Characterization of DNA Binding and the Cysteine Rich Region of SRE, a GATA Factor in *Neurospora crassa* Involved in Siderophore Synthesis[†]

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ABSTRACT: Several homologous genes encoding proteins involved in regulating siderophore synthesis in fungi have been isolated, including the *sre* gene from the filamentous fungus *Neurospora crassa*. We present data that further characterize SRE and provide new insights into the regulation of iron homeostasis in *Neurospora.* SRE is a member of the GATA factor family, which is comprised of transcription factors that contain either one or two zinc finger motifs that recognize and bind to GATA-containing DNA sequences. Results from electrophoretic mobility shift assays demonstrate that SRE binds with high affinity to a DNA probe containing the iron response element from the *sid1* promoter from *Ustilago*. SRE binding to DNA was demonstrated to be zinc-dependent. Moreover, changes in the spacing between two GATA sites altered the DNA binding affinity of SRE. Mutants of highly conserved cysteine residues present in SRE and homologous proteins were created by site-directed mutagenesis. The combined results of mobility shift assays, siderophore synthesis assays, and ornithine oxygenase enzyme activity determinations demonstrate that these mutants with cysteine substitutions have a dominant repressor phenotype.

In the filamentous fungus *Neurospora crassa,* a total of five GATA factors have been identified, each functioning in a specific metabolic pathway. NIT2 is a global regulator that positively controls the expression of genes involved in nitrogen metabolism (*1*). WC-1 (*2*) and WC-2 (*3*) regulate genes expressed in response to blue light induction and also play a role in controlling the circadian rhythm (*4*). ASD4 controls ascus development and ascospore maturation (*5*). SRE plays a rather undefined role in the negative regulation of iron transport (*6*).

Like all organisms with the exception of certain lactobacilli, *Neurospora* requires iron for a variety of functions. Iron plays a central role in many reduction−oxidation reactions ranging from respiration to ribonucleotide synthesis. Though iron is one of the most abundant elements on earth, it is relatively unavailable to biological systems due to the intrinsic insolubility of ferric iron (10^{-18} M) in an aerobic environment. This nearly insoluble form of iron predominates in nature as oxohydroxide polymers. Therefore, acquiring essential iron poses a serious challenge to most organisms for their growth and survival. Although iron is a strict requirement, too much intracellular iron leads to toxicity as it readily reacts with oxygen to produce hydroxyl radicals. Hydroxyl radicals react at extremely high rates with organic molecules found within cells, leading to oxidative damage to DNA, lipids, and proteins. Organisms are thus faced with the daunting task of acquiring essential iron, but must also necessarily employ mechanisms to avoid toxicity that results from too much intracellular iron.

Various elaborate biological systems have evolved to coordinate iron transport and homeostasis. Bacteria and fungi sequester iron by producing and secreting low-molecular weight ferric iron chelators called siderophores, which bind ferric iron very efficiently and tightly (*7*, *8*). Iron acquisition via siderophores must be tightly regulated to conserve energy, which is required for siderophore transport, and, more importantly, to avoid iron-mediated injury to cells.

In the basidiomycete *Ustilago maydis*, the *sid1* gene encodes L-ornithine N^5-oxygenase, which catalyzes the first step in siderophore synthesis. Under low-environmental iron conditions, the extent of transcription of *sid1* increases 16-fold (*9*). This increase in extent of transcription of *sid1* is reasonable since, when environmental iron is present in small amounts, the level of siderophore synthesis must increase to sequester needed iron. Equally important is the need to abate siderophore synthesis when environmental iron is abundant to avoid overloading of cellular iron that would lead to toxicity. URBS1 in *Ustilago* is a GATA factor protein that has been shown to negatively regulate siderophore synthesis by repressing the expression of the *sid1* gene (*10*). URBS1 inhibits *sid1* expression by binding to an upstream region of the *sid1* promoter, known as the iron responsive region, which contains two GATA elements (*11*). This sequence is necessary for iron-regulated expression of *sid1*.

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A few years ago, using a PCR[1]-mediated method specific for the highly conserved zinc finger sequence in GATA factors, a new GATA factor gene in *Neurospora*, *sre*, was identified (*6*). The amino acid sequence of *sre* is homologous to sequences of other fungal two-zinc finger GATA factors such as URBS1 of *Ustilago* (*10*), SREP of *Penicillium* (*12*), and SREA of *Aspergillus* (*13*). In addition to the zinc finger domains, a high degree of homology is seen in the region found between the zinc fingers. Within this zinc finger region are four highly conserved cysteine residues. Disruption of *sre* in *Neurospora* by the RIP mutation methodology results in the partial constitutive production of siderophores. SRE represses expression of L-ornithine N^5-oxygenase, the activity of which catalyzes the first step in siderophore biosynthesis (*6*). These results suggest that SRE may be a negative regulator of siderophore synthesis, acting to regulate transcription of the ornithine oxygenase gene and perhaps other genes in this biosynthetic pathway.

The work presented here was undertaken in an effort to characterize the DNA binding properties of SRE with electrophoretic mobility shift assays (EMSAs). Additionally, the importance of conserved cysteine residues found between the two zinc fingers was examined using site-directed mutagenesis. A better understanding of the action of SRE will assist our goal of understanding how the various GATA factors differentially regulate specific pathways. Additionally, a more detailed model of the regulation of siderophore synthesis in *Neurospora* will afford valuable insight into the understanding of the iron transport pathway in fungi.

MATERIALS AND METHODS

Bacterial Strains and Chemical Reagents. Escherichia coli XL-1 Blue and DH5α cells were used for general bacterial transformations. BL21 DE3 pLysS cells were utilized for GST fusion protein expression. *N. crassa* wild-type strains 740R23 A/a from the Fungal Genetics Stock Center (Kansas City, KS) and the *sre*-RIP mutant strain T4-4 (*6*) were used for most procedures. *Neurospora* cultures were grown in Vogel's minimal medium. For experiments requiring low-iron media, $Fe(NH_4)_2SO_4 \cdot 6H_2O$ was excluded. For experiments requiring high-iron media, $FeSO_4$ was added to a final concentration of 10 μM. Restriction endonucleases and DNA-modifying enzymes were purchased from Gibco BRL (Gaithersberg, MD) or New England Biolabs (Beverly, MA). Chemical reagents were purchased from Sigma (St. Louis, MO) or Boehringer-Mannheim (Mannheim, Germany).

Expression and Purification of the GST Fusion Protein. A single colony of BL21 *E. coli* containing the pGEX-2T vector with the *sre* gene insert was used to inoculate 50 mL of LB medium with ampicillin (25 μg/mL) and chloramphenicol (25 μg/mL), which was incubated at 37 °C overnight with shaking at 180−200 rpm. After being shaken for 16−20 h, the overnight culture was used to inoculate 1 L of LB containing antibiotics. The culture was grown to an OD_{600} of 0.6−0.8 at which time IPTG was added to a final concentration of 2 mM. Following additional growth for 3−4 h, the cell culture was chilled on ice for approximately 15 min and then the cells were harvested by centrifugation at 4 °C. The pellet was resuspended in 20 mL of MTPBS [150 mM NaCl, 16 mM Na_2HPO_4, and 4 mM NaH_2PO_4 (pH 7.3)] and frozen overnight at −20 °C. The cells were thawed in cool water and transferred to ice. Following addition of DNase (50 μg/mL), RNase (25 μg/mL), and PMSF (2 mM), cells were lysed by sonicating them on ice for 2 min. Triton X-100 was added to a concentration of 1% (v/v) and cell debris pelleted from the cell lysate by centrifugation at 4 °C.

Affinity Chromatography Using Glutathione−Agarose Beads. Glutathione−agarose beads were swelled overnight, washed, and transferred to a column to yield a bed volume of 10 mL. The cell extract obtained above was loaded on the column and washed with approximately 500 mL of MTPBS. The GST fusion protein was eluted with 50 mM Tris-HCl (pH 8.0) containing 5 mM reduced glutathione. Fractions (1 mL) were collected and analyzed by SDS−PAGE (12% gel). Protein concentrations were determined using a modified Bradford assay (Bio-Rad, Hercules, CA).

End Labeling of the DNA Probe. Synthetic oligonucleotides lacking a phosphate group at the 5′ end (Intergrated DNA Technologies, Coralville, IA) were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Unincorporated radioactive nucleotides were removed using a Sephadex G-50 spin column, and the amount of radioactivity (counts per minute) was measured by scintillation counting.

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift reactions were set up by mixing the appropriate radiolabeled DNA probe with various amounts of the purified protein in 12 mM HEPES, 4 mM Tris-HCl (pH 7.5), 60 mM KCl, 1 mM EDTA, 1 mM DTT, 2 μg of poly(dI-dC), and 10% glycerol. Approximately 10 000 cpm of the labeled DNA probe was used per reaction. Reaction mixtures were incubated at room temperature for 15−20 min and loaded onto a 4% native polyacrylamide gel which had been prerun for 1 h. The gel was run in 0.25× TBE (22.5 mM Tris, 22 mM boric acid, and 0.5 mM EDTA) for approximately 1 h at 25 mA. The gel was removed from the gel apparatus, transferred onto Whatman paper, covered with saran wrap, and dried for 45 min at 80 °C. X-ray film was exposed directly to the gels overnight at −80 °C.

PCR-Mediated Site-Directed Mutagenesis. A PCR-mediated site-directed mutagenesis method was used to create point mutations within wild-type gene sequences. The method consists of two rounds of PCR followed by subcloning. For both rounds of PCR, plasmid DNA containing the wild-type gene was used as the template. The first round of PCR utilized a 5′ primer containing a *Bam*HI restriction site and one of the following mutagenic primers (the altered nucleotides are indicated in bold): C204S and C210S, CCGTCAGGATCGT**C**CCCTGGAGGCGGAAGAT**C**CAACGGCACAGG; and C219S and C222S, GCGGAGGGGT**C**TGGTGG**AT**CCCCCGCATACAACAACCGCG.

The second round of PCR used a 3′ primer containing an *Nco*I site and the gel-purified mutated product from the first round (980 bp). The first PCR product was polymerized by high-fidelity *Pfx* polymerase (Gibco BRL, Gaithersberg, MD), and the final PCR product was polymerized using *Taq*

[1] Abbreviations: SRE, siderophore regulatory protein; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; IPTG, isopropyl 1-thio-β-D-galactopyranoside; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, hydroxyethylpiperazine ethanesulfonic acid; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol.

polymerase. For the in vivo wild-type control, the DNA insert was produced by a single round of PCR using the two wild-type end primers. The final product (~2300 bp) was ligated into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). The resulting pCR2.1 constructs were purified, sequenced, and used for transforming *Neurospora*.

For the protein expression vectors, the pGEX-2T glutathione *S*-transferase fusion protein vector containing the wild-type *sre* sequence (lacking introns) was used as the template for PCR. The PCR strategy described above was employed using primers designed to introduce a 5′ *Bam*HI site and a 3′ *Eco*RI site, which were then utilized for subcloning into pGEX-2T.

Neurospora Assays. Siderophore ferric perchlorate assays and ornithine oxygenase activity assays were carried out as previously described (*6*). Transformation of a *sre* mutant strain with plasmids carrying wild-type and manipulated *sre* genes was carried out by cotransformation with a plasmid that contained the benomyl resistant gene, using the protocol described previously (*6*).

UV−Visible Spectrophotometry. UV−visible spectra were collected for the purified wild-type and mutant GST fusion proteins in 50 mM Tris-HCl (pH 8.0) in a MultiSpec-1501 (Shimadzu) spectrophotometer.

RESULTS

Electrophoretic Mobility Shift Assays with SRE. The DNA binding specificity of SRE was characterized by conducting electrophoretic mobility shift assays. A GST fusion protein consisting of a 267-amino acid region of SRE that contained both zinc fingers and the region between them was expressed using the GST fusion protein expression vector pGEX-2T (Pharmacia, Piscataway, NJ) and purified as described in Materials and Methods. The SRE fusion protein was used for DNA binding studies.

To examine the specificity of SRE binding, mobility shift assays were conducted with a synthetic DNA probe containing two GATA elements and compared with a similar probe containing two GAAA elements. SRE bound to the DNA probe containing two GATA sequences, but not to the otherwise identical probe with GAAA sites (Figure 1). The shift obtained with the GATA-containing probe produced two bands with the higher-molecular weight band becoming more prominent with increasing SRE concentrations. These results may be due to the binding of two separate SRE proteins to the probe since it contains two GATA sites.

To examine binding to a natural DNA sequence, a DNA probe consisting of the "iron response element" of the *sid1* gene promoter of *U. maydis*, which contains two GATA sequences, was utilized (*11*). EMSA results indicate that SRE efficiently binds to the *sid1* promoter as demonstrated by a significant shift of the probe by the SRE fusion protein (Figure 2).

To further examine the DNA binding specificity of SRE, DNA fragments containing GATA sequences from the *sid1* and *asd4* promoters (both containing two GATA sites) were used for mobility shift assays. The *asd4* gene is involved in ascospore development and has no role in siderophore synthesis. We find that SRE binds the *sid1* promoter probe with a higher affinity than to the *asd4* promoter probe (Figure 3). To ensure that the difference in binding was not simply a function of the purity of the probe, binding to the two different probes by NIT2, another GATA factor in *Neurospora*, was examined. In contrast to SRE, NIT2 binds to the *asd4* probe with a similar or slightly higher affinity than to the *sid1* probe. Thus, the recombinant SRE−GST fusion protein shows a greater affinity in binding to a promoter sequence of a siderophore biosynthetic gene than to the unrelated asd4 promoter.

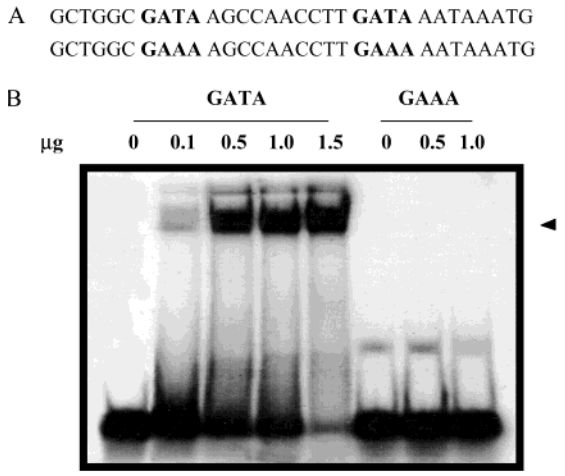

FIGURE 1: SRE binding to GATA-containing DNA. (A) Sequences of the top strand of the double-stranded DNA probes containing either two GATA or two GAAA sites. (B) Results of EMSA experiments showing the free probe at the bottom of the gel, and the shifted probe, denoted with the arrow. The amount of the GST−SRE fusion protein (micrograms) used in each lane is indicated above the lanes.

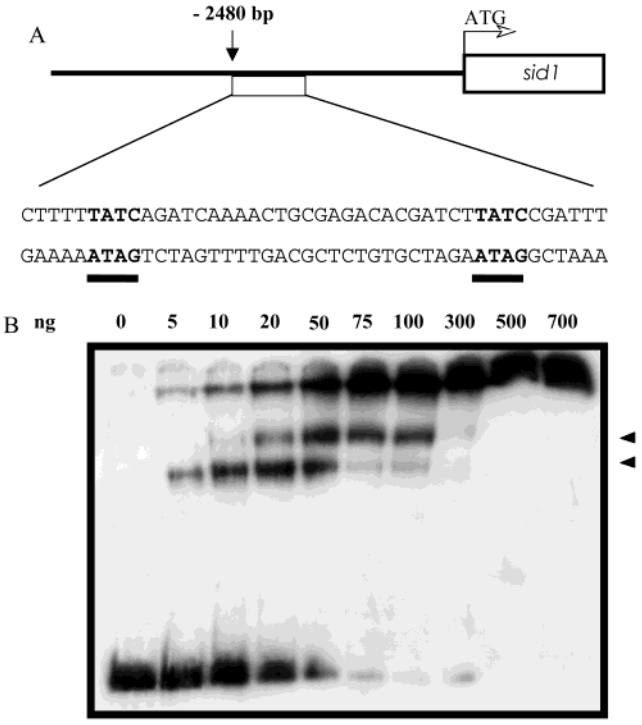

FIGURE 2: SRE binding to the *sid1* promoter of *U. maydis*. (A) Illustration of the iron responsive region and sequence of the *sid1* promoter. (B) EMSA with the region of the *sid1* promoter. The amount of protein (nanogram) used in each reaction is indicated above each lane. The free probe is located at the bottom of the gel, and the arrows identify shifted bands.

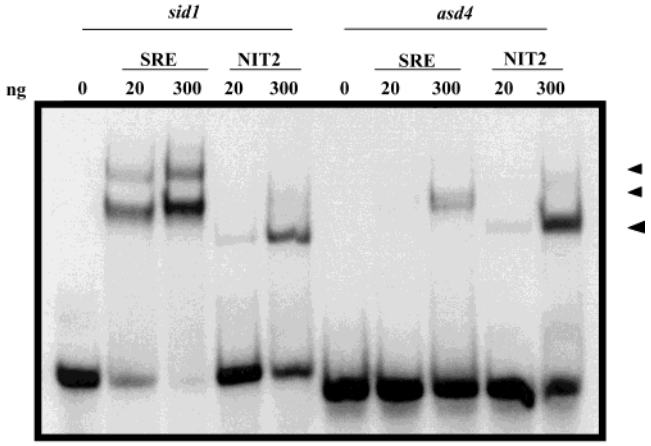

FIGURE 3: SRE binding to the *sid1* promoter in comparison to the *asd4* promoter. An EMSA was carried out using the region of the *sid1* promoter and a region of the *asd4* promoter using the GST−SRE fusion protein and the NIT2 GATA factor from *N. crassa*. The amount of protein (nanogram) used in each reaction is indicated above the lane along with the respective GATA factor protein used in the reaction. The arrows identify shifted bands which represent DNA−protein complexes; free DNA has run near the bottom of the gel.

Effects of Spacing between GATA Sites on DNA Binding. Both the *asd4* and *sid1* promoter DNA fragments contain two GATA sequences, so the difference in SRE binding to them is not due to a different number of potential binding sites. The major difference between these two probes is the spacing between their two GATA elements, with a greater spacing in the *sid1* promoter probe than in the *asd4* promoter fragment (25 and 10 bp, respectively). Probes were prepared in which the spacing between the two GATA sequences was increased or decreased. First, the spacing between the GATA sites of the *sid1* probe was decreased by 5 and 10 nucleotides (Figure 4A). EMSAs demonstrated that the extent of SRE binding decreased as the spacing between the two GATA sites was decreased (Figure 4B). Next, the spacing between the GATA sites of the *asd4* probe was increased by 5, 10, or 15 nucleotides (Figure 5A). The inserted nucleotide sequences correspond to the sequence found between the GATA sites of the *sid1* probe. The extent of DNA binding increased as the spacing between the GATA sites of the *asd4* probe was increased (Figure 5B). These results indicate that SRE has a clear preference for binding to DNA in which two GATA sites are a certain distance apart, with increasing affinity observed as the spacing is increased from 10 to 25 bp.

Zinc Dependence on DNA Binding. It has been shown that mutation of either one or both of the putative zinc fingers in SRE results in a decrease in the level of or complete loss of binding of SRE to GATA-containing DNA sequences, respectively (*14*). To investigate the importance of zinc for DNA binding of SRE, the effect of EDTA, a zinc chelator, on binding was examined. EDTA was added to SRE fusion protein samples to a final concentration of 1 mM, and a sample without EDTA was processed as a control. The protein samples were heated to 42 °C to enhance the accessibility of the predicted coordinated zinc atoms to EDTA. The samples were then allowed to cool to room temperature and subsequently used for DNA binding assays. As seen in lane 3 of Figure 6, heating of the protein sample alone does not diminish the level of DNA binding. In

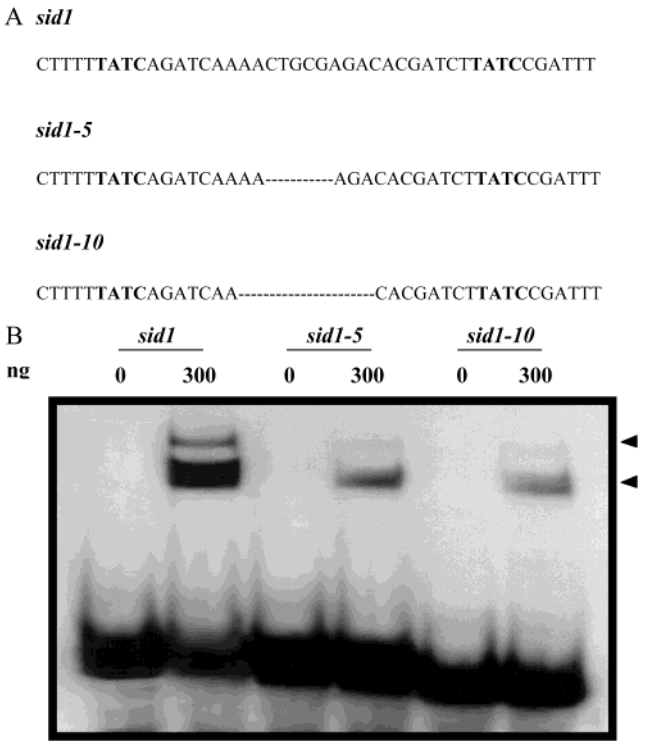

FIGURE 4: Effects of spacing between the GATA sites of the *sid1* promoter. (A) Sequences of the *sid1* promoter probe (*sid1*) and the probes with 5 (*sid1−5*) and 10 base pairs (*sid1−10*) removed from the sequence between the two GATA sites. (B) Mobility shift assay using 0 or 300 ng of the GST−SRE fusion protein with the different *sid1* probes. The arrows identify the shifted bands.

contrast, treatment with 1 mM EDTA completely eliminates DNA binding by SRE as shown in lane 4. To investigate the possible effect of various divalent cations in rescuing DNA binding, Zn^{2+}, Cd^{2+}, Mg^{2+}, or Ca^{2+} was individually added to EDTA-treated protein samples prior to incubating them with the DNA probe. Once again, protein samples were heated and allowed to slowly cool to room temperature. The results show that zinc and cadmium are capable of partially restoring DNA binding (Figure 6). Lower levels of cadmium were required to restore a functional zinc finger. It has been shown that certain zinc fingers display higher affinities for cadmium than for zinc (*15*, *16*). The inability of magnesium and calcium to restore DNA binding indicates that SRE requires specific cations to allow its finger motifs to assume a conformation functional in DNA binding.

In Vivo Studies of the Cysteine Mutants. The region between the two zinc fingers of SRE contains four cysteine residues that are conserved in the homologous proteins. Site-directed mutagenesis was carried out to examine potential roles of the cysteine residues by mutating either the first two (C204S and C210S) or the last two (C219S and C222S) cysteine residues located between the finger motifs of SRE. Mutant fragments consisting of the entire *sre* coding region and ~500 bp of the 5′ upstream region were obtained and subcloned into pCR2.1. A similar construct was also produced in which no mutations were introduced, thus yielding the wild-type sequence. The wild-type plasmid was used as a positive control to be certain that any phenotypes resulting from the mutated SRE genes were direct effects of the introduced mutations and not a result of the carrier vector or the lack of sufficient 5′ upstream sequence.

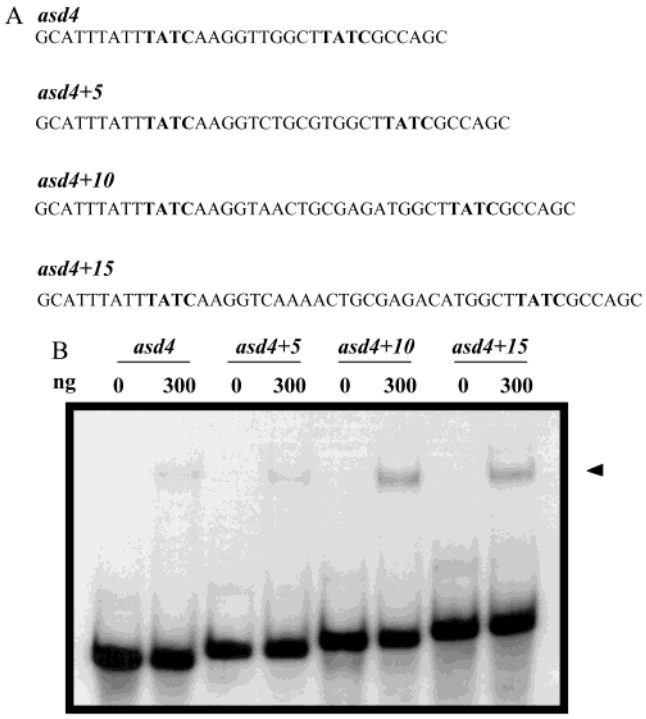

FIGURE 5: Effects of spacing between the GATA sites of the *asd4* promoter. (A) Sequences of the top strand of the *asd4* probe along with the probes containing 5 (*asd4+5*), 10 (*asd4+10*), or 15 additional base pairs (*asd4+15*) between the two GATA sites. (B) Mobility shift results using the four above-mentioned probes with 0 or 300 ng of the GST−SRE fusion protein. The arrow identifies the shifted band.

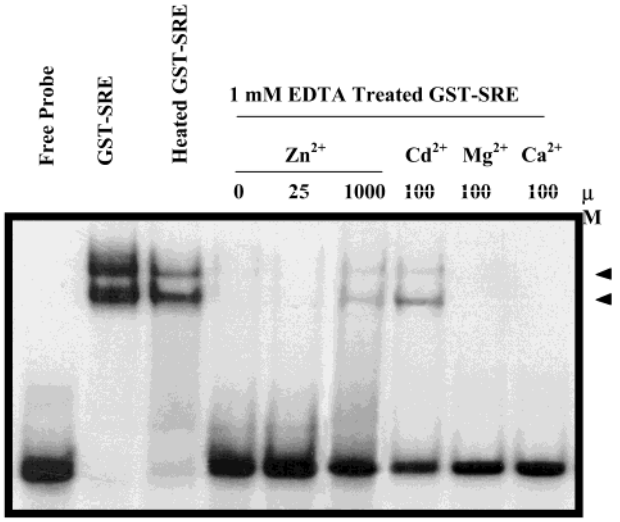

FIGURE 6: Effects of EDTA on DNA binding. The GST−SRE fusion protein (300 ng) was heated to 42 °C for 20 min with or without 1 mM EDTA. Samples were incubated at room temperature for 20 min prior to gel filtration to remove excess EDTA. The EDTA-treated GST−SRE fusion protein was incubated with various divalent cations (Zn^{2+}, Cd^{2+}, Mg^{2+}, and Ca^{2+}) at 42 °C for 20 min and cooled to room temperature. The concentration (micromolar) of cations used is indicated above each lane. Following various treatments, the GST−SRE fusion protein was incubated with the *sid1* promoter probe at room temperature for 20 min and loaded on a 4% native gel. Free DNA is at the bottom of the gels. The arrows identify the shifted bands that represent DNA−protein complexes.

As shown previously, in wild-type *Neurospora*, siderophore production and L-ornithine N^5-oxygenase activity are repressed under high-environmental iron conditions and derepressed under low-iron conditions (*6*). The *sre* RIP mutant strain shows an increase in the extent of siderophore synthesis and enzyme activity under both high- and low-iron conditions in comparison to the wild type, although iron-dependent regulation is not completely lost. To check the ability of the plasmid containing the wild-type *sre* gene to restore normal regulation of siderophore production and ornithine oxygenase activity, the pCR2.1 plasmid containing the wild-type *sre* gene insert was transformed into the *Neurospora sre* RIP mutant, as described in Materials and Methods. The transformed colonies were grown in high- and low-iron media, and the amount of siderophores produced and secreted in each case was measured using the ferric perchlorate assay. The transformed wild-type *sre* gene was capable of complementing the *sre* RIP mutant cells as determined by colorimetric assays (Table 1). Additionally, transformant cell extracts were used for ornithine oxygenase activity assays, which revealed that, as expected, the wild-type *sre* gene restored normal regulation of ornithine oxygenase activity (Table 1).

Next, plasmids containing the SRE mutant genes with substitutions for the targeted cysteine residues were transformed into *sre* RIP mutant spheroplasts. To be certain that the wild-type and mutant *sre* genes had been integrated into the transformed strains, genomic DNA was isolated and probed by Southern blot analysis for the presence of the transformed *sre* DNA. All colonies that were assayed possessed the transformed *sre* gene (data not shown). The presence of the cysteine mutant *sre* genes resulted in strong repression of siderophore synthesis under both high- and low-iron conditions (Table 1). Additionally, ornithine oxygenase activity was repressed in transformed colonies containing the mutated *sre* genes with replacements of the cysteine residues in media with both high and low iron concentrations (Table 1). To determine how the mutant genes would function in the presence of a wild-type *sre* gene, plasmids containing the cysteine mutants were transformed into wild-type *Neurospora*. The results show that as in the RIP mutant, the presence of the mutant gene results in repression of siderophore synthesis and ornithine oxygenase activity under both high- and low-iron conditions (Table 1). The results suggest that the mutant protein acts as a dominant negative and competes with endogenous wild-type SRE, resulting in complete repression of siderophore synthesis and enzyme activity. Since SRE acts as a repressor of siderophore synthesis, it appears that the mutant proteins are constitutively active regardless of the intracellular iron status.

In Vitro Studies of Cysteine Mutants. To elucidate specifically how the mutated cysteines alter regulation of siderophore synthesis, in vitro studies were carried out using a recombinant protein containing serine residues in place of the first two cysteine residues of the region (Cys204 and Cys210). GST−SRE fusion proteins containing the wild-type or mutated amino acid sequences were individually expressed and purified. The first obvious difference between the wild-type and cysteine mutant SRE proteins was observed during the purification steps. Upon elution of the bound proteins during column chromatography, an orange band was clearly observed migrating down the column containing wild-type SRE; however, the mutant SRE migrated as a colorless band.

Table 1: Siderophore Synthesis and Ornithine Oxygenase Enzyme Activity[a]

	siderophore synthesis		ornithine oxygenase activity	
strain	High iron	Low iron	High iron	Low iron
wild type	0.070 (0.010)	0.200 (0.010)	0.025 (0.001)	0.067 (0.003)
sre−	0.150 (0.010)	0.300 (0.010)	0.100 (0.010)	0.257 (0.010)
sre− WT comp	0.075 (0.010	0.260 (0.010)	0.159 (0.003)	0.319 (0.003)
sre− C12S comp	0.053 (0.010	0.093 (0.005)	0.004 (0.002)	0.005 (0.003)
sre− C34S comp	0.080 (0.008)	0.080 (0.008)	0.003 (0.001)	0.006 (0.002)
C12S	0.015 (0.010)	0.0567 (0.003)	0.026 (0.004)	0.030 (0.003)
C34S	0.015 (0.005)	0.045 (0.010)	0.003 (0.002)	0.003 (0.001)

[a] The relative amounts of siderophore synthesis and enzyme activity were determined for the various strains. The wild type, the *sre* RIP mutant strain (*sre*−), the RIP mutant strain complemented with the pCR2.1 vector containing the wild-type (*sre*− WT comp) or mutated *sre* gene (*sre*− C12S comp or *sre*− C34S comp) sequences, and the wild type transformed with the mutant genes (C12S and C34S) are shown. Mutant strains are labeled as having the first two cysteine residues (C12S) or the third and fourth cysteine residues (C34S) of the cysteine rich region mutated to serine residues. Data for the strains grown in high- and low-iron media are indicated. The absorbance values were normalized against absolute protein concentration and represent the average from three determinations with the standard deviation shown in parentheses.

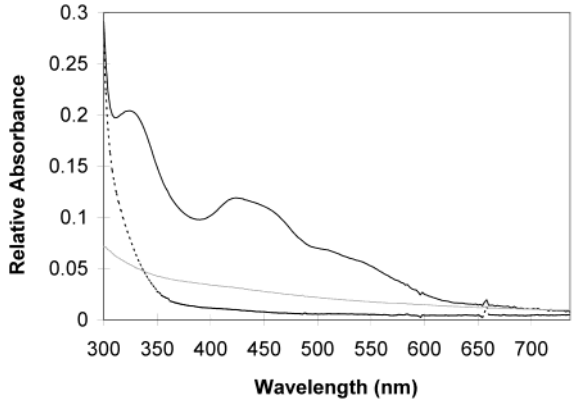

FIGURE 7: UV−visible spectrum of wild-type and mutant SRE proteins. UV−visible absorption spectra of the oxidized cysteine mutant protein (gray line) compared with the wild-type oxidized (black line) and reduced (dotted line) SRE protein.

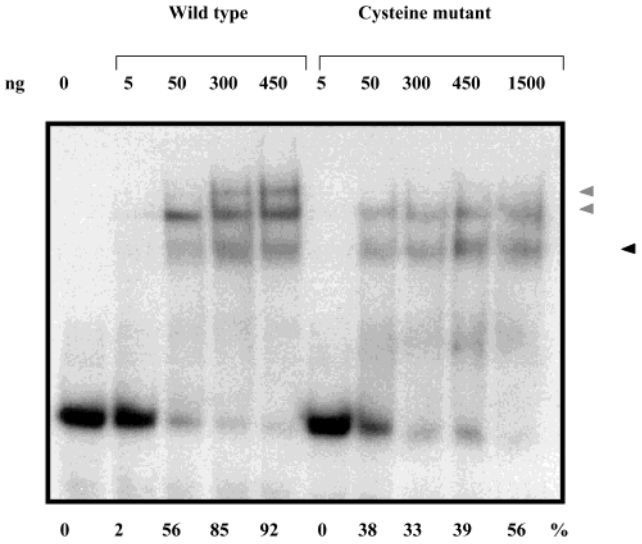

FIGURE 8: Comparison of DNA binding of the wild-type and cysteine mutant proteins. An electrophoretic mobility shift assay was used to examine the binding of SRE proteins to the *sid1* 44 bp oligonucleotide probe. The amount of protein used in each binding reaction is listed above the lanes. The percentage of the DNA bound in each reaction is indicated below each lane (as determined by densitometric values). The arrows identify shifted bands that indicate protein−DNA complexes.

To examine the color difference between the mutant and wild-type SRE, visible spectra were obtained for each. The purified wild-type SRE protein yields a spectrum with an absorption maximum located at approximately 420 nm with a shoulder at 540 nm (Figure 7), features broadly characteristic of iron-binding proteins. In contrast, the visible spectrum for the purified cysteine mutant SRE protein lacks the absorption bands exhibited by the wild-type SRE protein. To compare the spectra of oxidized (as purified) and reduced wild-type SRE, dithionite was added to a concentration of 1 mM to reduce the wild-type SRE protein. Dithionite-mediated reduction results in a complete bleaching of the absorbance spectrum of oxidized (as isolated) SRE (Figure 7).

The DNA binding properties of the cysteine mutant protein were examined by employing mobility shift assays using the *sid1* 44 bp probe. Increasing equivalent amounts of wild-type and cysteine mutant SRE proteins were added to binding reaction mixtures with a constant amount of labeled DNA. The results show that the mutant protein retains the ability to bind to DNA; however, the mutant protein appears to have a lower binding affinity than the wild-type protein (Figure 8). The percentage of DNA bound for each reaction was calculated on the basis of the apparent densities of the shifted bands. The results show that the wild-type protein shifted 92% of the DNA probe when 450 ng of the protein was added to the reaction mixture, whereas the mutant protein even at a higher concentration of 1.5 μg was only able to shift 56% of the DNA probe. These results indicate that the cysteine mutant protein is capable of significant DNA binding, however, with a slightly lower affinity than the wild-type SRE protein.

DISCUSSION

The mechanism by which SRE regulates genes involved in siderophore synthesis is still not completely understood. Indeed, each of the five known *Neurospora* GATA factors possesses the ability to bind to DNA containing core GATA sequences via their zinc finger domains, a motif in which all GATA factors share a high degree of homology. However, each GATA factor protein is responsible for regulating specific and distinctly different cellular processes. It seems unlikely that the basis for this differentiation arises from differences in their ability to access DNA since these proteins are not compartmentalized into different cellular locations. Thus, the exact mechanism by which each GATA factor specifically regulates a distinct set of downstream genes is not clear.

Results presented here provide evidence that the spacing between multiple GATA sites may constitute one possible mechanism for GATA factor specificity. Specificity in the case of SRE may also result from its possession of two zinc fingers, whereas the other GATA factors found in *Neurospora* contain only a single zinc finger. A single molecule of SRE may be capable of binding to two correctly spaced GATA sites simultaneously due to the presence of its dual zinc fingers. It has been shown that mutant SRE constructs possessing only one of each of the individual zinc fingers of wild-type SRE can bind to DNA containing GATA sites; however, each of the mutants does so with an affinity lower than that of the wild-type protein (*14*). Thus, it seems likely that SRE's two zinc fingers may be crucial in positioning the protein on the DNA.

SRE may also bind to DNA as a dimer. As in vertebrate GATA factors, one zinc finger of SRE may be involved in DNA binding while the second carries out protein−protein interactions (possibly with a second SRE molecule) (*17*, *18*). The carboxyl-terminal region of SRE, which shows conservation among its homologues, is another region that may promote dimerization. Analysis of the amino acid sequence of SRE using the MultiCoil analysis algorithm reveals a putative carboxyl-terminal coiled-coil motif (*19*). This feature indicates that SRE has a great potential to exist as a homodimer, which may be required for its function, since it may bind to adjacent GATA sites as a homodimeric complex. It is also quite possible that SRE may be involved in significant interactions with one or more other proteins which modulate its function. It has been clearly established that the activity of many GATA factors is dependent upon their interactions with additional protein factors (*18*, *20*, *21*). It is noteworthy that examination of the *Neurospora* genome reveals that all GATA factors of *Neurospora* have been identified.

Whether SRE directly senses iron, for example, by a reversible iron binding mechanism dependent on intracellular iron concentration or if SRE is an element of a more elaborate signal transduction cascade is not yet known. Interestingly, purified SRE is reddish-brown in color with a significant absorption band centered at 420 nm and a shoulder at approximately 540 nm. Site-directed mutants in which conserved cysteine residues were substituted yielded a mutant SRE protein that, upon purification, displayed no significant absorption, suggesting that these cysteine residues may serve to bind one or more iron atoms. In addition, in vivo studies were performed in *Neurospora* using the cysteine mutant *sre* genes. The results from ornithine oxygenase activity assays and siderophore synthesis determinations suggest that mutation of the cysteine residues results in what appears to be a dominant repressor phenotype where cells containing the mutant protein fail to exhibit iron-mediated regulation of siderophore synthesis even in the presence of the endogenous wild-type SRE protein. In these mutants, siderophore synthesis is strongly repressed, indicating that the mutant SRE protein has lost the ability to sense and respond to different environmental iron concentrations, but instead remains constitutively active.

Considering all of the information now available, one can propose several possible mechanisms for the action of SRE in iron homeostasis. The partial constitutive siderophore production in the *sre* RIP mutant strain suggests that other unidentified factors, acting either positively or negatively, also function in iron-mediated regulation of siderophore biosynthesis. Iron homeostasis may be governed at many levels and involve multiple factors to implement a failsafe means of ensuring proper intracellular levels of iron. To acquire essential iron while avoiding toxic levels, a delicate balance must be sustained. The results presented here provide a foundation for further studies aimed at elucidating the molecular mechanisms responsible for the elaborate control of iron homeostasis in *Neurospora*.

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