Functional Analysis of the Two Zinc Fingers of SRE, a GATA-Type Factor That Negatively Regulates Siderophore Synthesis in *Neurospora crassa*†

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ABSTRACT: Multiple GATA factors, zinc finger DNA binding proteins that recognize consensus GATA elements, exist in *Neurospora crassa*. One of them, SRE, is involved in controlling the iron metabolic pathway of *N. crassa*. In *N. crassa*, iron transport is mediated by a number of small cyclic peptides, known as siderophores. The siderophore synthesis pathway is negatively regulated by SRE; a loss-of-function *sre* mutant strain showed partial constitutive synthesis of siderophore. In the research presented here, the negative function of SRE was further confirmed by a heterokaryon test and by gene complementation. SRE was expressed as a GST fusion protein. *In vitro* EMSA revealed that SRE binds specifically to DNA molecules containing GATA sequence elements. Autoregulation of *sre* gene expression appears possible because the *sre* gene promoter itself contains GATA sequences. Mutations were introduced into *sre* that lead to amino acid substitutions in each of the zinc fingers that will disrupt their function. *In vitro* EMSA revealed that both N-terminal and C-terminal zinc fingers of SRE are involved in DNA binding. This feature is different from that found with the vertebrate two zinc finger GATA factors. *In vivo* tests, accomplished by transforming the mutant *sre* genes into *sre* rip mutant, showed that SRE with mutations in either or both zinc fingers still maintained its function under low-iron conditions. In contrast, these mutant SRE proteins fail to function under high-iron conditions. Our results predict the presence of other positive or negative regulators of the siderophore synthetic pathway.

Iron, an essential element, is an important component of many enzymes in all cells. However, iron is not readily available in aquatic or terrestrial environments or in animal hosts. 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 involves production of siderophores during conditions of iron limitation. Siderophores, cyclic hexapeptides, can solubilize ferric hydroxides and extract iron from the environment and even from other organisms. Most microorganisms synthesize several kinds of siderophores (*1*, *2*). *Neurospora* elaborates four different siderophores: coprogen, coprogen-B, ferrichrome, and ferricrocin. Their synthesis is highly regulated by iron availability within the environment (*3*). Through controlling the synthesis of siderophores, microbes regulate the uptake of iron into the cells.

Members of the GATA factor (GATA element binding proteins) family, transcription factors that recognize a consensus GATA DNA element, share high identity within their DNA binding motif, a Cys_2/Cys_2-type zinc finger with a central loop of 17 or 18 amino acids (*4*, *5*). DNA binding proteins of the GATA family occur in widely different organisms including mammals (*6*−*9*), birds (*10*), *Xenopus* (*11*), plants (*12*), *Drosophila* (*13*), *C. elegans* (*14*), and fungi (*15*, *16*). These GATA factors all have either one or two consensus Cys_2/Cys_2-type zinc fingers which determine their DNA binding ability.

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 (*17*−*21*). The zinc finger of NIT2, a GATA-type positive regulator of nitrogen metabolism of *Neurospora crassa*, is also involved in a protein−protein interaction of NIT2 with NIT4, a pathway-specific regulator (*22*), and of NIT2 with NMR, a global repression protein (*23*, *24*).

sid1, the gene which encodes L-ornithine-N^5-oxygenase, the first enzyme for siderophore synthesis, and *urbs1*, a negative regulator of the siderophore synthesis pathway, have been isolated and characterized from *Ustilago maydis* (*16*, *25*). URBSl, a GATA factor, negatively controls the siderophore synthesis pathway in *Ustilago*. URBSl binds at GATA elements in the promoter region of sid1, and regulates its expression (*26*). Only the C-terminal zinc finger of URBS1 is involved in the DNA binding-mediated regulation (*27*).

We previously reported the isolation of a new GATA factor gene, *sre*, from *Neurospora crassa* (*28*). SRE is a negative regulator of the iron metabolic pathway of *Neurospora*, and negatively controls siderophore synthesis. In the research reported here, heterokaryon tests and analysis of new *sre* mutants *in vitro* and *in vivo* were used to further understand the function of SRE. We present results which

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show that both zinc fingers of SRE are involved in DNA binding *in vitro* and in controlling siderophore synthesis *in vivo*.

MATERIALS AND METHODS

Bacterial and Fungal Strains. The *Neurospora crassa* wild-type strain 74OR23, a *his-3* mutant strain (Y234M723), and an *arg-12* mutant strain (CD3) were obtained from the Fungal Genetics Stock Center (University of Kansas Medical Center). The *sre* rip mutant was created during this study. These strains were grown on Vogel's minimal medium supplemented as required with histidine (his^-) or arginine (arg^-). *Escherichia coli* XL1Blue (supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac^- F′ [$proAB^+$ lacIq lacZ M15 Tn10(tet^r)]) was used as the host strain for DNA manipulation.

Media and Reagents. Low-iron (LI) medium was Vogel's minimal medium with iron omitted. High-iron (HI) medium was supplemented with 1 mM $FeSO_4$ for solid cultures and 10 μM $FeSO_4$ for liquid cultures. Crossing medium was 1× Westergaard's plus 0.1% sucrose and 4% agar noble (*29*, *30*). Restriction enzymes, T4 DNA polymerase, Klenow, and a random primer labeling kit were from BRL (Bethesda Research Laboratories). An *in vitro* mutagenesis kit was from Bio-Rad, and the Thermo sequencing kit was from Amersham.

Heterokaryon Formation. A heterokaryon was formed by mixing mycelia of two different mutant strains of the same mating type on Vogel's minimum medium, on which neither strain alone could grow (*30*). The *sre* mutant strain was crossed with a *his3* mutant to get the *his-3 sre* double mutant, which required histidine and also showed the *sre* mutant phenotype. Mycelia of the *his-3 sre* mutant plus mycelia of an *arg-12* mutant were inoculated together into minimum medium, and after several days incubation at 30 °C, a heterokaryon which grew vigorously on minimal medium was obtained.

Neurospora Transformation. Neurospora crassa spheroplast preparation and transformation were done as described by Akins and Lambowitz (*31*) with the following modifications. Following Novozyme digestion, spheroplasts were washed extensively with STC (1 M sorbitol, 50 mM Tris, pH 8.0, 50 mM $CaCl_2$). Then 5 μL of heparin (5 mg/mL in STC, made freshly) was mixed with 1 μg of DNA and incubated on ice for 5 min, before addition of 100 μL of spheroplasts. After incubation on ice for 30 min, 1 mL of PTC (40% PEG 4000, 50 mM Tris, pH 8.0, 50 mM $CaCl_2$) was added, followed by incubation at room temperature for another 20 min. The spheroplasts were added to 10 mL of top agar (1× Vogel's, 2.8% agar noble, 18.2% sorbitol, 2% sorbose, 0.05% fructose, 0.05% glucose), and immediately poured onto 25 mL of bottom agar (1× Vogel, 1.5% agar, 2% sorbose, 0.05% fructose, 0.05% glucose). Transformants were picked after 2 days incubation at 30 °C.

DNA Procedures. Neurospora genomic DNA was isolated as described (*32*) and used for Southern blot analysis. pBlue Script KS(+) (from Stratagene) was used as the vector for DNA manipulations, and the pGEX-2T vector was used to express proteins. DNA fragments for subcloning were purified with gene clean (Bio101, Inc., La Jolla, CA). DNA cloning and Southern blots followed standard procedures (*33*). The probe for Southern blots was labeled with a random primer labeling kit (from Bethesda Research Laboratories). 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 termini of DNA had a 5′ overhang. Site-directed mutagenesis and *E. coli* transformations were done by following the instruction manual from the Bio-Rad Muta-Gene Phagmid *in vitro* mutagenesis Kit.

Ferric Perchlorate Assay. Neurospora was grown for 48 h in liquid media (low-iron or high-iron). The culture was centrifuged, the supernatant was collected, and an equal volume of perchlorate assay solution [5 mM $Fe(ClO_4)_3$, 0.1 M $HClO_4$] was added. The absorbance was determined at 495 nm using fresh medium as the control (*34*).

Protein Expression and Purification. The pGEX-2T vector was used 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 DNA sequence to be expressed was subcloned into pGEX-2T so that it encoded a chimeric protein with a GST tag 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 5 mL of LB medium containing ampicillin. After growth overnight 500 mL of fresh medium was inoculated with the overnight culture. The culture was grown at 37 °C until the A_{600nm} reached 0.6, when IPTG was added to a final concentration of 0.1 mM. The culture was further grown for 3−7 h; then the cells were pelleted and suspended in 1/50 to 1/100 culture volume of MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3). 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 was added 800 μL of glutathione−agarose beads. 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 5 times with 10 mL of MTPBS. Finally, 200 μL of freshly prepared 50 mM Tris-HCl (pH 7.5) containing 5 mM reduced glutathione was added to the bead−protein mixture to elute the fusion protein. The purified protein was examined by gel eletrophoresis, and its concentration was determined with the Bio-Rad protein assay solution.

Gel Electrophoresis Mobility Shift Assay (EMSA).[1] 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 12 mM Hepes, 4 mM Tris-HCl, pH 7.9, 110 mM KCl, 1 mM EDTA, 3 mM $MgCl_2$, 1 mM DTT, 10% glycerol, and 0.3 mg/mL BSA. The reaction mixture was loaded onto 4% native polyacrylamide gels and electrophoresed in 0.5 × TBE (0.0445 M Tris−borate, pH 8.2, 0.001 M EDTA) for about 2 h at 200 V until the bromophenol blue reached the bottom of the gel. The gel was dried and exposed to X-ray film, which was scanned by a densitometer to determine the ratio of free and shifted DNA probes.

[1] Abbreviation: EMSA, electrophoretic mobility shift analysis.

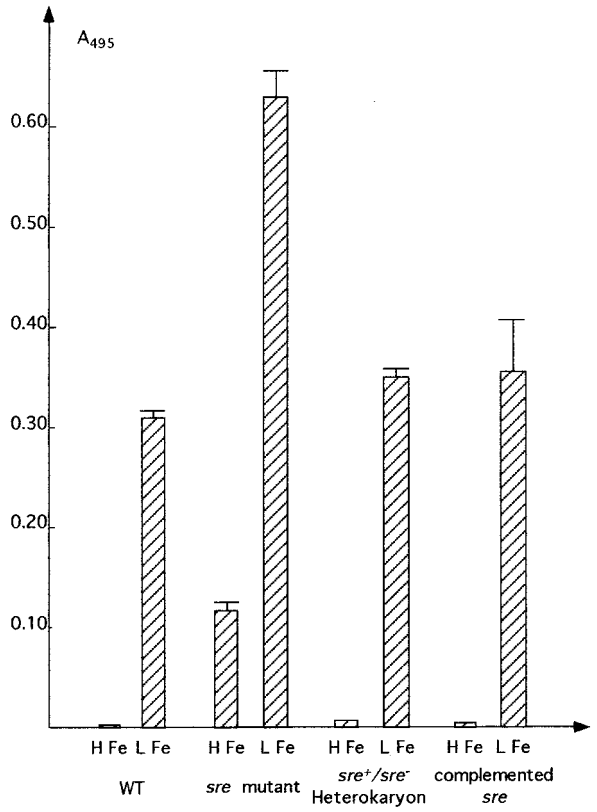

FIGURE 1: Ferric perchlorate assay for siderophore production by a [*sre*$^+$*his*$^-$+*sre*$^-$*arg*$^-$] heterokaryon and *sre*$^+$ completed *sre* rip mutant strain under different growth conditions. The wild-type strain, the *sre* rip mutant, the *sre*$^+$/*sre*$^-$ heterokaryon, and the completed *sre* rip mutant were grown either in the high iron or in the low iron condition. After treatment with ferric perchlorate, the absorbance of each sample at 495 nm was taken and standardized by the amount of mycellia used. The error bars were derived by averaging the deviation of five parallel experiments for each assay.

RESULTS

Heterokaryon Test and Gene Complementation. Neurospora normally grows as a multinucleated syncytium. This make it possible to put a wild-type gene and its mutant form together in one cell to determine the phenotype of the heterokaryon. If the wild-type gene is dominant, the heterokaryon will show the wild-type phenotype; otherwise, the heterokaryon will display the mutant phenotype. A heterokaryon was formed as described under Materials and Methods and examined for control of sidrophore synthesis with the ferric perchlorate assay, together with the wt and the *sre* mutant as controls (Figure 1). The results showed that the phenotype of the heterokaryon is wild type, demonstrating that the *sre* rip mutant is recessive to the wt *sre*$^+$ gene. Because rip leads to many G/C to A/T transition mutations, it is virtually certain that the mutated gene created by this method lacks all function; i.e., it will be a null mutant (*28*). It should be noted that the *sre* mutant differs from wild type in two ways: under high iron conditions, the *sre* mutant displays partial constitutive expression, i.e., is not fully repressed. In addition, under low iron conditions, the *sre* mutant strain overexpresses siderophores at least 2-fold in comparison with wild type (Figure 1).

In order to further test the function of the *sre* gene, a 6.5 kb *Hin*dIII−*Sac*I DNA fragment, which covers the entire coding and promoter regions of the gene, was inserted into a plasmid along with the benomyl-resistant B-tubulin gene. This plasmid was transformed into the *sre* rip mutant strain with selection for benomyl resistance. Benomyl-resistant clones were isolated and genomic DNA was prepared from these transformants for Southern analysis (data not shown). A transformant which had a full-length *sre*$^+$ gene was chosen for the ferric perchlorate assay (Figure 1). The results showed that the phenotype of this transformant is wild type. This demonstrated that the transformed wt copy of the *sre*$^+$ gene 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*.

In Vitro EMSA Study of SRE Protein. Site-directed mutagenesis was applied to remove two small introns from the genomic copy of the *sre* gene. A *Bam*HI restriction site was also introduced at the start and at the end of the *sre* coding region. DNA sequencing was used to ensure that the correct construct was obtained. This DNA fragment, which contains the full-length *sre* gene coding region, was inserted into the pGEX-2T expression vector in-frame. The recombinant pGEX-2T-SRE, which has the entire *sre* gene, was introduced into *E. coli BL21(DE3)pLysS*, and expressed to obtain a SRE−GST function protein. The full length SRE−GST fusion protein was purified as described under Materials and Methods. GST was also expressed and purified by the same method.

The purified GST−SRE fusion protein was analyzed by a gel mobility shift assay for binding to a DNA region from the *sre* gene promoter, nucleotides 1−400, which has three GATA sequences. Another DNA fragment from the *sre* promoter region, which lacks any GATA sequences, was used as a negative control. The results are shown in Figure 2A.

Mobility shift experiments were also done with the GST−SRE fusion protein and with GST alone, using a double-stranded oligonucleotide with the sequence 5′aattctatggtacgtaGATAagtaccacccgtg3′ and a second oligo with the identical DNA sequence except with GATA changed to GAAT. The results are shown in Figure 2B.

The results of these gel mobility shift assays demonstrated that the full-length SRE protein binds specifically only to DNA fragments which have GATA sequences. GST itself did not show any binding to DNA fragments with or without GATA sequences. These experiments show that SRE acts as a sequence-specific DNA binding protein, specifically recognizing DNA molecules containing GATA elements.

In Vitro EMSA of Truncated wt 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 fingers by site-directed mutagenesis to change at least one of the four critical cysteines and one or more adjacent amino acids in each zinc finger. In some cases amino acid codons were changed to create a unique restriction site to simplify the identification of mutants (Figure 3). The constructs, encoding truncated forms consisting of 267 aa of both the wt and mutant SRE proteins, were subcloned into the pGEX-2T expression vector. These truncated forms of the SRE protein were each expressed as GST fusion proteins and purified by affinity chromatography.

The truncated SRE fusion proteins were used in gel mobility shift assays using the same DNA probes as used in

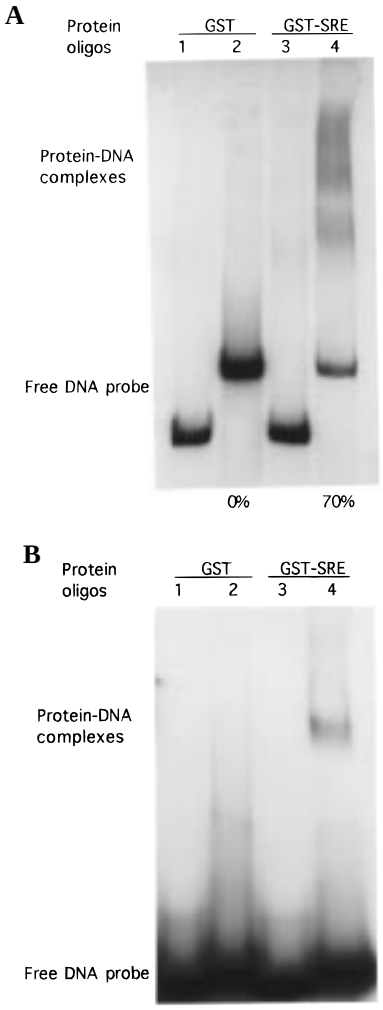

FIGURE 2: Electrophoretic mobility shift analysis (EMSA). SRE was expressed as a full-length GST fusion protein. GST was used as a negative control in the assay. GST protein used in the assay was about 1 μg, while GST−SRE fusion proteins were about 1.4 ng. Lanes 1 and 3, DNA fragment lacking GATA sequences. Lanes 2 and 4, DNA fragment containing GATA sequences. The extent of the mobility shift was estimated by comparing the amount of free DNA probe in lanes 2 and 4. (A) The DNA probes used were taken from *sre* gene promoter; the multiple shifted bands may be due to the presence of three GATA sequences. (B) The DNA probes used were aattctatggtacgtaGATAagtaccacccgtg (lanes 2 and 4) and aattctatggtacgtaGAATagtaccacccgtg as a negative control (lanes 1 and 3).

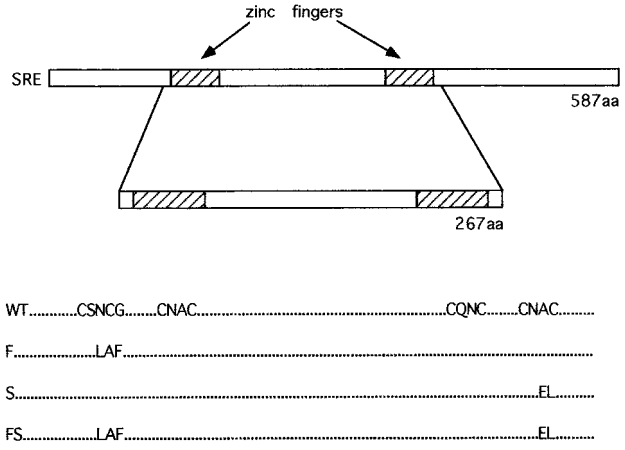

FIGURE 3: Diagram of the truncated SRE proteins which were expressed and different mutations created that altered one or both of the two zinc fingers of the SRE protein.

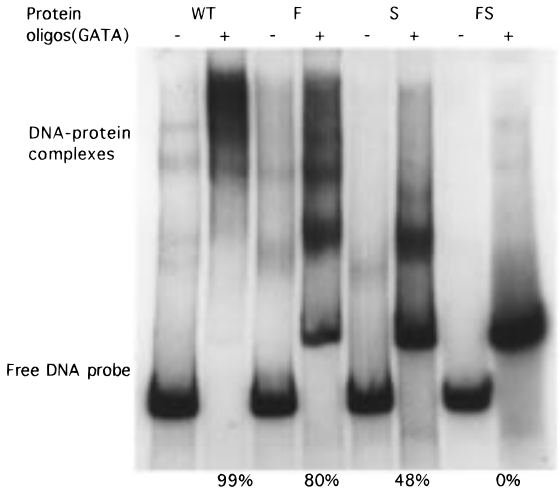

FIGURE 4: Electrophoretic mobility shift analysis (EMSA) of expressed truncated wt and mutant forms of the SRE protein using *sre* gene promoter as probe. WT, wild-type SRE proteins. F, S, and FS indicate SRE proteins with mutation in the first, second, or both first and second zinc fingers of SRE. +, DNA probe with GATA sequences. −, DNA lacking GATA sequences. 1 ng of WT SRE was used in EMSA. 3 ng of the F, S, FS mutant proteins was used in EMSA. The extent of the mobility shifts (shown at bottom) was estimated by comparing the amount of free DNA probe in each lane.

the full-length SRE EMSA. The results revealed that the wild-type truncated SRE protein binds specifically to GATA-containing DNA fragments as well as did the full-length SRE protein. Mutation in either of thhe two zinc fingers significantly weakened the DNA binding. When both of the zinc fingers of SRE were mutated, DNA binding was completely eliminated (Figures 4 and 5).

Different amounts of each protein were also applied to EMSA, and the fraction of each DNA probe which was shifted was determined (data not shown). The wild-type SRE protein binds to GATA-containing DNA fragments 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 DNA binding, although the C-terminal finger plays a more important role in the binding. These results appear to be somewhat different than found with other two zinc finger GATA factors, in which the C-terminal finger alone is sufficient for DNA binding.

In Vivo Study of sre Mutants. In order to compare the function of mutant SRE proteins with the wt SRE protein *in vivo*, the same mutations altering the finger regions were also introduced into the full-length *sre* gene. The benomyl-resistant B-tubulin gene was also engineered into the same plasmids. These plasmids with the B-tubulin gene and the wild-type and different mutant *sre* genes were transformed into the *sre* rip mutant strain. 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 the *sre* gene and the B-tubulin gene as DNA probes (data not shown). Transformants which received a single intact copy of the *sre* gene were selected for analysis.

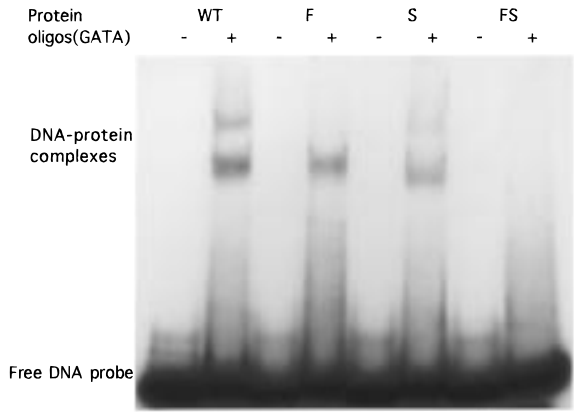

FIGURE 5: Electrophoretic mobility shift analysis (EMSA) of expressed truncated wt and mutant forms of the SRE protein using 33mer oligo nucleotide as probes. WT, wild-type SRE protein. F, S, and FS indicate SRE proteins with mutation in the first, second, or both first and second zinc fingers of SRE. +, DNA probe with GATA sequences. −, DNA lacking GATA sequences. 1 ng of WT SRE and 3 ng of the F, S, FS mutant proteins were used in EMSA.

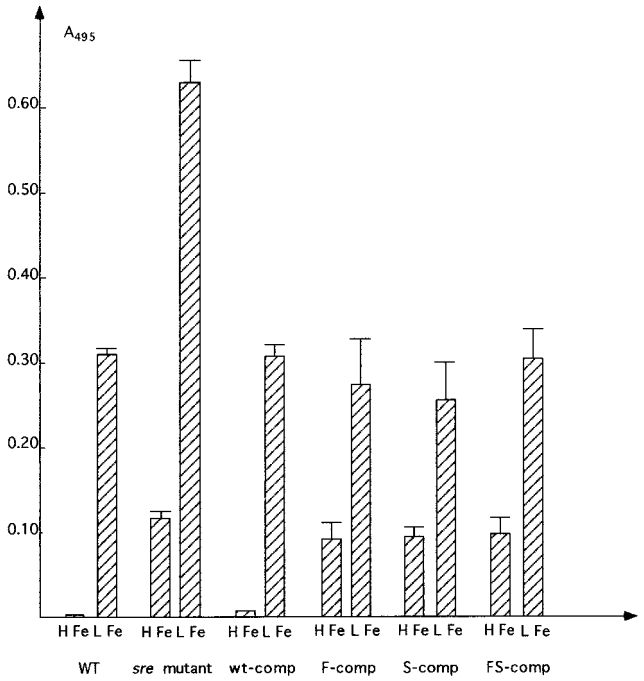

FIGURE 6: Ferric perchlorate assay for siderophore production by wild-type, the *sre* rip mutant, and the rip mutant strain transformed with different *sre* genes, under different growth conditions. The wild-type, *sre* rip mutant, and transformed strains were grown either in the high iron or in the low iron condition. WT, wild type; *sre* rip host transformed with wild type and mutated *sre* genes are as follows: wt-comp, wild-type *sre* gene; F-comp, *sre* gene with mutation in the first zinc finger; S-comp, *sre* gene with mutation in the second zinc finger; FS-comp, *sre* gene with mutation in both the first and second zinc fingers. The absorbance of each sample at 495 nm was taken and standardized by the amount of mycelia used. The error bars are derived by averaging the deviation of five parallel experiments for each assay.

The ferric perchlorate assay was used to test the phenotype of these transformants. If the inserted copy of *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 6) demonstrate that the transformed wt *sre* is fully functional. In contrast, the *sre* gene with mutations that alter either the first finger, the second finger, or both zinc fingers appears 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 contrast, the transformants with mutations affecting either or both SRE zinc fingers produced substantial amounts of siderophores under high iron, as found with the *sre* mutant. These results, which reveal that each of the mutants behaves as the wild type when on low iron, but display the *sre* mutant phenotype when on high iron, will be discussed below.

DISCUSSION

In this study, the proposed negative regulatory function of SRE controlling the iron metabolic pathway in *Neurospora* was further investigated and confirmed. The wt sre^+ gene complemented a *sre* mutant strain yielding the wild-type phenotype. SRE was expressed as a GST fusion protein, and *in vitro* gel mobility shift assays revealed that SRE binds specifically to DNA molecules containing GATA sequence elements. One of the DNA probes used in the gel mobility assays was derived from the *sre* gene promoter itself, which suggests a possible autoregulation. However, this must be considered with caution, and further research is needed to address the possibility of autoregulation of *sre* gene expression.

The *in vitro* mobility shift results demonstrated that SRE is a sequence-specific DNA binding protein whose binding activity depends upon the presence of GATA sequences. The presence of the multiple shifted bands may reflect the fact that the DNA fragment used has three GATA elements and thus might bind different numbers of SRE molecules, and may also be due to SRE−SRE dimer formation as found in GATA-1 homotypic interactions (*35*). It should also be noted that one of the DNA fragments used in these studies was derived from the *sre* gene promoter itself. However, we do not know whether *sre* is autoregulated, and do not have available any genes that are subject to SRE control. SRE may bind with even greater affinity to promoters which it regulates.

Since SRE is the only GATA factor isolated from *Neurospora* that has two zinc fingers, it was of interest to study their functional significance. Mutations were introduced into *sre* that lead to amino acid substitutions in each of the fingers that will disrupt their function. Gel mobility shift assays revealed that both the N-terminal and C-terminal zinc fingers of the SRE protein are involved in DNA binding. Although the wild-type protein showed the strongest DNA binding, SRE proteins in which only the C-terminal finger or the N-terminal finger were functional were capable of significant DNA binding. It is important to note that the wild-type SRE protein not only retards a greater amount of the DNA probes than do the mutant proteins, but also results in DNA−protein complexes of significantly high mass in the binding with *sre* gene promoter as DNA probes (see Figure 4). We interpret this feature to indicate that in the case of the wild-type protein, with its strong binding affinity, multiple SRE proteins have bound the DNA molecule, which contains three GATA elements. With the single GATA site DNA probe, only one mobility shift band was formed for the

mutant SRE proteins but two bands appear with the wild-type SRE protein (Figure 5). We interpret this to result from dimer formation by the wild-type protein. It appears that only a single protein molecule has bound to the majority of the DNA molecules in the case of the mutant SRE proteins in which one of the two fingers is nonfunctional. These observations reinforce the concept that both zinc fingers of SRE are involved in DNA binding and both are required to achieve high-affinity binding. This feature is different than found with the vertebrate two zinc finger GATA factors (*36*, *37*) and URBS1 from *Ustilago* (*27*). In all of these other GATA factors, the N-terminal zinc finger is primarily involved with DNA binding specificity and interactions with other regulatory proteins, while the C-terminal finger alone 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. These experiments revealed that, unlike transformants that received a sre^+ gene yielding the wild-type SRE protein, each of the mutant SRE proteins failed to repress siderophore synthesis during high iron growth conditions, but instead showed the same level of constitutive expression as found with the complete loss of function *sre* rip mutant. These *in vivo* results differ somewhat from the *in vitro* results, in which SRE mutant proteins with alterations in either of the zinc fingers retained considerable DNA binding affinity, although significantly reduced in comparison with that of wild type.

One particularly intriguing finding is that siderophore synthesis is only partially constitutive in the *sre* rip mutant; i.e., control of siderophore synthesis was not totally lost even in the complte absence of the 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 (*26*). Based on this result, together with the finding that SRE displays sequence-specific DNA binding, one can readily predict that an important function of SRE is executed by its 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 is tempting to speculate that an as yet unidentified positive regulator, which competes with SRE for GATA DNA binding sites, might be another GATA factor. In yeast, Gln3P, a positive GATA factor, and Da180P, a negative GATA factor, compete in DNA binding, and thereby together regulate the same metabolic pathway (*38*, *39*).

Some GATA factors function not only by binding to the promoters of their regulated genes but also through protein−protein interactions with other positive or negative regulators. The vertebrate GATA-1 protein interacts with SP1 (a general transcriptional factor), with EKLF (an erythroid Kruppel-like factor), and with FOG (a cofactor of GATA-1) in order to cooperatively regulate cell-specific erythroid gene expression at the transcriptional level (*37*, *40*). Our results suggest that in addition to its DNA binding activity, the SRE protein may be involved in one or more functional protein−protein interactions. Under low iron conditions, the *sre* loss-of-function rip mutant shows nearly double the wild-type level of siderophore synthesis (Figure 1). Significantly, the SRE mutant protein with mutations in both zinc fingers, which completely lacks detectable DNA binding, maintains siderophore synthesis at the normal level under the low iron condition. A reasonable speculation is that a protein−protein interaction, which does not require SRE's DNA binding activity, between SRE and a positive factor is required to maintain siderophore synthesis at an optimal level. The *sre* rip mutant, which completely lacks any *sre* mRNA or protein, presumably lacks this feature and shows unrestrained siderophore synthesis.

It is important to emphasize that the striking regulation of siderophore synthesis is dependent upon the iron concentration within the growth medium. It is unknown whether the cells sense intracellular or extracellular iron, and it will be very informative to identify the factor or signal pathway that is sensitive to iron. Under conditions of low iron, SRE appears to help maintain siderophore synthesis at an optimal level. The *sre* gene is expressed constitutively (*28*). If, as expected, the SRE protein is present in cells whether growing on high or low iron, it will be very informative to identify the mechanism that modulates the functional activity of SRE, which almost certainly reflects the concentration of iron available to the cells. A highly conserved cysteine-rich amino acid sequence is located between the two zinc fingers or SRE and URBS1 (*16*). It would not be surprising if this conserved motif is involved in the cellular reponse to iron; e.g., it might even constitute an iron binding domain. Mutagenesis of this region and both *in vitro* and *in vivo* tests may be helpful in determining the functional role, if any, of this conserved region. These considerations suggest that SRE may sense or at least respond to the cellular iron concentration and to function in at least two different ways, that involve interactions with other regulatory factors, to control siderophore synthesis.

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REFERENCES

1. Neilands, J. B. (1995) *J. Biol. Chem. 270*, 26723−26726.
2. Guerinot, M. L. (1994) *Annu. Rev. Microbiol. 48*, 743−772.
3. Huschka, H., Naegeli, H. U., Leuenberger-Ryf, H., Keller-Schierlein, W., and Winkelmann, G. (1985) *J. Bacteriol. 162*, 715−721.
4. Omichinski, J. G., Clore, G. M., Schaad, O., Felsenfeld, G., Trainor, C., Appella, E., Stahl, S. J., and Gronenborn, A. M. (1993a) *Science 261*, 438−446.
5. Omichinski, J. G., Trainor, C., Evans, T., Gronenborn, A. M., Clore, G. M., and Felsenfeld, G. (1993b) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1676−1680.
6. Evans, T., and Felsenfeld, G. (1989) *Cell 58*, 877−885.
7. Tsai, F.-Y., Keller, G., Kuo, F.-C., Weiss, M. J., Chen, J.-Z., Rosenblatt, M., Alf, F., and Orkin, S. H. (1994) *Nature 371*, 221−226.
8. Trainor, C. D., Evans, T., Felsenfeld, G., and Boguski, M. S. (1990) *Nature 343*, 92−96.
9. Fujiwara, Y., Browne, C. P., Cunniff, K., Goff, S. C., and Orkin, S. H. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 12355−12358.
10. Laverriere, A. C., MacNeil, C., Mueller, C., Poelmann, R. E., Burch, J. B. E., and Evans, T. (1994) *J. Biol. Chem. 269*, 23177−23184.

11. Xu, R.-H., Kim, J., Taira, M., Lin, J.-J., Zhang, C.-H., Sredni, D., Evans, T., and Kung, H.-F. (1997) *Mol. Cell. Biol. 17*, 436−443.
12. Chang, W.-T., Newell, P. C., and Gross, J. D. (1996) *Cell 87*, 471−481.
13. Ramain, P., Heitzler, P., Haenlin, M., and Simpson, P. (1993) *Development 119*, 1277−1291.
14. Speith, J., Shim, Y.-H., Lea, K., Conrad, R., and Blumanthal, T. (1991) *Mol. Cell. Biol. 11*, 4651−4659.
15. Fu, Y.-H., and Marzluf, G. A. (1990a) *Mol. Cell. Biol. 10*, 1056−1065.
16. Voisard, C., Wang, J., McEvoy, J. L., Xu, P.-L., and Leong, S. A. (1993) *Mol. Cell. Biol. 13*, 7091−7100.
17. Fu, Y.-H., and Marzluf, G. A. (1990b) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5331−5335.
18. Ballario, P., Vittorioso, P., Magrelli, A., Talora, C., Cabibbo, A., and Macino, G. (1996) *EMBO J. 15*, 1650−1657.
19. Kudla, B., Caddick, M. X., Langdon, T., Martinez-Rossi, N. M., Bennett, C. F., Sibley, S., Davies, R. W., and Arst, H. N. (1990) *EMBO J. 9*, 1355−1364.
20. Chae, K.-S., Kim, J.-H., Choi, Y., Han, D.-M., and Jahng, K.-Y. (1995) *Mol. Cell 5*, 146−150.
21. Haas, H., and Marzluf, G. A. (1995) *Curr. Genet. 28*, 177−183.
22. Feng, B., and Marzluf, G. A. (1998) *Mol. Cell. Biol. 18*, 3983−3990.
23. Xiao, X.-D., Fu, Y.-H., and Marzluf, G. A. (1995) *Biochemistry 34*, 8861−8868.
24. Pan, H. G., Feng, B., and Marzluf, G. A. (1997) *Mol. Microbiol. 26*, 721−729.
25. Mei, B.-G., Budde, A. D., and Leong, S. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 903−907.
26. An, Z.-Q., Mei, B.-G., Yuan, W. M., and Leong, S. A. (1997a) *EMBO J. 16*, 1742−1750.
27. An, Z.-Q., Zhao, Q., McEvoy, J., Yuan, W. M., Markley, J. L., and Leong, S. A. (1997b) *Proc. Natl. Acad. Sci. U.S.A. 94*, 5882−5887.
28. Zhou, L.-W., Haas, H., and Marzluf, G. A. (1998) *Mol. Gen. Genet. 259*, 532−540.
29. Singer, M. J., Kuzminova, E. A., Tharp, A., Margolin, B. S., and Selker, E. U. (1995) *Fungal Genet. Newsl. 42*, 74−75.
30. Davis, R. H., and DeSerres, F. (1970) *Methods Enzymol. 17A*, 79−143.
31. Akins, R. A., and Lambowitz, A. M. (1985) *Mol. Cell. Biol.* 5, 2272−2278.
32. Leach, J., Finkelstein, B., and Rambosek, J. A. (1986) *Neurospora Newsl. 33*, 32−33.
33. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
34. Atkin, C. L., Neilands, J. B., and Phaff, H. J. (1970) *J. Bacteriol. 103*, 722−733.
35. Yang, H.-Y., and Evans, T. (1995) *Mol. Cell. Biol. 15*, 1353−1363.
36. Crossley, M., Merika, M., and Orkin, S. H. (1995) *Mol. Cell. Biol. 15*, 2448−2456.
37. Merika, M., and Orkin, S. H. (1995) *Mol. Cell. Biol. 15*, 2437−2447.
38. Minehart, P. L., and Magasanik, B. (1991) *Mol. Cell. Biol. 11*, 6216−6228.
39. Cunningham, T. S., and Cooper, T. G. (1991) *Mol. Cell. Biol. 11*, 6205−6215.
40. Tsang, A. P., Visvader, J. E., Turner, C. A., Fujiwara, Y., Yu, C.-N., Weiss, M. J., Crossley, M., and Orkin, S. H. (1997) *Cell 90*, 109−119.

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