 3

ISOLATION OF A NEW GENE, *sre*, FROM *NEUROSPORA*

3.1. Introduction

A total of four GATA factors have been isolated so far from *Neurospora*. NIT2 is the master positive regulator of the nitrogen circuit (Fu and Marzluf, 1990a), WC-1 and WC-2 are involved in light responses and circadian rhythm (Ballario *et al.*, 1996; Linden and Macino, 1997). The function is still unknown for a fourth GATA factor found in our lab (Feng, personal communication). These GATA factors regulate different metabolic pathways or cellular activities, presumably without interfering with each other.

These GATA factors all contain a single vertebrate C-terminal like zinc finger. This zinc finger determines the DNA binding ability of these GATA factors. The zinc finger of NIT2 is also involved in protein-protein interactions (Feng and Marzluf, 1998). Although the loop region of the zinc fingers of WC-1 and WC-2 has eighteen amino acids, while that of NIT2 has seventeen amino acids, the zinc fingers of these GATA factors share a high amino acid identity (about 50-60%). Other than the zinc finger regions, these GATA factors show

very little identity, probably due to their distinct functional roles.

It is possible that additional GATA factors exist in *Neurospora* other than NIT2, WC-1, and WC-2. Such unknown GATA factors might be expected to share at least one property with the existing GATA factors: A high identity in their zinc finger region, while other regions of any new GATA factors could be totally different and determine the functional specificity.

In order to study further the regulatory mechanisms of these GATA factors and to identify any possible additional GATA factors of *Neurospora*, a PCR mediated method was employed to isolate GATA factor genes from *Neurospora* genomic DNA. A pair of degenerate primers (Figure 2), which cover the conserved zinc finger region of the existing GATA factor genes was used to amplify any additional sequences using *Neurospora* genomic DNA as template. A DNA fragment, which contained a unique sequence, was used to screen a *Neurospora* genomic DNA library. One clone, which showed a strong signal, was isolated and characterized. This identified a new GATA factor gene, which was totally sequenced.

RT-PCR was used to determine the existence of any introns in the coding region of this newly found GATA factor gene. The deduced amino acid sequence of this new GATA factor contains a total of 587 residues. The new GATA factor has two zinc fingers and two small introns, one in each of the zinc

finger coding regions. The new GATA factor sequence was used to search GenBank and led to the identification of two homologues: URBS1 and SREP, GATA factors in the fungi *Ustilago* and *Penicillium*, respectively.

3.2. Results

3.2.1. Identification of a new GATA factor by PCR

For PCR amplification of a GATA-factor-specific DNA sequences from *Neurospora crassa* genomic DNA, two degenerate primers derived from the peptide sequences TPLWR and CNACGL (Figure 2), which are conserved in the zinc fingers of most fungal GATA factors, were employed, as described in Materials and Methods. The products were cloned and sequenced. The sequence of one of the PCR products identified a new zinc finger sequence, different from that of any previously known GATA factors from *Neurospora*.

3.2.2. Isolation of the gene which encodes the new *Neurospora* GATA factor

The PCR product with the new zinc finger sequence was used as a probe to screen a *Neurospora* cosmid DNA library. Several positive cosmid clones were recovered. One of them, which gave the strongest signal in the screening, was chosen for further analysis. Cosmid DNA from the positive clone was prepared and digested with different restriction enzymes and various combinations of restriction enzymes. Southern blots were used to analyze the restriction enzyme digestion products and several hybridizing fragments were subcloned into pBlueScript KS(+) vector (Figure 3,4).

Genomic DNA was also isolated from *Neurospora crassa* and digested with two different restriction enzymes, BamHI and

HindIII, along with the cosmid DNA. Southern blots were employed to analyze the restriction enzyme digestion products (Figure 5). The probe used was a DNA fragment which has the new GATA factor gene. Two fragments of the same size appear in both genomic DNA and cosmid DNA for both enzyme digestions. This suggests that no obvious rearrangement or recombination occurred in the cosmid DNA during construction of the library.

A restriction map is given in Figure 6. Further restriction enzyme analysis and sequencing revealed that one of the subclones contained a 1.7kb EcoRI fragment covering almost the entire coding region and part of the 3' untranslated region of the new GATA factor. A second subclone contained a 2.5kb BamHI-SacI fragment, which covered the whole 1887bp coding region, 3' untranslated region, and a small portion of the promoter region of the new GATA factor. A third subclone had a 6.5kb HindIII-SacI fragment which covered the whole 1887 coding region, 400bp 3' untranslated region, and 3.5kb of the 5' region of the new GATA factor. This 5' region includes the promoter and the 5' untranslated region of the new GATA factor mRNA.

3.2.3. Nucleotide sequence and sequence comparison

The nucleotide sequence of the 3478bp KpnI-SacI fragment containing the 1887 bp sre coding region, 1151 bp 5'-non coding region and 407 bp 3'-non coding region was determined

for both strands. RT-PCR (Figure 8) was used to examine the intron/exon structure of this gene, revealing the presence of two small introns. The open reading frame deduced from the nucleotide sequence reflected a putative protein of 587 amino acids (Figure 7). This new gene was named *sre* (for siderophore regulation) based on its function (see below).

The sequence analysis revealed the presence of six GATA sequences in the promoter region of *sre* gene. Three of them are closely clustered. Based on comparison with known GATA factor binding sites, these GATA sequences may represent an element for binding of a GATA factor. This suggests a possible autoregulation of the *sre* gene or that *sre* is regulated by other GATA factors.

The sequence surrounding the translation start site (AGGAATGGCA) of the *sre* gene matches the consensus sequence for translation start sites in *N. crassa*, with GC following and an A immediately preceding the ATG initiation codon.

Both introns start with GT and end with AG, the conserved nucleotides that occur at the border of eukaryotic nuclear gene introns.

Unlike several other *Neurospora* GATA factors which have a single zinc finger, the SRE protein contains two zinc finger motifs. The deduced amino acid sequence of SRE was compared with other GATA factors from both vertebrates and invertebrates. The C-terminal zinc finger of the SRE exhibits high homology with the C-terminal zinc finger of the

vertebrate GATA factors and also with the single zinc finger region of the fungal GATA factors. On the other hand, the N-terminal zinc finger of SRE showed high homology with the N-terminal zinc finger of the vertebrate GATA factors (Figure 9).

Except for the zinc finger region, the other parts of the SRE protein do not show significant homology with other GATA factors. The only exception to this is a stretch of 28 amino acids located between the two zinc fingers of SRE which can be found in the same region in the fungal GATA factors SREP (Haas et al., 1997) and URBS1 (Voisard et al., 1993), both of which also contain two zinc fingers (Figure 9). This amino acid sequence shows an overall identity of about 50% and contains four conserved Cys-residues (Figure 10). Additionally, the positions of the two introns found in *Neurospora sre* are perfectly conserved in *sreP* from *P. chrysogenum* (Haas et al., 1997). The two zinc fingers in these three fungal GATA factors are separated by about 120 amino acids, greater than that between the vertebrate two zinc finger GATA factors. This finding suggests that these three fungal GATA factors may have a similar function.

3.2.3. Discussion

In this study, a PCR mediated method led to the isolation of a new gene, designated *sre*. Sequencing of the gene revealed that it encodes a new GATA factor, which contains two zinc fingers, similar to GATA factors from vertebrates. This is the first two zinc finger GATA factor gene isolated from *Neurospora*.

The amino acid sequences of the two zinc fingers show high identity with the zinc fingers from vertebrate GATA factor and also the single zinc finger of other invertebrate GATA factors. In addition, a Cys-rich region between the two zinc fingers of the SRE protein showed high identity to the same region of the siderophore regulator URBS1 from *Ustilago maydis* (Voisard et al., 1993) and SREP from *P.chrysogenum* (Haas et al., 1996). This high identity within the protein coding region of SRE and URBS1 suggested that the new *Neurospora* GATA factor may be involved in controlling iron uptake as found with URBS1 for *Ustilago*.

SRE, together with its homologue URBS1 from *Ustilago* (Voisard et al., 1993), SREP from *Penicillium* (Haas et al., 1997), and Gaf2p from *Schizosaccharomyces pombe* (Hoe et al., 1996), appear to comprise a new sub-family of GATA factors. Instead of the two zinc fingers closely following each other as with the GATA factors from vertebrates, the two zinc fingers in this new group of GATA factors are separated by a long stretch of amino acid sequence, which displays

significant identity in SRE, URBS1 and SREP. Two of them, SRE (this study) and URBS1 (Voisard et al., 1993), have been demonstrated to be negative regulators of iron uptake, and this analysis suggests a similiar function for the other two homologues.

There are a total of five putative GATA factor binding sites in the promoter region of *sre*, the new GATA factor gene. This suggests the possibility that transcription of *sre* is under control of another GATA factor or possibly is subject to autoregulation.

CHAPTER 4

FUNCTIONAL STUDY OF THE NEW GATA FACTOR SRE

4.1. Introduction

A total of five GATA factors have been isolated so far from *Neurospora crassa*. Four of them are involved in different cellular processes or metabolic pathways (Fu and Marzluf, 1990a; Ballario et al., 1996; Linden and Macino, 1997) although they share high identity in their zinc finger regions. These GATA factors all can interact with the same type of DNA fragments in gel mobility shift assays without significant differences in their DNA binding ability (Feng and Marzluf, 1998). This makes it difficult to predict the function of a new GATA factor only from sequence information.

The most effective method to identify the function of a new gene is a genetic analysis, namely to disrupt the endogenous copy of the gene to study the phenotype of the mutant strain. This method is very informative if disruption of the gene results in a discernible phenotype. Mutation analysis is particularly effective for prokaryotic and lower eukaryotic species because of the haploid nature and single cell characteristics of these species.

Mutational analysis has been used very successfully in molecular studies of *Neurospora*. *Neurospora* grows as a haploid, so that most genes are maintained only as a single copy in the genome. If the gene whose function is to be determined is not essential for viability of *Neurospora* and the mutant strain disrupted for the gene shows a distinct phenotype, it is possible to infer the function of the gene.

A method called rip (repeat induced point mutation) (Selker and Ganet, 1988; Cambareri et al., 1989), has been used very successfully in *Neurospora* molecular biology to disrupt endogenous genes, see Materials and Methods (Figure 11). By including an extra copy of the target gene in a parental strain, crossing it with wild type will create multiple G/C to A/T transition mutations and methylation along the entire duplicated region. Rip normally results in loss of function for any genes which reside within the duplicated region. Rip mutant strains can be isolated and detected by Southern blot analysis, due to the fact that the large number of transition mutations will decrease the annealing affinity of the mutated copy of the gene with a DNA probe containing the wild type gene sequence. If the mutated gene is not an essential gene but has a regulatory function, the metabolic pathway under its control will suffer loss of its regulation. In this chapter, the rip mutation method was employed to disrupt the newly found GATA factor gene, *sre*.

The sre rip mutant strain was isolated and confirmed by Southern blot analysis.

Further confirmation of the function of a new gene can be achieved by gene complementation, which can be examined in *Neurospora* by transforming the wild type gene back into the rip mutant strain. Rip mutants with multiple base changes will usually be null (loss-of-function) mutations. Null mutations are expected to be recessive to the wild type gene, which can be tested by a heterokaryon analysis that is possible because of the multinuclear property of *Neurospora* vegetative cells.

Sequence comparison of the new SRE GATA factor gene with other GATA factor genes revealed that it showed high identity with URBS1 and SREP (Voisard *et al.*, 1993; Haas *et al.*, 1996). URBS1 is involved in iron uptake in *Ustilago maydis*, and is a negative regulator which controls siderophore synthesis in *Ustilago*. The function of the *Penicillium* SREP factor is still unclear. This comparison suggested that the new GATA factor may be involved in iron homeostasis in *Neurospora*.

Iron uptake is achieved by a highly conserved biosynthetic pathway among all kinds of fungi (Figure 13). They all use the same substrate, ornithine, to produce small peptides, called siderophores, by a synthetic pathway that does not employ ribosomes (Figure 13). URBS1 negatively controls the synthesis of siderophores in *Ustilago*. The

function of *URBS1* was determined by detecting the activities of this pathway, by simply analyzing the final products of this pathway, the siderophores, or by detecting the expression level of any of the genes or enzymes that are involved in the synthesis of siderophores.

Several methods have been used to study iron metabolism of fungi. Among them, the ferric perchlorate assay and thin layer chromatography detect the production of siderophores, and an enzyme assay is used to detect the first enzyme in this pathway, that catalyzes the oxidation of ornithine. In this study, all of these methods were employed to study the phenotype of the new GATA factor gene using the *rip* mutant strain and wild type *Neurospora*. The results suggest that the new GATA factor is a negative regulator of iron uptake in *Neurospora*. It negatively regulates the synthesis of siderophores in *Neurospora*.

The expression of any new gene itself can be interesting and informative, such as the expression pattern of the gene in different types of cells, at different stages of development both spatially and temporally, or under different growth conditions. The GATA type transcription factor, NIT2, has a different expression pattern under different nitrogen conditions, which is reflected by northern blot analysis. The expression pattern of *urbs1*, the homologue of the new *Neurospora* GATA factor gene, is not dependent on the availability of iron in the growth medium even though this

gene is a negative regulator of the siderophore synthetic pathway, which is controlled by the cellular iron concentration. In this study, I employed northern blot analysis to study the expression pattern of the new *Neurospora* GATA factor gene under different growth conditions.

In order to study the function of this new found GATA factor gene, rip mutation was created for this gene and further phenotypic tests were employed to study the function of wild type and the created rip mutant strain. The new found GATA factor gene was determined to be a negative regulator of the iron metabolic pathway in *N. crassa*.

4.2. Results

4.2.1. Gene disruption of *sre* and phenotype of *sre* null mutant.

To decipher the function of SRE, Rip (repeat induced point mutation) was used to disrupt the *sre* gene (Figure 11) (Singer *et al.*, 1995; Davis and DeSerres, 1970; Selker and Ganet, 1988). The 1.7 kb EcoRI fragment, which covers almost the entire coding region and part of 3' untranslated region of the *sre* gene was linked to a truncated *his-3* gene in the pDEI vector. The new construct was targeted to the *his3* locus of *his3⁻* *Neurospora* strain by transformation as described in Materials and Methods. Genomic DNA was isolated and Southern blots were used to identify a transformant which carried one extra copy of the new gene. It was crossed with wild type to obtain a *rip* mutant strain. A *sre rip* mutant, which does not contain an intact *sre* gene, as proved by Southern analysis using the β -tubulin gene as an internal control (Figure 12), was chosen for further phenotypic analysis.

After several days of growth on iron-rich plates, the *sre rip* mutant colonies were orange colored while the wild type colonies remained white (Figure 16). This phenotype suggested that this new *Neurospora* GATA factor encoding gene is involved in iron metabolism (Voisard *et al.*, 1993).

4.2.2. Ferric perchlorate assay and thin layer chromatography

To further confirm the phenotype of the *sre* mutant, siderophore production was examined by a ferric perchlorate assay and by thin layer chromatography. Both methods have been used to study iron transport in *Ustilago* (Voisard et al., 1993).

The results of both tests revealed that synthesis and excretion of siderophores is suppressed under high-iron conditions in wild type *N. crassa*. In contrast, the *SRE* loss of function mutant expresses the siderophore biosynthesis pathway irrespective of the iron content of the growth medium (Figures 17 and 19). For both the ferric perchlorate assay and thin layer chromatography, a triple mutant, *arg-5, ota, aga*, was used as a control. This mutant cannot synthesize ornithine, which is the substrate for L-ornithine-N⁵-oxygenase, the first committed step in fungal siderophore biosynthesis (Figure 15). This triple mutant produced siderophore only under low iron conditions and only when ornithine was supplied in the growth medium, confirming that both tests detect specifically siderophores (Figures 17 and 19). *Neurospora* wild type and the *sre* mutant were also grown in low iron and high iron liquid media, and the culture media were taken at different times. The ferric perchlorate assay was used to determine the production of siderophores relative to the growth time (Figure 18). The *sre* *rip* mutant accumulated more siderophores compared to wild type in the

low iron condition. Moreover, the *sre* mutant produced a significant amount of siderophores even in the high iron condition.

In conclusion, the perchlorate assay and thin layer chromatography demonstrated that SRE plays a negative role in controlling siderophore biosynthesis in *Neurospora*. Moreover, since siderophore biosynthesis was only partially derepressed in the SRE loss of function mutant, the existence of additional regulatory elements involved in this control circuit can be suggested.

4.2.3. Regulation of L-ornithine-N⁵-oxygenase

Regulation of the first enzyme specific for siderophore biosynthesis, L-ornithine-N⁵-oxygenase was examined for wild-type and *sre* mutant cells grown under conditions of low iron and high iron. After growth of the wild-type and mutant strains for 39 and 45 hours, the cells were collected, homogenized, and assayed for L-ornithine-N⁵-oxygenase activity as described in Materials and Methods. The results demonstrated that expression of this enzyme is regulated by the iron concentration of the growth medium. In wild-type, the enzyme was expressed at a very high level in cells grown with low iron, and only in trace amounts in cells growing with high iron. In contrast, cells of the *sre* mutant strain possessed significant L-ornithine-N⁵-oxygenase activity whether grown in low iron or high iron medium (Table 2).

4.2.4. Analysis of *sre* mRNA

To study the expression of the *sre* gene, northern blots were used to analyze total RNA from both wild type and the *sre* mutant grown on low iron and high iron media.

Using the 1.7kb *EcoRI* *sre* fragment as the probe, a 3 kb transcript was detected for the wild type strain (Figure 20). This transcript was present in identical amounts in wild type cells grown in both low iron and high iron medium. This indicated that *sre* gene expression itself is not regulated by the iron concentration. In contrast, this transcript was not detected in RNA from the *sre* *rip* mutant, whether grown on low iron or high iron medium; equivalent loading of RNA was demonstrated using the β -tubulin transcript as an internal control (Figure 20). This result is consistent with the genomic Southern blot analysis of the *sre* *rip* mutant, which confirmed that the gene had been disrupted (Figure 12). A weak band, which appeared below the 3 kb *sre* transcript, is an artifact due to trapping of the probe by a large amount of rRNA. Comparison of the level of *sre* mRNA with the β -tubulin transcript revealed that the transcription level of *sre* is very low, as expected for a regulatory factor.

4.2.5. Heterokaryon test

Neurospora normally grows as a multinucleated syncytium. This makes it possible to put the wild type gene and its mutant form together in one cell to test the phenotype of the

heterozygote. If the wild type gene is dominant, the heterokaryon will show the wild type phenotype, otherwise the heterokaryon will display the mutant phenotype.

In order to do this heterokaryon test, the *sre* *rip* mutant strain was crossed with *his3⁻* strain and the double mutant was isolated. This double mutant cannot grow on Vogel's minimum medium without adding histidine. Another mutant strain, *arg-12*, which can not grow without arginine, was chosen to form a heterokaryon with the *his3⁻*, *sre⁻* double mutant. Selection for a heterokaryon was on Vogel's minimum medium without histidine and arginine, on which neither of them can grow. An *arg-12⁺his3⁻sre⁻* *rip* heterokaryon was obtained which grew vigorously on this medium. The heterokaryon was used to conduct the ferric perchlorate assay, together with the wild type and the *sre* mutant as controls (Figure 21). The results showed that the phenotype of the heterokaryon is wild type. This suggests that the *sre* *rip* mutant is recessive to the wild type *sre* gene.

4.2.6. Gene complementation

The heterokaryon test showed that the *sre* *rip* mutant is recessive to wild type *sre*. Because *rip* leads to many G/C to A/T transition mutations, it is highly probable that the mutated gene created by this method will lack any function, i.e. it will be a null mutant.

In order to further test the function of the *sre* gene, a 6.5 kb HindIII-SacI DNA fragment, which covers the entire coding region and promoter region of *sre* gene, was inserted into a plasmid along with the benomyl-resistant β -tubulin gene. This plasmid was transformed into the *sre* *rip* mutant strain, by selection for benomyl resistance. Benomyl resistant clones were isolated and genomic DNA was prepared from these transformants for Southern analysis. The genomic DNAs were digested with PstI, which has a single site in the *rip* mutation region of the *sre* gene. The PstI recognition sequence, CTGCAG, is GC rich. The probe used was a 200 bp fragment from the *sre* gene 3' region, which is far away from the β -tubulin gene (Figure 22). Wild type and *sre* *rip* mutant strain genomic DNAs were used as controls in the Southern blot. A typical Southern blot (Figure 23) showed that there is one copy of the *sre* gene in the wild type strain, and also one copy of the *sre* gene in the *rip* mutant strain, visible because the probe used covered a region of the *sre* gene outside of the *rip* mutation region. The critical difference is that the fragment in the *rip* mutant is bigger than that from wild type, due to the disappearance of the PstI site in the *rip* mutation region that would have resulted from any G/C to A/T change in the PstI recognition site. One transformant examined in this blot showed an extra copy of the *sre*⁺ gene. The other transformants either didn't receive a wild type copy of *sre* or obtained a truncated copy which did not give a

signal in the Southern blot. The transformant which had a full length *sre*⁺ gene was saved for the ferric perchlorate assay (Figure 24). The result showed that the phenotype of this transformant is wild type. This demonstrated that the transformed wild type copy of *sre* fully complemented the *sre* null mutant. This result further confirmed that the new GATA factor gene, *sre*, is a negative regulatory gene of the siderophore synthetic pathway in *Neurospora crassa*.

4.3. Discussion.

The rip procedure is a valuable technique to disrupt endogenous genes in *Neurospora*. This requires crossing a wild type strain with another strain that contains a duplicated region of more than 500bp of *Neurospora* genomic DNA (Selker and Ganet, 1988; Cambareri et al., 1989). Although the mechanism of rip mutation is not clear, a premeiotic event, such as chromosome alignment, may be the event which leads to the methylation and many G/C to A/T transition mutations along both copies of the duplicated DNA segment, whether it is a promoter region or a protein coding region. Thus, rip will silence any genes that reside within the duplicated region, and a rip mutation is anticipated to be recessive to wild type.

Recently, a new phenomenon was found in *Neurospora*, called quelling. Quelling requires the integration of multiple copies of extra DNA into the *Neurospora* genome. The integrated DNA should contain at least 200bp of a protein coding region. The gene which resides in this region loses its function. Heterokaryon tests revealed that this condition is dominant to wild type. However, the affected gene is not mutated and a gradual return of function can occur after vegetative growth in concert with loss of much of the duplicated DNA copies. Further research suggested that an aberrant RNA-mediated mRNA degradation was the reason for

this type of gene silencing in *Neurospora* (Romano and Macino, 1992; Cogoni et al., 1996; Cogoni and Macino, 1997).

In this study, the rip procedure was employed to create a mutant strain of the new GATA factor gene. The mutant strain was tested for its phenotype using several different methods. These assays revealed that the new GATA factor is a negative regulator of the iron metabolic pathway in *Neurospora*. The assay results showed a similar pattern as found with the *urbs1* gene of *Ustilago*, which suggests that the iron metabolic pathway and its control is conserved among these fungal species.

Siderophore production is only partially derepressed in the SRE loss-of-function mutant indicating that this pathway is not regulated exclusively by SRE. It is possible that one or more other negative factors are involved in the control of this pathway or that a positive regulatory protein is needed to increase expression of siderophore biosynthetic enzymes during iron limitation. An example for such a regulatory mechanism exists in yeast: the negative acting GATA factor Ash1p controls, in concert with the positive regulator Swi5p, mating type switching in *S. cerevisiae* (Sil and Herskowitz, 1996). It is even possible that SRE acts more generally as a negative regulator and carries out particular control roles in concert with pathway-specific regulators.

Northern blot analysis indicated that *sre* is expressed constitutively and that its expression is not regulated by

the iron content of the growth medium. This finding is analogous to the situation in *U. maydis* where expression of URBS1 is also constitutive, indicating that a posttranscriptional event must occur to modulate activity of these siderophore biosynthesis transcription factors (Voisard et al., 1993). Two possibilities exist for this posttranscriptional regulation. One is that the regulation occurs at the translational level. An example of this kind of regulation is the translational regulation of the ferritin protein, which depends on the concentration of iron in the cell. A cluster of RNA hairpins in the 5' untranslated region of ferritin mRNA controls mRNA translation even though the level of ferritin mRNA is the same under both high and low iron conditions. A different case is that of differential mRNA stability as exemplified in control of the transferrin receptor. A cluster of similar hairpins are located in the 3' region of the transferrin receptor mRNA and regulate its stability. A Western blot analysis can be used to detect the level of the SRE protein in cells subjected to high and low iron conditions to investigate possible translational control (DeSilva et al., 1996). Another possibility is that same amount of SRE protein exists in *Neurospora* cell whether on high or low iron conditions. In this case, SRE is inactive during high iron conditions and is active in cells under a low iron condition. A possible regulator that can modulate the activity of SRE is iron. Further experiments conducted

both *in vitro* and *in vivo* are needed to investigate the mechanism that regulates the siderophore synthetic pathway, depending on iron concentration in the cells.

CHAPTER 5
RELATIONSHIP OF SRE WITH OTHER GATA FACTORS, SUCH AS NIT2
FROM *NEUROSPORA*

5.1. Introduction

Multiple GATA factors exist in *Neurospora crassa*, NIT2 (Fu and Marzluf, 1990), WC-1 (Ballario et al., 1996), WC-2 (Linden and Macino, 1997), NFG-1 (Feng, personal communication) and SRE. NIT2, WC-1 and WC-2 are involved in controlling different cellular processes or metabolic pathways without interfering with each other. However, these factors all recognize the same kind of DNA binding site, namely the GATA sequence. *In vitro* gel mobility shift assays revealed that these GATA factors do not show significant differences in affinities to DNA fragments containing GATA sequences. Their DNA binding specificity overlaps and differences in the flanking region of the GATA sequences were not enough to distinguish between the GATA factors, at least *in vitro*. NIT2 interacts with a pathway-specific regulator NIT4, while WC-1 and WC-2 do not show any protein-protein interaction with NIT4 *in vitro* (Feng and Marzluf, 1998). This finding suggested that pathway-specific regulatory proteins

determine the regulatory specificity of the GATA factors *in vivo*.

Sequence analysis revealed that multiple GATA sequences exist in the promoter region of the *sre* gene. These GATA sequences resemble typical GATA factor binding sites. This suggested that *sre* gene expression might be under control of another GATA factor, or by autoregulation it may control its own transcription. The fact that GATA factors can bind GATA sequences without significant preferences brings up the question of whether *sre* could be under the control of any of the previously identified GATA factors. An *in vitro* gel mobility shift assay was conducted to examine a possible DNA-protein interaction between the *sre* gene promoter and NIT2 protein.

Nitrogen regulation circuit is a well studied metabolic pathway in *Neurospora crassa* (Marzluf, 1996, 1997). NIT2, a GATA factor, is a master positive regulator of nitrogen metabolism (Fu and Marzluf, 1990a). NIT4, a pathway specific regulator, is a Cys6/Zn2 type regulatory protein (Fu et al., 1989; Yuan et al., 1991), and NMR is a negative regulator of this pathway. There are multiple different downstream target genes, which encode different enzymes involved in nitrogen metabolism, regulated by NIT2. *nit3* encodes nitrate reductase, and its promoter contains multiple NIT2 and NIT4 binding sites. By determining the cellular nitrate reductase

activity, co-regulation of the *nit-3* gene by NIT2 and NIT4 has been ascertained (Marzluf, 1996, 1997).

Turning on of *nit3* requires the presence of the global regulator NIT2, the pathway-specific regulator NIT4, N-derepression and inducer, nitrate. The *nit2* null mutant lacks the positive regulator and cannot express the *nit3* gene, and thus lacks nitrate reductase activity even under the induction conditions (Marzluf, 1996, 1997).

All the GATA factors found in *Neurospora* may regulate their downstream genes by a similar mechanism, which involves their binding to the promoter region of their regulated genes. In order to study whether there is any interference between two GATA factors, SRE and NIT2, a possible effect of SRE on the regulation of another metabolic pathway, nitrogen assimilation, distinct from siderophore synthesis, was investigated. In parallel, a possible regulation by NIT2 of the iron metabolic pathway was also investigated by *in vivo* assays and by *in vitro* gel mobility shift assays.

5.2. Results

5.2.1 Nitrate reductase assay of *sre* mutant

In order to test whether SRE has any influence on the nitrogen circuit, the following experiments were conducted. The *sre* mutant was grown under nitrogen derepression and induction conditions (presence of nitrate) or repression conditions (presence of glutamine), and nitrate reductase assays were conducted to determine whether SRE influences the nitrogen metabolic pathway.

The *sre* *rip* mutant, *nit2* mutant, and wild type conidia were inoculated in medium containing the inducer (nitrate) or the inducer plus the repressor, glutamine, at final concentrations of 20mM. Total cell proteins were isolated and assayed to determine nitrate reductase activity. The results (Figure 25) showed that the phenotype of *sre* mutant is identical to that of wild type with regard to nitrogen metabolism, indicating that SRE does not play any role in control of *nit-3* expression.

5.2.2 Gel mobility shift assay of NIT2 protein and *sre* gene promoter.

In order to study the DNA-protein interaction of NIT2 with *sre* gene promoter, *in vitro* gel mobility shift assays were conducted using *in vitro* expressed NIT2 protein. The DNA probes used in the assay were the *sre* gene promoter with GATA

sequences, and another region of the *sre* promoter without GATA sequences as a negative control.

The results of EMSA demonstrated that the NIT2 protein does interact *in vitro* with the DNA fragment from *sre* gene promoter with the GATA sequence. NIT2 did not bind to the other DNA fragment that lacks any GATA sequences (Figure 26).

5.2.3 Ferric perchlorate assay of *nit2* mutant

In vitro EMSA revealed that NIT2 protein can interact with the *sre* gene promoter. It was interesting to see whether this protein-DNA interaction has any functional significance. In order to test whether NIT2 has any regulatory function on the iron metabolic pathway, the following experiments were conducted. Both wild type and *nit2* *rip* mutant were grown under high and low iron conditions, and in addition, nitrogen induction and repression conditions were also examined with wild type. Ferric perchlorate assays were conducted to test for activity of the pathway for production of siderophores.

The *nit2* *rip* mutant and wild type were inoculated on low iron and high iron media, and 20mM KNO₃ or 20mM Gln were added to the wild type growth media to apply nitrate induction or N-repression conditions. After 48 hours of growth in liquid medium, the culture media were taken for the ferric perchlorate assay. The assay results (Figure 27) showed that the phenotype of the *nit2* mutant is wild type with respect to the iron metabolic pathway. Moreover, the

wild type showed the same pattern of control of siderophore expression by iron whether nitrogen induction or repression conditions were applied.

Based on both of these tests, it is concluded that NIT2 does not have any influence on the iron metabolic pathway and SRE does not have any influence on the nitrogen metabolic pathway, even though they are both GATA type regulatory factors and both recognize the same kind of GATA DNA sequences. The NIT2 protein interacts with *sre* gene promoter very well in the gel mobility shift experiment *in vitro* but apparently has no effect on *sre* expression *in vivo*.

5.3. Discussion

Eukaryotes possess multiple GATA factors which may exist in the same cells at the same time or under the same growth conditions. There are cases in which different GATA factors are involved in the same process or metabolic pathway, such as Gln3p and Dal80p in *Saccharomyces cerevisiae* (Cunningham et al., 1994). Vertebrate GATA-1 and GATA-2 have some functional overlap (Xu et al., 1997). However, most GATA factors are involved in different cellular activities or metabolic pathways.

In *Neurospora*, a total of at least five GATA factors coexist in the same cells, NIT2 (Fu and Marzluf, 1990), WC-1 (Ballario et al., 1996), WC-2 (Linden and Macino, 1997), SRE (this study), and NGF-1 (Feng, personal communication). The function of these GATA factors are known except for NGF-1. They control different processes of cell metabolism apparently without interfering with each other, even though their DNA binding activities are identical or at least quite similar.

In this study, the possible involvement of NIT2 in iron metabolism and of SRE in nitrogen regulation was studied by an assay for siderophore production and the nitrate reductase assay, respectively. The results revealed that neither of these GATA factors was involved in control of the other metabolic pathway even though the NIT2 protein can bind to

the same DNA probe, the promoter of *sre* gene, as well as the SRE protein.

All of GATA factors in *Neurospora*, including NIT2 (Fu and Marzluf, 1990), WC-1 (Ballario et al., 1996), WC-2 (Linden and Macino, 1997), and SRE, have similar zinc finger DNA binding motifs. These different GATA factors regulate distinct metabolic pathways within the same cells. At least two mechanisms could allow these GATA factors to specifically regulate only their own target genes. The flanking region of the GATA core binding sites might determine the DNA binding specificity of each GATA factor. A second possibility is that each GATA factor interacts with one or more pathway-specific proteins, which determines the regulatory specificity of that GATA factor, the mechanism by which NIT2 regulates its downstream gene, *nit3* (Feng and Marzluf, 1998). It is also quite possible that both mechanisms are involved in providing specific regulatory functions to particular GATA factors. Further analysis of the mechanism of SRE-function will help to elucidate this basic question of regulatory specificity.

CHAPTER 6

MUTATIONAL STUDY OF SRE PROTEIN *IN VITRO* AND FUNCTION *IN VIVO*

6.1. Introduction

All the GATA proteins found so far act as transcription factors. Both *in vitro* and *in vivo* experimental evidence suggest that these transcription factors bind to the promoter regions of their downstream regulated genes. The positive acting GATA factors appear to help to recruit the polymerase II transcriptional machinery into the promoter thereby enhancing the expression of the regulated genes. They can also interact with other general transcription factors to activate transcription of their regulated genes (Crossley et al., 1995; Merika and Orkin, 1995; Kawana et al., 1995; Tsang et al., 1997). On the other hand, the negative regulators can compete with the positive regulators for the binding sites in the promoters to prevent or at least reduce the positive regulator's activation of downstream gene transcription (Cunningham et al., 1994).

A basic feature of the promoter region of the GATA factor regulated genes is the presence of several GATA sequences. These GATA sequences exist alone or in clusters, both of which can be the binding sites of GATA factors. In

vivo evidence suggests that some GATA sequences are not physiologically relevant binding sites for regulatory GATA factors.

GATA factors bind to the GATA sequences in the promoter region of the regulated genes. Removal or mutation of GATA sequences within the promoters results in the loss of control of downstream genes by its regulatory GATA factors. Two mechanisms may determine regulatory specificity, the flanking sequences of the GATA elements (Merika and Orkin, 1993) or pathway-specific regulatory proteins, which can interact with a particular GATA factor (Feng and Marzluf, 1998). It is also possible that both of these mechanisms contribute to the regulatory specificity of GATA factors.

It is a common feature that GATA sequences exist in the promoter region of GATA factor genes themselves. This finding suggests the transcription of a GATA factor gene could be regulated by other GATA factors. It is also possible that autoregulation is involved in the transcriptional regulation of GATA factor genes. Several GATA sequences exist in the *sre* gene promoter. They could be binding sites of other GATA factors or for the SRE protein itself.

The GATA factors found in vertebrates all have two zinc fingers. The C-terminal finger of these GATA factors is involved in DNA binding, whereas the N-terminal finger contributes to DNA binding stability and specificity and to protein-protein interactions with other regulatory proteins

(Crossley et al., 1995; Merika and Orkin, 1995; Kawana et al., 1995; Tsang et al., 1997). Studies suggest that for URBS1, the GATA factor involved in iron metabolism of *Ustilago* and a homologue of SRE, its C-terminal finger is involved in DNA binding. Mutation of the C-terminal finger results in the loss of DNA binding of URBS1 and also a parallel loss of regulation of the iron metabolic pathway. On the other hand, mutation of the N-terminal zinc finger had no effect either on DNA binding or on regulation of the siderophore synthesis pathway (An et al., 1997). Mutational study of the two zinc fingers of SRE would be interesting to investigate this unique double zinc finger GATA factor from *Neurospora*.

It is a common feature that GATA factors can bind specifically to DNA fragments which have GATA sequences. In this study, SRE was expressed as a full length GST fusion protein. The purified GST-SRE fusion protein was used in gel mobility shift assays to test its binding to a DNA fragment that contains GATA sequences. The DNA fragment used in the EMSA was derived from the *sre* gene promoter itself.

In order to study possible functional differences of the two zinc fingers of SRE protein, mutations were introduced to alter each of the fingers of SRE protein, by changing a critical cysteine in the zinc finger to render it nonfunctional. Truncated forms of SRE-GST fusion proteins were expressed as wild type, with the N-terminal finger

mutated, the C-terminal finger mutated, and both zinc fingers mutated. EMSA was used to study the importance of each of the two zinc fingers for DNA binding.

To study the function of GATA factors, *in vitro* experimental evidence alone is not enough to draw decisive conclusions. In order to investigate the possible functional importance of the two zinc fingers of the SRE protein, full length *sre* genes, both the wild type and the identical mutants that affect each or both of the fingers, were transformed into the *sre* *rip* mutant strain, and transformants were picked. Southern blots were used to identify clones which received the full length *sre* gene. The ferric perchlorate assay was used to study the phenotype of these transformants.

6.2. Results

6.2.1. *In vitro* study of SRE protein

Site-directed mutagenesis was applied to remove two small introns from the genomic copy of the *sre* gene because of the possibility of introducing mutations in an RT-PCR product. DNA sequencing was used to check the sequences. A BamHI restriction site was also introduced into the start and the end of the *sre* coding region. This DNA fragment, which has the full length *sre* gene coding region, was inserted into pGEX-2T expression vector in frame.

The recombinant pGEX-2T, which has the full length *sre* gene was introduced into *BL21(DE3)pLySS*. The construct was then expressed to obtain a SRE-GST fusion protein. The full length SRE-GST fusion protein was further purified as described in Material and Methods. GST was also expressed and purified by the same method.

Purified GST-SRE fusion proteins were analyzed by a gel mobility shift assay using the *sre* gene promoter, nucleotides 1 to 400, which has three GATA sequences. Another DNA fragment from the *sre* promoter region which lacks any GATA sequences was used as a negative control.

From the results of gel mobility shift assays, the full length SRE protein can bind specifically only to the DNA fragment which has GATA sequences. GST itself doesn't show any binding to DNA fragments with or without GATA sequences (Figure 28). These experiments suggest that SRE acts in the

same way as other GATA factors, specifically binding to DNA containing GATA sequences.

6.2.2. EMSA of truncated wild type and mutant SRE proteins

In order to study the functional significance of the two zinc fingers of the SRE protein, amino acid substitutions were introduced into the zinc fingers by site-directed mutagenesis to change at least one of the four critical cysteines and several other amino acids in one zinc finger. In some cases amino acid codons were changed to create a unique restriction site to make identification of mutants easier (Figure 29).

Two extra BamHI sites were introduced into the coding region of both the wild type and the different mutant forms of the *sre* gene. The constructs encoding truncated forms consisting of 267 aa of both wild type and mutant SRE proteins were subcloned into the pGEX-2T expression vector. These truncated forms of SRE protein were expressed as GST fusion proteins and further purified by GST affinity chromatography.

The truncated SRE fusion proteins were used in gel mobility shift assays using the same probes as used in the full length SRE EMSA. The results revealed that the wild type truncated SRE protein binds as well as the full length SRE protein to GATA-containing DNA fragments. Mutations in either of the zinc fingers caused significantly weakened DNA

binding. When both of the zinc fingers of SRE were mutated, DNA binding was completely eliminated (Figure 30A,B,C).

Different amounts of protein were also applied to EMSA and a densitometer was used to determine the ratio of DNA probes which were shifted in the EMSA (Figure 30A, B, C; Figure 31). This ratio was compared between the wild type SRE protein and its different mutant forms. The wt SRE protein binds to the correct DNA fragment 5 times stronger than the mutant SRE protein altered in its first zinc finger. The DNA binding ability of the SRE protein with a mutation in its second finger was even lower than the one which had its first finger disrupted. These results suggested that both the N-terminal and C-terminal zinc fingers of SRE are involved in the DNA binding, although the C-terminal finger plays a more important role in the DNA binding. These results are different than found with other two zinc finger GATA factors, in which only the C-terminal finger is needed for DNA-binding.

6.2.3. *In vivo* study of mutant sre gene

In order to compare the function of mutant SRE proteins with the wild type SRE protein *in vivo*, the same mutations altering the finger regions were also introduced into the full length sre gene. The benomyl resistant β -tubulin gene was also engineered into the same plasmid. These plasmids with the β -tubulin gene and the wild-type and different

mutant *sre* genes were transformed into the *sre* *rip* mutant strain. Transformants were selected for benomyl resistance. *Neurospora* with only the wild type β -tubulin gene are benomyl sensitive. Benomyl-resistant transformants were obtained and genomic DNA was isolated from them. Southern blots were employed to detect the presence of the introduced *sre* gene using a distal region of *sre* gene and the β -tubulin gene as DNA probes (Figure 22). Transformants which received an intact copy of the *sre* gene were selected for analysis.

The ferric perchlorate assay was used to test the phenotype of these transformants. If the inserted copy of the *sre* gene is functional, the phenotype of the transformants will be wild type. On the other hand, if the inserted copy of the *sre* gene lacks function, the transformant will still have the *sre* mutant phenotype. The results (Figure 32) demonstrate that the transformed wild type *sre* is fully functional. In contrast, the *sre* genes with mutations that alter either the first finger, the second finger, or both zinc fingers appear to be only partially functional. In particular, the level of siderophores of all the transformants is similar to that of wild-type and distinct from that in the *sre* mutant when they were grown under low iron conditions. The wild-type has only trace amounts of siderophores when growing on high iron medium. In contract, the transformants with mutations affecting either or both SRE zinc fingers produce substantial

amounts of siderophores under high iron, as found with the
sre mutant.

6.3. Discussion

In this study, SRE was expressed as a GST fusion protein. *In vitro* gel mobility shift assays were employed to study the binding ability of this GATA factor. The results revealed that SRE binds specifically to GATA-sequence-containing DNA probes as found with other GATA factors. The DNA probes used in the gel mobility assay were derived from the *sre* gene promoter itself, which suggests a possible autoregulation. However, this possibility must be considered with caution, and further research is needed to address the possibility of autoregulation of *sre* gene expression.

Since SRE is the only GATA factor isolated from *Neurospora* that has two zinc fingers, it was of interest to study the functional significance of its two zinc fingers. Different *sre* mutations were introduced that affect each of the fingers that theoretically should disrupt their function. *In vitro* gel mobility shift assays revealed that both the N-terminal and C-terminal zinc fingers of the SRE protein are involved in DNA binding. This feature is different than found with the vertebrate two zinc finger GATA factors (Crossley et al., 1995; Merika and Orkin, 1995) and URBS1 from *Ustilago* (An et al., 1997). In all of these GATA factors, the N-terminal zinc finger is only involved with DNA binding specificity and interaction with other regulatory proteins, while the C-terminal finger is essential for DNA binding.

In vivo tests were also conducted by transforming the above mentioned different *sre* mutant genes into the *sre* *rip* mutant. SRE phenotypic testing by the ferric perchlorate assay revealed that each of the mutant SRE proteins maintained partial function *in vivo* depending on growth condition. These *in vivo* results are somewhat different from *in vitro* results, in which the different SRE mutant proteins with alterations in either of the zinc fingers retained considerable DNA binding affinity although clearly significantly reduced in comparison with wild-type.

CHAPTER 7

GENERAL DISCUSSION

Iron is an essential component of many enzymes of primary metabolism for nearly all living organisms (Guerinot, 1994). In vertebrates, transferrin and ferritin play a major role in the transport and storage of iron. Both proteins are regulated by the iron concentration inside the cells (De Silva et al., 1996). For many microorganisms, transport of iron is facilitated by small molecular weight compounds known as siderophores. The cells possess a membrane transport system which transports siderophore-iron complexes (Guerinot, 1994). Different fungal species use different kinds of siderophores for iron transport. *Aspergillus* uses ferrichrome and ferrichrome C, while *Penicillium* employs ferrichrome, ferrirubin and coprogen for this purpose (Huschka et al., 1985). In *Ustilago maydis*, both ferrichrome and ferrochrome-A are used for iron transport. As in bacteria, iron metabolism may be related to pathogenic behavior in filamentous fungi since iron is tightly sequestered in all host cells due to the toxicity of this metal. Two genes involved in iron metabolism have been cloned from *Ustilago*. One of them, *sid1*, encodes the first enzyme in the siderophore synthesis

pathway-L-ornithine-N⁵-oxygenase (Mei et al., 1993); the other, *urbs1*, is a negative regulator which controls this pathway (Voisard et al., 1993). A recent study showed that URBS1 binds to GATA sites in the promoter region of the *sid1* gene (An et al., 1997) and that its second zinc finger is required for DNA binding, which is essential for iron-mediated repression (An et al., 1997).

In this study, a PCR based method led to the isolation of a new gene, designated *sre*, from *Neurospora*. Sequencing of the gene revealed that it encodes a new GATA factor which contains two zinc fingers, similar to GATA factors found in vertebrates. The deduced amino acid sequence of SRE revealed significant identity in the two zinc finger regions and also in a Cys-rich zinc finger intervening region to the siderophore regulator URBS1 from *Ustilago maydis* (Voisard et al., 1993), SREP from *P.chrysogenum* (Haas et al., 1997), and Gaf2 of *Schizosaccharomyces pombe* (Hoe et al., 1996). An SRE mutant strain was created by the rip procedure (Singer et al., 1995) and its phenotype was analyzed. The results of growth tests, colormetric tests, thin layer chromatography and assays of L-ornithine-N⁵-oxygenase, the first committed step in siderophore biosynthesis, all indicated that *sre* encodes a negative regulator of siderophore biosynthesis. The negative regulatory function of SRE was further confirmed by a heterokaryon test and gene complementation. Both a *sre*⁺/*sre*⁻

heterokaryon and wt *sre*⁺ gene complemented *sre*⁻ strains showed the wild type phenotype.

SRE was also expressed as a SRE-GST fusion protein. *In vitro* EMSA showed that SRE can interact specifically with DNA fragments with GATA sequences. SRE with mutations in either the first finger, the second finger, or both zinc fingers were also expressed as SRE-GST fusion proteins, and EMSA with these mutated proteins revealed that mutations in either of the zinc fingers weakened its DNA binding. However, *in vivo* experiments revealed that *sre* gene with the same mutations partially maintained their function.

Northern blots revealed that *sre* mRNA is present at the same level in both high and low iron condition. In order to execute its regulatory function, SRE has to act differently under different growth conditions. There are at least two obvious possibilities: either through controlling the SRE protein level or by controlling SRE protein activity. The SRE protein level could be regulated by translational control of *sre* mRNA, which could be influenced by the iron concentration in the living cell, as found with translational control of ferritin protein. Another possibility is that there could be a different rate of SRE protein turnover, depending upon the cellular iron concentration; e.g. SRE might turnover rapidly when a low amount of iron is present. In both of these cases, there will be different amounts of the SRE protein depending on the iron concentration in the cell. Another possibility is

that the cell maintains the same amount of SRE protein, but SRE is only active during high iron conditions. In this case, the same amount of SRE protein would exist in both high iron and low iron conditions. Western blot analysis will be a useful way to determine the amount of SRE protein in cells subject to various iron concentrations, and thus will help to distinguish between these possibilities.

In vitro EMSA revealed that SRE binds specifically to a DNA fragment with GATA sequences, which was derived from its own promoter. This finding raises the possibility of autoregulation of *sre* gene transcription. Since SRE is a negative regulator, this regulation could be used to maintain an optimal SRE protein level in the cell. A reporter gene under control of *sre* gene promoter could be transformed into wild type or *sre rip* mutant strains to test this possibility.

All of the tests that were applied to wild type and the *sre rip* mutant revealed that siderophore synthesis is only partially constitutive in *sre rip* mutant; i.e., control of siderophore synthesis was not totally lost due to the complete absence of SRE protein. This finding suggests the existence of other positive or negative regulators in the same metabolic pathway. In *Ustilago*, URBS1 interacts with GATA sequences in the *sid1* gene promoter to negatively regulate *sid1* gene expression. Based on this result, together with the finding that SRE is very active in sequence-specific DNA binding, one can readily predict that SRE executes its

function by binding to GATA sequences in the promoter region of its downstream-regulated genes. By competing with a positive regulator for the GATA binding sites in these promoters, SRE could negatively control the siderophore synthesis pathway. It can also be speculated that an as yet unidentified positive regulator, which competes with SRE for GATA DNA binding sites, might be another GATA factor. Degenerate PCR or affinity chromatography can be employed to identify and isolate this postulated GATA factor. A good example of this kind of competition is that of Gln3P, a positive GATA factor, and Dal80P, a negative GATA factor, since both of them regulate the same metabolic pathway and have opposite functions (Minehart and Magasanik, 1991, Cunningham and Cooper, 1991).

Some of the previously found GATA factors execute their function not only by binding to the promoters of their regulated genes but also through protein-protein interactions with other positive or negative regulators. A reasonable speculation is similar protein-protein interactions may exist between SRE and other regulators. Because of the negative regulatory nature of SRE, it is possible that SRE can interact with a positive regulator and suppress its positive function. Mutational analysis could be applied to the SRE protein to identify any region of SRE that mediates such a function. The same region could be used in the yeast two

hybrid system to identify any possible regulatory proteins that can interact with SRE.

It is important to emphasize that the striking regulation of siderophore synthesis is dependent upon iron concentration in the cell. It will be of great interest to determine if iron is the critical regulator of the whole process of siderophore synthesis and how iron or some other primary regulator executes the control function. If the same SRE protein level is maintained in both high and low iron conditions, understanding the mechanism that modulates the functional activity of SRE will be very informative, and is probably related to the iron concentration in the cell. Sequence comparison between SRE and URBS1 revealed that a highly conserved Cys-rich region is located between the two zinc fingers of both proteins. This conserved region could reflect the functional similarity of these two proteins. Mutagenesis of this region and both *in vitro* and *in vivo* tests could be used to study the functional significance of this conserved region of SRE. It will not be surprising if a connection is found between this conserved region and SRE's response toward iron; e.g., this motif might be an iron binding domain of SRE protein.

Based on all the experimental evidence and the above analysis, I propose a simple working model to explain the regulatory mechanism of the SRE protein (Figure 33). SRE could carry out its function by both binding to the promoter

region of its regulated genes and by binding to other positive regulators that control the same pathway. By binding to the promoter region of its regulated genes, SRE could inhibit the binding of other positive regulators at the promoter so as to repress the expression of its regulated genes. By binding to one or more other pathway-specific or global positive regulators through protein-protein interactions, SRE could also directly inhibit the function of such positive regulators. SRE could keep the activity of the siderophore synthesis pathway at a certain level by the postulated protein-DNA and protein-protein binding.

The *sre* *rip* mutant produces much more siderophores than wild type in low iron condition, presumably due to the lack of SRE protein. It is possible that SRE with mutations in either or both of the two zinc fingers still maintains important protein-protein interaction, and in low iron conditions, the mutant SRE proteins may carry out the same functions as does the wild type SRE protein, thus maintaining the production of siderophores at a similar level as that in WT. Northern blots showed that *sre* gene expression is at the same level both during high and low iron conditions. However, SRE must function differently under high and low iron conditions. Iron itself is a probable regulator of this process. Iron might regulate the translation of *sre* mRNA or turnover of SRE protein thus controlling the cellular amount of SRE protein. Alternatively, iron might regulate an

activity of SRE, such as DNA binding, which would result in a different behavior of SRE during different growth conditions. Further experimental approaches are needed to test this working model and other critical features of the SRE protein. Or the possible existence of other SRE model that can better explain iron metabolism in *Neurospora*.

The vertebrate GATA factors all have two zinc fingers, and all appear to have a positive function. It was found that these transcription factors can interact with various proteins whereby the zinc finger regions, especially the N-terminal finger are not only necessary for DNA recognition but also for these interactions. For example, GATA-1 interacts with SP1, a general transcription factor, and with EKLF, an erythroid Kruppel-like factor, in order to cooperatively regulate cell-specific erythroid gene expression at the transcriptional level (Merika and Orkin, 1995). Most recently, a cofactor of GATA-1, FOG, was isolated utilizing the yeast two hybrid system employing the N-terminal zinc finger as bait. This finding underscores the importance of the zinc finger for "cross talk" with the basic transcription machinery (Tsang et al., 1997). Similar functions may be anticipated for the fingers of SRE from *Neurospora*.

The invertebrate single zinc finger GATA factors can act as positive regulators, like NIT2 from *Neurospora* (Fu and Marzluf) and AREA from *Aspergillus* (Kudla et al., 1990), or

as negative regulators, like DAL80p from *S. cerevisiae* (Cunningham and Cooper, 1991) and PANNIER from *Drosophila* (Ramain et al. 1993). A general mechanism derived from this research is that the negative GATA factors compete with positive GATA factors for the same DNA binding sites at their regulated genes. SRE has two zinc fingers and it has a negative function, while the two zinc finger GATA factors from vertebrates both have a positive function. It will be interesting to determine the mechanism by which SRE negatively regulates its downstream genes. If it competes with another positive-acting GATA factor for binding, it is of great interest to identify and determine the features of the other GATA factor.

For the vertebrate two zinc finger GATA factors, the N-terminal zinc finger is involved in protein-protein interactions with other general transcription factors to establish its positive function. In the case of SRE and URBS1, which are negative regulators, it will be interesting to find any protein-protein interactions between these GATA factors with other proteins, possibly mediated by the N-terminal zinc finger. It will be very informative to determine the nature and function of other proteins which interact with SRE.

Zinc type	Approximate consensus sequence	Function	Structure
Cys ₂ His ₂ (TFIIIA)	C-X ₂₋₄ -C-X ₁₂ -H-X ₃₋₅ -H	Nucleic acid binding	NMR, x-ray
Cys ₈ (steroid-thyroid receptor)	C-X ₂ -C-X ₁₃ -C-X ₂ -C-X ₁₅ -C-X ₅ -C-X ₁₂ -C-X ₄ -C	DNA binding Oligomerization	NMR, x-ray
Cys ₆ (GAL4)	C-X ₂ -C-X ₆ -C-X ₆ -C-X ₂ -C-X ₆ -C	DNA binding	NMR, x-ray
Cys ₃ HisCys ₄ (RING finger)	C-X ₂ -C-X ₉₋₂₇ -C-X ₁₋₃ -H-X ₂₋₃ -C-X ₂ -C-X ₄₋₄₈ -C-X ₂ -C	Protein-Protein interaction? Nucleic acid binding?	NMR
Cys ₂ HisCys (retroviral nucleocapsid)	C-X ₂ -C-X ₄ -H-X ₄ -C	Single-stranded nucleic acid binding	NMR
Cys ₂ HisCys ₅ (LIM domain)	C-X ₂ -C-X ₁₇₋₁₉ -H-X ₂ -C-X ₂ -C-X ₂ -C-X ₁₆₋₂₀ -C-X ₂₋₃ -C	Protein-protein interaction, DNA binding?	NMR
Cys ₄ (GATA-1)	C-X ₂ -C-X ₁₇ -C-X ₂ -C	DNA binding	NMR
Cys ₃ His (Nup475)	C-X ₆₋₁₄ -C-X ₄₋₅ -C-X ₃ -H	Unknown	Unknown
Cys ₄ HisCys ₃ (requium)	C-X ₂ -C-X ₁₁₋₁₂ -C-X ₂ -C-X ₄ -H-X ₂ -C-X ₁₄₋₁₇ -C-X ₂ -C	Unknown	Unknown

Table 1. Selected families of zinc-binding domains. C and H denote Cys and His, and X represents other amino acids. NMR, nuclear magnetic resonance (From Berg and Shi, 1996).

Strain	Relative enzyme activity			
	39hrs		45hrs	
	H-Fe	L-Fe	H-Fe	L-Fe
Wild Type	0.005 (0.002)	0.268 (0.067)	0.068 (0.021)	0.550 (0.107)
Sre mutant	0.242 (0.039)	0.127 (0.038)	0.128 (0.031)	0.234 (0.025)

Cells were grown on high iron or low iron medium for different period of time. Total protein from the cells was isolated and enzyme assay was done as described in Material and Methods.

The absorbance at 520nm was taken for each sample and the value was standardized by the amount of protein used in the assay. The number in parentheses is the deviation obtained by averaging the deviation of five independent parallel assays.

Table 2. L-ornithine N⁵-oxygenase enzyme assay in *N. crassa*

Figure 1. Sequence of cGATA-1 DNA binding domain and binding site. (A) Sequence of the cGATA-1 DNA binding domain (residues 158 to 223). The region forming the single helix is boxed. Open squares represent residues involved in maintaining the structural integrity of the zinc finger, whereas closed and open circles are residues that interact with the DNA in the major and minor grooves, respectively. (B) Sequence of the 16-bp DNA illustrating sites of contact with the cGATA-1 zinc finger DNA binding domain. Open squares represent the region of the oligonucleotide interacting with the cGATA-1 DNA binding domain. Closed and open circles are sites of interaction with bases in the major and minor grooves, respectively, and closed and open squares are sites of interaction with the sugar or phosphate or both in the major or minor grooves, respectively (from Omichinski et al., 1993).

Figure 1

Figure 2. Two degenerate primers with the conserved zinc finger region they amplified. Y=T/C; M=A/C; P=A/G; N=any NT.

5'ACNCCNYTNTGGMG 3'
 ----->
 5'acaccattgtggcgaagggacgaagccggtcataccatttgcaacgcttgcggtct 3'
 T P L W R R D E A G H T I C N A C G L
 3'tgtggtaccaccgcttccttgccttcggccagtatggtaaacgttgcgaacgccaga 5'
 <-----
 3' ACPTTPCGNACPCCNPA 5'

Figure 2

Figure 3. Southern blot analysis of single restriction enzyme digested cosmid DNA. The different enzymes used in the DNA digestion are shown. The DNA probe used is an ECORI fragment from the cosmid, which has the new GATA factor gene as confirmed by DNA sequencing.

Figure 3

Figure 4. Southern blot of double restriction enzyme digested cosmid DNA. The different combinations of restriction enzymes used in the digestion are shown. The DNA probe used is an EcoRI fragment from the cosmid, which has the new GATA factor gene as confirmed by DNA sequencing.

Figure 4

Figure 5. Southern blot of restriction enzyme digested cosmid DNA and *Neurospora* genomic DNA. C, cosmid DNA, G, genomic DNA. The two enzymes, BamHI and HindIII, employed are identified for the appropriate lanes. The DNA probe used is an EcoRI fragment from the cosmid, which has the new GATA factor gene as confirmed by DNA sequencing.

Figure 5

Figure 6. Restriction map of the 6kb HindIII-SacI fragment of the *N. crassa* genomic DNA containing the *sre* gene. The open reading frame is indicated by the box. The two black segments represent the two introns.

Figure 6

Figure 7. The entire DNA sequence and deduced amino acid sequence of the *N. crassa sre* gene. The amino acid sequence is represented by uppercase letters above the DNA sequence. GATA sites in the promoter region are indicated by arrows. The two zinc fingers are underlined and the region between the zinc fingers, which shows high homology with the same region of URBS1 and SREP is also underlined. Non-coding regions are represented by lowercase letters. The stop codon is indicated by an asterisk.

	ggt	accttgtggg	tcacttattc	ccttgcagca	-1152
tctggctccc	caagtcgagg	ggaccagtga	cactggacct	gagaaaggac	-1102
ctttttcatc	ctgtagccag	cccaattcca	cgtcccatga	tctcaaatac	-1052
ggtctcccaa	acaagtggga	aaacgggttt	gatttttgtt	gtgccaaatc	-1002
ctctctatgt	cgtactgtaa	aatcaaaccg	ctttgttctc	ttgtggccct	-952
ccttcagggt	aagacactgt	cgaaccttcc	tccttccag	acgtgtttgt	-902
ccttggcaac	gactacttag	cagacgaacc	cctgggtgaa	gccctcctgc	-852
gtacggtatc	caaccgtctc	tcagcccttt	ggttcgccag	gattatccgt	-802
****				****	
agatcagata	acagatctgt	ggagcagtgg	agtagtaatc	agaagcacaa	-752

cagagactct	tgaggcaaag	gggggaagga	tatgcagaca	ttcgtctgtc	-702
cactcaccta	gaagacgcaa	aaagacagaa	agacgacaga	aaacctcaag	-652
accagccttt	ggcccagacc	cgccctcttt	tttgctttgc	cttttccgtc	-602
caccaaccct	tcagttttat	ctgctacctc	ggatcccagc	cgacttcgcc	-552
	***	^			
atcctcgtgc	actctaccgg	agtacacaac	cgtccaggtc	ttgccaccaa	-502
cgcgaaaccg	ctcgtaccac	aacctggcgc	cgtgtccaag	ccttttcttc	-452
cagcataacc	aacttctgat	tcagcggcac	cctgctgcgg	tctgtagtac	-402
tcgcttcctc	tggccatttg	accccatcaa	tcgcctgttt	cggacaccca	-352
acacgtgctg	ttctcctggg	accggcgctc	tgtattggcc	ccggatcccg	-302
cctggtcctt	agcgcacatt	gcccagaagt	tgggggtggc	tacttgccat	-252
ccaagtacaa	ctccaacgat	cgatctcggc	atgtgtttga	ccgccgacct	-202
gcataaccgtt	tccgcctctt	gacgatcacg	gagcgatatt	gaagccagcc	-152

tgcgcatccc	caagactccc	ggcgccgtac	aacatccact	ttgtttttca	-102
acaggctttc	aaacacattg	tgtcccttct	caagacggat	aactagcctt	-52
			***	^	
gagcatacat	attgttaggt	tgagatccat	acgcaaacac	attgagaagg	-2
M A L	P D D T	R L M	N N Q	T S I E	(17)
aATGGCATTG	CCGGATGACA	CTCGCCTTAT	GAACAACCAA	ACTTCCATCG	49
G N S	A P D	R M A L	P S R	P P P	(33)
AAGGTAACTC	GGCACCTGAT	CGTATGGCTC	TTCCATCAAG	GCCACCCCCA	99
P T A S	N E P	P E H	E S T G	P V D	(50)
CCGACCGCAA	GTAACGAGCC	CCCGGAGCAC	GAGTCAACCG	GACCGGTGTA	149
E A A	S K S E	S G L	S P N	S K K A	(67)
CGAGGCGGCC	TCCAAGTCCG	AATCCGGGCT	CTCGCCGAAT	TCAAAGAAAG	199
G H A	A Q S	P S G R	K K S	P K I	(83)
CGGGGCATGC	TGCGCAGAGT	CCGTCTGGTC	GCAAGAAGTC	TCCAAAATC	249
Q N A Q	P A Q	S P A	E T V G	V V N	(100)
CAAAATGCC	AACCAGCACA	ATCTCCAGCC	GAAACCGTGG	GGGTGGTGAA	299
V A T	L G L V	P S V	Q G H	A G Q V	(117)
CGTTGCAACG	CTGGGACTTG	TGCCTTCTGT	TCAGGGTCAT	GCGGGTCAAG	349
C S					(119)

TCTGCAGgta	tgaatgtccc	tttcctaattg	actgaatctg	taggccagcg	399

(continued)

Figure 7

Figure 7 (continue)

			N	C		G	T	T		H	T	P		L	W	R	R	(131)

ctaacgactg	agtagCAATT	GTGGTACGAC	ACATACTCCT	CTTTGGCGAA														449
S P Q	G A I	I C N A	C G L	Y L K														(147)

GATCACCTCA	AGGAGCCATC	ATTTGCAACG	CTTGCGGACT	GTACCTAAAG														499
A R N A	A R P	A N I	R R P P	S V M														(164)
GCGCGTAACG	CGGCTCGCCC	CGCCAACATC	AGGCGCCCTC	CCAGCGTGAT														549
A S N	V R Q A	A A K	L S P	K K A T														(181)
GGCGAGCAAT	GTTAGGCAGG	CCGCGGCCAA	ATTGTCCCCC	AAAAAAGCAA														599
A P L	L P S	N P G A	T Y V	A A D														(197)
CCGCACCGTT	ACTGCCAAGC	AACCCGGGAG	CAACGTATGT	GGCCGCCGAT														649
Q T P S	G S C	P G G	G R C N	G T G														(214)

CAGACGCCGT	CAGGATCGTG	CCCTGGAGGC	GGAAGATGCA	ACGGCACAGG														699
G A E	G C G G	C P A	Y N N	R V S K														(231)

TGGTGCGGAG	GGGTGTGGTG	GTTGCCCCGC	ATACAACAAC	CGCGTTTCCA														749
S A S	L N V	L K C Q	G A A	A A S														(247)

AGAGTGCGAG	TCTCAACGTG	CTAAAGTGTC	AGGGCGCAGC	AGCCGCGTCT														799
S K K P	Q A A	E G S	G E E P	T E M														(264)
TCCAAGAAAC	CTCAAGCAGC	AGAGGGCAGC	GGGGAAGAAC	CGACTGAGAT														849
D I T	A L H V	Q S Q	N T T	V V I A														(281)
GGACATCACC	GCCTTACACG	TACAATCGCA	AAACACAACC	GTTGTTATTG														899
C Q N	C G T	T I T P	L W R	R D E														(297)

CTTGCCAAAA	CTGCGGTACT	ACCATTACTC	CGCTCTGGCG	AAGGGACGAA														949
A G H T	I C N	A C G																(307)

GCCGGTCATA	CCATTGCAA	TGCGTGCGgt	aagtgcatga	acttatggcc														999
				L Y														(309)
tgaaaagtta	caaatgggat	ggaatttgct	aactcgagta	aagGTCTCTA														1049
Y K L	H G V H	R P V	T M K	K A I I														(326)
TTACAAGTTG	CACGGTGTGC	ATCGGCCAGT	GACCATGAAA	AAGGCAATAA														1099
K R R	K R V I	P A A	G G D	A E I														(342)
TCAAACGGAG	AAAACGTGTG	ATTCCTGCCG	CCGGAGGTGA	TGCCGAAATT														1149
E P S E	A P D	S P P	A T S E	P P M														(359)
GAGCCAAGTG	AGGCTCCAGA	TTCACCACCC	GCGACATCAG	AACCTCCGAT														1199
E K G	T V N E	D G S	V N L	G I R R														(376)
GGAGAAAGGT	ACGGTCAACG	AAGATGGATC	TGTTAATCTC	GGCATCCGCC														1249
R S V	R P L	T L V P	E D E	L R R														(392)
GACGTTTCAGT	GCGACCATTG	ACGCTCGTGC	CTGAGGACGA	ATTGCGGCGA														1299

(continued)

Figure 7

Figure 7 (continue)

N R Q A S P L S S A A L G Q Y H S	(409)
AACAGACAGG CATCCCCGCT GTCTTCAGCC GCCTTGGGAC AGTACCACTC	1349
S H T N Q P H H G A H A S L T Y E	(426)
ATCTCATACA AATCAACCAC ACCACGGGGC TCATGCATCT CTCACTTACG	1399
N R L A P I H S L A L P V D R Q	(442)
AGAATCGACT GGCTCCCAT T CATTCACTGG CGCTCCCAGT TGATCGTCAA	1449
A S I S P A S F L S P S R K R S I	(459)
GCGTCCATTT CACCAGCATC ATTTCTGTCC CCATCGAGAA AGCGGTCGAT	1499
S A V E N E P S S H N D N E S H K	(476)
ATCTGCTGTA GAGAACGAAC CTTCTGTCTCA TAATGACAAC GAAAGTCATA	1549
R L S S I K S I L N P M P S S E	(492)
AGCGCCTGTC TTCCATCAAA TCCATTCTCA ACCCGATGCC GAGTTCTGAG	1599
I H R E V S P A D Q L R M Q P A S	(509)
ATACACCGAG AGGTTTCGCC GGCAGACCAG TTACGGATGC AACCAGCCTC	1649
R S P A M S S L T P A H S P G S F	(526)
CCGGTCCCCA GCCATGAGCA GTCTGACTCC AGCACACAGT CCAGGGTCAT	1699
S N S T V I G T P T S A P I L R	(542)
TTTCAAACAG TACGGTTATT GGGACACCAA CATCAGCTCC GATTTTGCGG	1749
S A S R D T H N D S E R M K A E R	(559)
AGCGCCTCGA GAGATACCCA CAATGATAGC GAGCGGATGA AGGCAGAGCG	1799
R A A L E R E A E M M R E L L A A	(576)
AAGAGCAGCG TTGGAGCGCG AGGCCGAAAT GATGAGAGAG TTGCTGGCCG	1849
K E R E L A E L G Y D *	(587)
CAAAGGAACG GGAGCTTGCC GAGTTGGGT T ATGATTGAca tgcggaggct	1899
gaaaaagcgg catgctgtga attcggggga aacaactgga gttattcggg	1949
agagcatacc catggaattt gacttgtgca tagatagata gcaacggaag	1999
gcatagtctt tttgaggaaa tcccaatgac agttggagcc agagacctt	2049
agtcatagta tccgcatagt ggttatgcgt ctattcatcg atgccgtcca	2099
gtggatttgg ttttgcatag taatagccgt ctcatggctg taaaccctaa	2149
ttgcaacacc ctcgctgcaa tggcgaagtg tgatgcggca atggaagttt	2199
caaagttcca gacggaaagc ccctgccttg acatgccgta ggagccagtg	2249
acaagaggcc gcgcacgctt gcatcagcgt cgtagggcgg agctc	2299

Figure 7

Figure 8. RT-PCR amplification of *sre* mRNA covering the two small introns.

Figure 8

Figure 9. Comparison of amino acid sequences of *N.crassa* SRE protein with *P. chrysogenum* SREP protein. The top sequence is SREP, and the bottom sequence is SRE. Four conserved regions are found, which include the two zinc fingers, a cysteine-rich region between the two zinc fingers, and a small segment in the 3' region. Identical amino acids in both proteins are denoted by black dots.

```

      10      20      30      40      50
      |      |      |      |      |
      MLASLPQMEALHSLPRNQVVPMPRQPSAEDLDAAQQLISSAQAGREHLADHYRDDGM
      .
MALPDDTRLMNQTSIEGNSAPDRMALPSRPPPTASNEPPEHESTGPVDEAASKESGLSPNSKKAGHAAQSPSGRKKS
      |      |      |      |      |
      10      20      30      40      50      60      70
      |      |      |      |      |      |      |
      60      70      80      90      100     110     120     130
      |      |      |      |      |      |      |      |
      VQRSGQPGSNQWNGHNETPSEKTS PSSSQKDTTFLGHSCSNCGTKSTPLWRRSPTGAMICNACGLYLKARNVARPTKRNRM
      .
      PKIQNAQPAQSPAETVGVVNVATLGLVPSVQGHAGQVCSNCGTTHTPLWRRSPQGAIICNACGLYLKARNAARPANIRRP
      |      |      |      |      |      |      |      |
      80      90      100     110     120     130     140     150
      |      |      |      |      |      |      |      |
      140     150     160                               170     180     190     200
      |      |      |      |      |      |      |      |      |      |
      QSEGAEKPPPPASSHCGGSSEGGGCKGS-----CPRGGSCNGTGGAEAGCDGCPAYNNRIYKSATRGAAAI
      .
      PSVMASNVQAAAKLSPKKATAPLLPSNPGATYVAADQTPSGSCPGGRCNGTGGAEAGCGCPAYNNRVSKSASLNLVKC
      |      |      |      |      |      |      |      |      |      |
      160     170     180     190     200     210     220     230
      |      |      |      |      |      |      |      |      |      |
      210     220     230                               240     250     260     270
      |      |      |      |      |      |      |      |      |      |
      QSSWGRHQAHEPEPATQEETPVKAPAPADAS-----NTLVACQNCGTTVTPWRRDEQGHPICNACGLYYKLHG CYRPTN
      .
      QGAAAASSKKPQAAEGSGEETEMDITALHVQSQNTTVVIACQNCGTTITPLWRRDEAGHTICNACGLYYKLHG VHRPVT
      |      |      |      |      |      |      |      |      |      |
      240     250     260     270     280     290     300     310
      |      |      |      |      |      |      |      |      |      |
      280     290     300     310     320     330     340     350
      |      |      |      |      |      |      |      |      |      |
      MKKSIKRRKRVPALRDQSPTAGTLSSNGSSTSPEASPAALAHGHDDHYRMSSEPGDHYQMMPGKTEDAPRALPFAPP
      .
      MKKAIKRRKRVI PAAGDAEIEPSEAPDSPPATSEPPMEKGTVNEDGSVNLGIRRRSVRPLTLVPEDELRNRQASPLS
      |      |      |      |      |      |      |      |      |      |
      320     330     340     350     360     370     380     390
      |      |      |      |      |      |      |      |      |      |
      360     370     380     390     400     410     420     430
      |      |      |      |      |      |      |      |      |      |
      PVDFTGYNVSSVPLAHGPSIPHGLPKLLEVERLGHSPVSQYGRRSVSPNTLNVPKKRTLAEAAATEALPAPSTLEAGSNQL
      .
      SAALGQYHSSHTNQPHHGAHASLTyenRLAPIHSLALPVDRQASISPASFLSPSRKRSISAVENEPSSHNDNESHKRLSS
      |      |      |      |      |      |      |      |      |      |
      400     410     420     430     440     450     460     470
      |      |      |      |      |      |      |      |      |      |
      440     450     460     470     480     490     500
      |      |      |      |      |      |      |      |      |      |
      PPIMSSANPTPPGRLSSIILNQSDVRGEPRSDNNLGRPPPIHHSVSPSPHPPQPLPGLASLGDSWD-----RR
      .
      IKSILNPMPSSEIHREVSPADQLRMQPASRSPAMSSSLTPAHSPGSFSNSTVIGTPTSAPILRSASRDTHNDSERMKAERR
      |      |      |      |      |      |      |      |      |      |
      480     490     500     510     520     530     540     550
      |      |      |      |      |      |      |      |      |      |
      510     520     530
      |      |      |
      ARLEREAEQMREMLRAKERELAEMRR•
      .
      AALEREAEEMRELLAAKERELABLGYD•
      |      |      |
      560     570     580

```

Figure 9

Figure 10. Comparison of aa sequences of *N.crassa* SRE protein with other GATA factors. (A) Comparison of N-terminal zinc finger of *N.crassa* SRE with the N-terminal zinc finger from other GATA factors. (B) Comparison of C-terminal zinc finger from *N.crassa* SRE protein with the C-terminal zinc finger of other GATA factors and with the zinc finger of the single finger GATA factors. (C) Comparison of the highly conserved region between the two zinc fingers from *N.crassa* SRE, *U.maydis* URBS1, and *P.chrysogenum* SREP. The consensus sequences deduced from each comparison are shown at the bottom. hGATA-1 is human GATA-1, cGATA-1 is chicken GATA-1, and mGATA-1 is mouse GATA-1, AREA is from *Aspergillus nidulans*, NIT2 is from *Neurospora crassa*, Dal80p and Gln3p are from yeast, and Gaf2p is from *Schizosaccharomyces pombe*.

(A)

hGATA-1 (1) CVNCGATATPLWRRDRTGHYLCNACGLYHKMNGQNRPLIRPKRLIVSKR

cGATA-1 (1) CVNCGATATPLWRRDGTGHYLCNACGLYHRLNGQNRPLIRPKRLLVSKR

mGATA-1 (1) CVNCGATATPLWRRDRTGHYLCNACGLYHKMNGQNRPLIRPKRMIVSKR

URBS1 (1) CSNCGVTSTPLWRRAPDGSTICNACGLYIKSHSTHRSASNRLSGSKASPP

SREP (1) CSNCGTKSTPLWRRSPTGAMICNACGLYLKARNVARPTKRNRMQSEGAEK

SRE (1) CSNCGTTHSTPLWRRSPQGAII CNACGLYLKARNAARPANIRRPSPVMASN

Consensus. C.NCG...TPLWRR...G...CNACGLY.....R.....

(B)

hGATA-1 (2) CTNCQTFTTTTLWRRNASGDPVCNACGLYYKLHQVNRPLTMRKDG IQTRNR

cGATA-1 (2) CSNCQTSTTTTLWRRSPMGDPVCNACGLYYKLHQVNRPLTMRKDG IQTRNR

mGATA-1 (2) CTNCQTFTTTTLWRRNASGDPVCNACGLYYKLHQVNRPLTMRKDG IQTRNR

AREA CTNCFTQTTPPLWRRNPEGQPLCNACGLYEKLHGVRPLSLKTDVIKKRNR

Da180p CQNCFTVKTPPLWRRDEHGTVLCNACGLFLKLHGEP RPISLKTDTIKSRNR

Gln3p CFNCKTFKTPLWRRSPEGNTLCNACGLFQKLHGTMRPLSKSDVIKKRISK

NIT-2 CTNCFTQTTPPLWRRNPDGQPLCNACGLFLKLHGVRPLSLKTDVIKKRNR

URBS1 (2) CTNCQTFTTTPLWRRDEDGNNICNACGLYHKLHGTHRPIGMKKS VIKRRKR

SREP (2) CQNCGTTVTPLWRRDEQGHPICNACGLYYKLHGCYRPTNMKKSIIKRRKR

SRE (2) CQNCGTTITPLWRRDEAGHTICNACGLYYKLHGVRPVTMKAIIKRRKR

Consensus C.NC.T..T.LWRR...G...CNACGL..KLHG..RP.....

(C)

URBS1 GSCP GDGLCNGTGGTASCSGCPAYNNNL SHAL

SREP GSCP GGGSCNGTGG AEGCDGCPAYNNRIYKSA

SRE GSCP GGGRCNGTGG AEGCGGCPAYNNRVSKSA

Gaf2p GTCAGD GFCNGTGG SASCTGCPALNNRIRSLN

Consensus G C G G CNGTGG...C.GCPA NN.....

Figure 10

Figure 11. Schematic representation of the RIP procedure in *Neurospora crassa*. Fertilization results in formation of a diploid cell which has two parental haploid nuclei. The cell will undergo several rounds of mitosis, then karyogamy and meiosis. The four possible progeny of meiosis are shown. The open box represents the target gene, which is duplicated in the transformed strain, which contains both the resident gene and a second copy shown on a separate chromosome. Both copies are subject to multiple G/C to A/T mutations (RIP). The hatched box indicate the mutated gene. The open box with a lower case m indicates the marker used for the transformation.

Figure 11

Figure 12. Southern blot analysis of genomic DNA from wt, wt with one extra copy of *sre* gene, and progeny from a cross of the above two strains. P1 is wt *Neurospora* with one extra copy of the transformed *sre* gene in the genome. P2 is wt. The other three lanes are genomic DNA isolated from three different progeny. All of these DNA samples were digested with BamHI. The β -tubulin gene was used as an internal control.

Figure 12

Figure 13. Iron transport pathway in fungi. The right half of the figure represents the iron transport system in the cell membrane. The left half displays the steps that lead to the synthesis of siderophores, and several assay methods to detect the activities of this biosynthetic pathway.

Figure 13

Figure 14. Structure of siderophores. (A) Structure of ferrichrome-type siderophores showing the *cis* configuration about the iron center and three ornithyl-*N*-acyl groups, which surrounds iron. Ferrichrome: $R = -CH_3$, $R' = R'' = R''' = -H$; ferricrocin: $R = -CH_3$, $R' = R''' = -H$, $R'' = -CH_2OH$; ferrichrysin: $R = -CH_3$, $R' = -H$, $R'' = R''' = CH_2OH$; ferrirubin: $R = -CH = C(CH_3) - CH_2 - CH_2OH(trans)$, $R' = -H$, $R'' = R''' = -CH_2OH$. (B) Structure of ferrichromes synthesized from D-ornithine and glycine or D-serine, showing a Δ -*cis* configuration about the iron center and iron-surrounding residues. R, R', R'', and R''' are assigned as in (A). (C) Ferricrocin derivatives. O-(phenyl-carbamoyl)-ferricrocin: $R'' = -CH_2 - O - CO - NH - Ph$; O-(sulfanilyl-carbamoyl)-ferricrocin: $R'' = -CH_2 - O - CO - NH - SO_2 - Phe - NH_2$. (D) Structure of coprogen-type siderophores containing a Δ -*trans* configuration about the iron center and iron-surrounding residues. CoprogenB: $R_1 = R_2 = -CH = C(CH_3) - CH_2 - CH_2OH(trans)$, $R_3 = H$; Coprogen: $R_1 = R_2 = -CH = C(CH_3) - CH_2 - CH_2OH(trans)$ (From Huschka et al., 1985).

Figure 14

Figure 15. Metabolic pathway of *arg-5,ota,aga* triple mutant. Biosynthetic pathways for arginine, proline, polyamines, and associated intermediary metabolism in *Neurospora*, showing genes responsible for steps in biosynthesis and arginine catabolism. Three mutations, *arg-5*, *ota*, and *aga*, occur in the triple mutant strain which cannot synthesize ornithine (from Perkins et al., 1982).

Figure 15

Figure 16. Colony color of wild type (WT) and *sre* rip mutant grown on high iron medium.

WT

sre mutant

Figure 16

Figure 17. Ferric perchlorate assay for siderophore production under different growth conditions. The wild type strain, *sre* *rip* mutant and the *arg-5,ota,aga* triple mutant were grown either on high iron or low iron condition. For the triple mutant, the medium was supplied with either 1mM ornithine or with 1mM arginine and 1mM spermidine as indicated. The absorbance of each sample at 495nm was taken and standardized by the amount of mycellia used. The error bars are derived by averaging the deviation of five parallal experiments for each assay.

Figure 17

Figure 18. Siderophore production with time under different growth conditions. The ferric perchlorate assay was used to determine the amount of siderophores. The wild type strain and *sre rip* mutant were grown either on high iron or low iron condition, and 1ml of liquid growth medium was taken at various times for the ferric perchlorate assay.

Figure 18

Figure 19. Thin layer chromatography assay for siderophore production under different growth conditions. The wild type strain, *Sre rip* mutant and the *arg-5,ota,aga* triple mutant were grown on either high iron or low iron medium. For the triple mutant, the medium was either supplied with 1mM ornithine or with 1mM arginine and 1mM spermidine. The siderophore was extracted from the growth medium, and the Fe-siderophore complex was separated on silica plate as described in Materials and Methods.

Figure 19

Figure 20. Northern analysis of *N. crassa sre* gene expression. Total RNA was isolated from wild type and the *sre* *rip* mutant under either the high iron or low iron growth condition. 50ug of total RNA was loaded on each lane, and after electrophoresis, blotted onto a nitrocellulose filter. The filter was probed with a ³²P-labeled EcoRI fragment of *sre* gene. Expression of the β -tubulin gene was used as an internal control.

Figure 20

Figure 21. Ferric perchlorate assay for siderophore production by a [*sre⁺his⁻+sre⁻arg⁻*] heterokaryon under different growth conditions. The wild type strain, *sre⁺* mutant and the *sre⁺/sre⁻* heterokaryon were grown either on the high iron or low iron condition. After treatment with ferric perchlorate, the absorbance of each sample at 495nm was taken and standardized by the amount of mycelia used. The error bars were derived by averaging the deviation of five parallel experiments for each assay.

Figure 21

Figure 22. Diagram of the location of the DNA probe used in the Southern blot of genomic DNA from *sre* gene complemented strains. B-tubulin and *sre* genes are represented as boxes. The DNA probe is shown as a small hatched box.

Figure 22

Figure 23. Southern blot analysis of genomic DNA from WT, *sre* rip mutant, and *sre*⁺ gene complemented *sre* rip mutant. All genomic DNAs were digested with PstI. RM, *sre* rip mutant. T1 to T4, different transformants. T4 has one extra copy of the intact *sre* gene.

Figure 23

Figure 24. Ferric perchlorate assay for siderophore production from *sre*⁺ complemented *sre* *rip* mutant strain under different growth conditions. The wild type strain, *sre* *rip* mutant and the complemented *sre* *rip* mutant were grown either on high iron or low iron condition. The absorbance of each sample at 495nm was taken and standardized by the amount of mycellia used. The error bars are derived by averaging the deviation of five parallel experiments for each assay.

Figure 24

Figure 25. Nitrate reductase assay of wt strain, *nit2* rip mutant, and *sre* rip mutant. Mycelia from all of the strains were grown overnight in Vogel's minimum medium with 20mM GLN as the nitrogen source. Then the mycelia were collected, washed with water and transferred to media with either 20mM KNO₃ as inducer, or 20mM KNO₃+20mM GLN as induction/repression condition. The mycelia were allowed to grow for another 4 hours before the they were collected and nitrate reductase assays were conducted. The absorbance at 540nm was standardized by the protein concentration of each sample.

Figure 25

Figure 26. Electrophoretic mobility shift analysis (EMSA) of NIT2 protein with *sre* gene promoter region as DNA probe. DNA fragments containing and lacking GATA sequences were from the *sre* gene promoter region. The NIT2 protein binds *in vitro* to DNA fragments containing GATA sequence elements

Protein	NIT2	
GATA	-	+

DNA probe

Figure 26

Figure 27. Ferric perchlorate assay for siderophore production from *nit2* rip mutant and wild type strain grown in nitrate induction and N-repression conditions. The wild type strain, *sre* rip mutant, and *nit2* rip mutant were grown either on high iron or low iron condition. The wild type strain was also supplied with 20mM KNO₃ as nitrate induction or with 20mM Gln as nitrogen repression conditions. The absorbance of each sample at 495nm was taken and standardized by the amount of mycellia used. The error bars are derived by averaging the deviation of five parallal experiments for each assay.

Figure 27

Figure 28. Electrophoretic mobility shift analysis (EMSA) of full length SRE protein. SRE was expressed as a full length GST fusion protein. GST was used as negative control in the assay. The DNA probes used were taken from sre gene promoter; +, DNA fragment containing GATA sequences; -, DNA fragment lacking GATA sequences. GST-SRE protein used in the assay was 0.0014ug.

Figure 28

Figure 29. Diagram of the region of SRE protein which was expressed and different mutations created around the two zinc fingers of SRE protein.

WT.....CSNCG.....CNAC.....CQNC.....CNAC.....
 F.....LAF.....
 S.....EL.....
 FS.....LAF.....EL.....

Figure 29

Figure 30. Electrophoretic mobility shift analysis (EMSA) of expressed truncated wild type and mutant forms of the SRE protein. WT wild type SRE protein. F, S, and FS indicate SRE proteins with mutation in first, second, or both first and second zinc fingers of SRE. +, DNA probe with GATA sequences. -, DNA lacking GATA sequences. 1ng WT SRE was used in all three EMSAs. 8ng F,S,FS mutant SRE protein were used in EMSA-A. 4ng F, S, FS mutant SRE protein were used in EMSA-B. And 3ng F, S, FS mutant protein in EMSA-C.

Figure 30

Figure 30 (continue)

Figure 30

Figure 30 (continue)

Figure 30

Figure 31. EMSA of different amounts of truncated wild type SRE protein. The amount of protein used in each assay is shown. DNA probes were derived from *sre* gene promoter with and without GATA sequences.

Protein(X10 ⁻⁴ ug)	6.6	6.6	5.0	4.0	3.3	2.8	2.5	2.2
GATA	-	+	+	+	+	+	+	+

DNA probe

Figure 31

Figure 32. Ferric perchlorate assay for siderophore production from different mutated *sre* gene transformed into the *sre* *rip* mutant strain under different growth conditions. The wild type, *sre* *rip* mutant, and transformed strains were grown either on high iron or low iron condition. WT, wild type; *sre* *rip* host transformed with wild type and mutated *sre* genes as follows: wt-comp, wild type *sre* gene; F-comp, *sre* gene with mutation in first zinc finger; S-comp, *sre* gene with mutation in second zinc finger; FS-comp, *sre* gene with mutation in both first and second zinc finger. The absorbance of each sample at 495nm was taken and standardized by the amount of mycellia used. The error bars are derived by averaging the deviation of five parallel experiments for each assay.

Figure 32

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Figure 33. Working model for SRE protein. ACTIVATOR is a positive regulator, whose concentration or activity is regulated by iron concentration in the cell. SRE binds to the promoter of its regulated genes and turns off the genes under high iron condition. SRE can interact with ACTIVATOR and inhibit its activity in both high iron and low iron condition so as to maintain the transcription of its regulated genes below a certain level. SRE activity could be regulated by iron concentration in the cell, which is not indicated in the figure.

High Iron

Low Iron

Figure 33

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IMAGE EVALUATION TEST TARGET (QA-3)

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