SRE1 Regulates Iron-Dependent and -Independent Pathways in the Fungal Pathogen Histoplasma capsulatum

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Regulation of iron acquisition genes is critical for microbial survival under both iron-limiting conditions (to acquire essential iron) and iron-replete conditions (to limit iron toxicity). In fungi, iron acquisition genes are repressed under iron-replete conditions by a conserved GATA transcriptional regulator. Here we investigate the role of this transcription factor, Sre1, in the cellular responses of the fungal pathogen Histoplasma capsulatum to iron. We showed that cells in which SRE1 levels were diminished by RNA interference were unable to repress siderophore biosynthesis and utilization genes in the presence of abundant iron and thus produced siderophores even under iron-replete conditions. Mutation of a GATA-containing consensus site found in the promoters of these genes also resulted in inappropriate gene expression under iron-replete conditions. Microarray analysis comparing control and SRE1-depleted strains under conditions of iron limitation or abundance revealed both iron-responsive genes and Sre1-dependent genes, which comprised distinct but overlapping sets. Iron-responsive genes included those encoding putative oxidoreductases, metabolic and mitochondrial enzymes, superoxide dismutase, and nitrosative-stress-response genes; Sre1-dependent genes were of diverse functions. Genes regulated by iron levels and Sre1 included all of the siderophore biosynthesis genes, a gene involved in reductive iron acquisition, an iron-responsive transcription factor, and two catalases. Based on transcriptional profiling and phenotypic analyses, we conclude that Sre1 plays a critical role in the regulation of both traditional iron-responsive genes and iron-independent pathways such as regulation of cell morphology. These data highlight the evolving realization that the effect of Sre1 orthologs on fungal biology extends beyond the iron regulon.

Iron acquisition is essential for the survival of most microorganisms, as it is an important cofactor in a number of essential metabolic processes. However, excess iron within a cell can result in toxicity due to the production of cell-damaging reactive oxygen species (9, 10). Therefore, the acquisition and storage of iron within cells are tightly regulated processes. Iron availability and restriction are also important aspects of pathogen host interactions (32). Restriction of iron within the mammalian host is a crucial part of the mammalian defense system. In addition, some pathogens sense low iron levels as an environmental signal that results in the expression of iron uptake genes as well as virulence determinants.

In previous work, we studied the role of iron acquisition genes in the pathogenesis of the fungus Histoplasma capsulatum (18). H. capsulatum is a dimorphic fungal pathogen that causes respiratory and systemic disease in both healthy and immunocompromised humans (2, 7, 25, 30, 40). Inhalation of fungal spores or mycellal fragments from contaminated soil initiates a respiratory infection. Once in the mammalian host, H. capsulatum grows in a budding yeast form that parasitizes alveolar macrophages.

Iron limitation in H. capsulatum induces the production and secretion of hydroxamate siderophores, which act as low-molecular-weight ferric iron chelators (3, 16). Additionally, low iron levels have been shown to induce a reductive iron assimilation pathway (36, 37). Previously, we identified a genomic cluster of coregulated genes involved in the biosynthesis and utilization of siderophores (18). Regulation of siderophore production in H. capsulatum, as with many other microorganisms, appears to occur at the level of transcription. In many fungi, a GATA family transcriptional repressor negatively regulates siderophore biosynthesis gene expression under iron-replete conditions (5, 8, 11, 23, 29, 39, 42). Using bioinformatics analysis, we and others identified H. capsulatum Sre1 as an ortholog of these regulators (4). A truncated version of Sre1 was shown in vitro to bind to a consensus sequence found in the promoters of all of the siderophore biosynthesis genes (4).

In this study, we determined the in vivo role of Sre1 in iron-responsive gene expression. Since gene disruption is technically challenging in H. capsulatum, we depleted levels of Sre1 in H. capsulatum by the use of RNA interference. We showed that Sre1 is essential for the iron-dependent regulation of siderophore biosynthesis genes and determined that a consensus site in the promoters of these genes is required for proper regulation by Sre1. Finally, we performed whole-genome microarray analysis in SRE1 RNA interference (RNAi) and vector control strains to identify both those genes whose expression is regulated by iron and those genes whose expression is regulated by Sre1. Interestingly, we found that whereas Sre1 is essential for appropriate regulation of genes involved in H. capsulatum iron acquisition, it also appears to regulate other cellular processes, including optimal filamentous growth.

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MATERIALS AND METHODS

Strains and culture conditions. *Histoplasma capsulatum* strains G217B (ATCC 26032) and G217BBara5A (WU15), all kind gifts from the laboratory of William Goldman, Washington University, St. Louis, were grown in HMM (*Histoplasma*-macrophage medium) broth or plates (41) or in mRPMI broth (RPMI 1640 medium without phenol red or bicarbonate (Invitrogen) supplemented with 1.8% dextrose (Fisher Scientific), 25 mM HEPES (Fisher Scientific) (pH 6.5), and both penicillin and streptomycin (UCSF Cell Culture Facility [http://www.ccf.ucsf.edu/]) (100 U/ml each) and modified from the method described in reference 36. Unlike HMM, mRPMI contained no added iron. Cultures were grown in mRPMI medium in plastic flasks to reduce trace iron contamination, but trace amounts of iron were presumably not completely eliminated. Media were supplemented with various concentrations of FeSO4 (Fisher Scientific) as described below. HMM and mRPMI media were supplemented when necessary with hygromycin (Roche) (200 μg/ml) and uracil (Sigma-Aldrich) (200 μg/ml). Cultures were grown at 37°C under 5% CO2.

For quantitative reverse transcriptase PCR (qRT-PCR), strains were grown in HMM to the log phase, subjected to passage at 1:25 into 50-ml HMM, and grown for 24 h. The culture (5 ml) was harvested at the zero time point, and then the cultures were pelleted, washed with phosphate-buffered saline (PBS), and resuspended in mRPMI media. The cultures were split into two sets, and 5 μM FeSO4 was added to one set. Samples (5 ml) were harvested at various time points.

For microarray studies, initial cultures of HcLH120 (control RNAi-1), HcLH121 (control RNAi-2), HcLH122 (SRE1 RNAi-1), or HcLH123 (SRE1 RNAi-2) yeast cells were grown in 5 ml of HMM medium and then subjected to passage at 1:25 into 100 ml of HMM medium. After 2 days of growth, the cultures were pelleted, washed in 100 ml of PBS, resuspended in 100 ml of mRPMI (pH 6.5), and diluted to an optical density at 600 nm (OD600) of approximately 2 in 1 liter of mRPMI medium. After 24 h of growth, 200 ml of culture was harvested for each zero time point. The cultures were then split into 2 400-ml fractions, and 10 μM FeSO4 (final concentration) was added to one set of cultures. At each time point, 100 ml of culture was harvested and processed as described below.

Siderophore production assays. Cells were grown in 5 ml of HMM medium to the late log phase, sonicated twice for 3 s to disperse cells, and then subjected to passage into 25 ml of HMM medium. After 24 h of growth, they were pelleted, washed in 50 ml of PBS, and resuspended in 25 ml of mRPMI medium (pH 6.5). To measure growth, triplicate samples of 1 ml of cells were taken at each time point and sonicated twice for 3 s, and dilutions were measured by spectrophotometer at OD600. Quantification of siderophores was performed as described previously (18). Briefly, 0.5 ml of culture supernatant was mixed with 0.5 ml of CAS assay solution (600 μM hexadecyltrimethyl ammonium [Sigma-Aldrich], 15 μM FeCl3 [Sigma-Aldrich], 150 μM CAS [Sigma-Aldrich], 500 mM anhydrous piperazine [Fluka], 750 mM HCl [Fisher Scientific], and 4 mM 5-sulfosalicylic acid [Sigma-Aldrich]) and incubated for 1 h, and the OD530 was measured with a spectrophotometer. Quantification of siderophores was normalized to OD600.

Construction of SRE1 RNAi. A 526-bp region of the SRE1 coding sequence was amplified by PCR using G217B cDNA and oligonucleotides OAS1442 and OAS1443. The product was used to generate pLH202, containing a hairpin repeat of SRE1 in backbone vector pFANTAIa4. The vector control (pVN69) and pLH202 were integrated into the *H. capsulatum* strain G217B by the use of an *Agrobacterium*-mediated gene transfer method as described previously (26, 35).

Construction of gfp fusions. Wild-type and mutant versions of the SID4 promoter driving expression of the green fluorescent protein (gfp) reporter gene were generated as follows. The wild-type and mutant versions of the SID4 promoter were amplified using oligonucleotides OAS1293 and OAS1295. The promoter was mutated using fusion PCR, introducing the mutations with oligonucleotides OAS1296 and OAS1297 to mutate the GATA site, OAS1298 and OAS1299 to mutate the consensus site, and OAS1300 and OAS1301 to mutate both. PCR products were cloned into pCR2.1 by the use of TOPO-TA cloning (Invitrogen). Restriction sites were introduced into all versions of the SRE1 promoter by the use of PCR with oligonucleotides OAS2001 and OAS2002, and the products were again cloned into pCR2.1. The promoters were then cloned into pD96 (gift from Diane Inglis) in which the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) promoter was removed and replaced with versions of the SID4 promoter to drive expression of gfp.

The OXR1 promoter driving expression of the gfp reporter gene was generated by fusion PCR. The promoter of OXR1, including 1,046 bp upstream of the start codon, was amplified using oligonucleotides OAS1573 and OAS1574. OAS1575 and OAS1576 were used to amplify 592 bp immediately 3′ to the OXR1 coding sequence, including the stop codon. OAS1574 and OAS1575 also contain sequences homologous to gfp. The gfp coding sequence was amplified with OAS1398 and OAS1399. All three PCR products were gel purified and then combined and amplified with OAS1573 and OAS1576. The final product was recombined into pDONR/Zeо by the use of a Gateway system (Invitrogen).

Site-directed mutagenesis of the OXR1 promoter was performed using QuikChange site-directed mutagenesis protocols (Stratagene). The oligonuclotides used are listed in Table S6 in the supplemental material.

The wild-type and mutant versions of the SID4 and OXR1 promoters were all confirmed by sequencing. The constructs were then recombined with pVN47 by the use of Gateway cloning (Invitrogen), transformed into A. fumigatus strain LBA1100, and integrated into H. capsulatum strain G217B by the use of *Agrobacterium*-mediated gene transfer.

*Agrobacterium* transformation. *H. capsulatum* yeast cells were transformed using *Agrobacterium*-mediated gene transfer as described previously (26, 35). Briefly, the A. *fumigatus* strain (LBA1100; a kind gift of Thomas Sullivan and Bruce Klein with permission from Paul Hooeyks [Leiden University, Leiden, The Netherlands]) transformed with the desired plasmid was induced overnight with 200 μM acetyrsingone (AS; Sigma-Aldrich). *H. capsulatum* yeast cells were harvested from 4-day patches on HMM plates and diluted to 5 × 105 cells/ml. Equal volumes of the *H. capsulatum* and A. *fumigatus* cultures were mixed, and 400 μl of the mixture was spread onto Biodyne A nylon membranes ( Pall Gelman) on IM (induction medium) agarose plates containing 200 μM AS. Co-cultivation plates were incubated at 28°C for 3 days. The membranes were then transferred onto HMM plates with the appropriate selection and incubated at 37°C for 2 to 3 weeks.

Quantitative RT-PCR. Total RNA isolated for microarray studies was treated with DNase I (Promega). cDNA was synthesized using 3.3 μg of DNase I-treated RNA, Stratascript reverse transcriptase (Stratagene), and oligo(dT). Quantitative PCR was performed using 1:100 dilutions of cDNA, 1.5 mM MgCl2, 1× AmpliTaq Gold buffer, 0.6 U of AmpliTaq Gold (Applied Biosystems), 1× SYBR green (Molecular Probes), and 200 nM primers. Reactions were performed using an MX3000P qPCR system (Stratagene) with the Comparative Quantitation (with dissociation curve) program. Cycling parameters were 95°C for 10 min and then 40 cycles of 95°C (30 s), 57°C (1 min), and 72°C (30 s); cycling was followed by dissociation curve analysis. All reactions were performed in triplicate. A calibrator sample made from a mix consisting of equal amounts of all RNA samples was included on each plate. Reactions were analyzed using MxPro software. Primer sequences are included in Table S6 in the supplemental material.

Microarrays. Cultures of *H. capsulatum* were harvested by filtration, and total RNA was isolated using a guanidine thiocyanate lysis protocol as previously described (27). CDNA was synthesized from 30 μg of total RNA from strains HcLH120 (control RNAi-1) and HcLH123 (SRE1 RNAi-2), and fluorescent labeling was performed as described previously (27). Due to difficulties in generating cDNA from iron-starved *H. capsulatum* strains, amplified RNA (aRNA) was synthesized from poly(A)-selected RNA from strains HcLH121 (control RNAi-2) and HcLH122 (SRE1 RNAi-1) by the use of an Ambion MessageAmp II kit. RNA or cDNA from each individual time point was labeled with Cy5 and competitively hybridized against a reference pool generated from either aRNA or cDNA.
Samples were hybridized to *H. capsulatum* G217B 70-mer oligonucleotide microarrays. Each microarray contained one or two 70-mer oligonucleotides for each predicted gene in the G217B genome (11,088 gene predictions and a total of 14,418 oligonucleotides per array).

Arrays were scanned on a GenePix 4000B scanner (Axon Instruments/Molecular Devices) and analyzed using GenePix Pro, version 6.0 (Molecular Devices), Spotreader (Niles Scientific), NOMAD 2.0 (http://nomad2.ucsf.edu), MeV (MultiExperiment Viewer; available at http://www.tm4.org/mev.html), Cluster (6), and Java Treeview 1.0.12 (available at http://sourceforge.net/project/showfiles.php?group_id=84593). To eliminate elements with low signal, we analyzed only elements for which the sum of the medians for the 635-nm and 532-nm channels was ≥500 intensity units. Data from microarray probes corresponding to 3,810 genes met these criteria and were analyzed as follows. Data from all of the time points were normalized relative to the control zero time point. The two time courses were independently examined in MeV by SAM (Significance Analysis of Microarrays) (38) for iron dependence, SRE1 dependence, or iron and SRE1 dependence, with a false-discovery-rate cutoff of 5%. Microarray spots that met those criteria for both time courses were clustered and further analyzed. The only exceptions to the requirement for significance in both time courses were for *NIT3* and *NIT2*, which were found to be Sre1 dependent by the SAM criteria described above in only one of the two time courses. Nonetheless, given their clear regulation by both iron and Sre1 (see Fig. 4B), they were included in the set of iron-dependent genes (see Table S2 in the supplemental material) and Sre1-dependent genes (see Table S3 in the supplemental material). With respect to the tables in the supplemental material, microarray data from one representative time course are included. Some genes are represented by two oligonucleotides (rather than one) on the microarray, and all available data for each gene from the representative time course are present in the tables.

**Assessment of filamentous growth.** Yeast form cultures of vector control strains (HcLH120 and HcLH121) and SRE1 RNAi strains (HcLH122 and HcLH123) were grown to the early log phase in HMM media–hygromycin (200 μg/ml) at 37°C. Cells were washed once in PBS, and 10-fold serial dilutions of each strain were spotted onto HMM medium–hygromycin (200 μg/ml) plates and grown at 37°C to monitor yeast-phase growth. Concurrently, yeast cell dilution series were spotted onto HMM medium–hygromycin (200 μg/ml) plates as well as Sabouraud dextrose (Difco)–hygromycin (200 μg/ml) plates and grown at room temperature to assess mycelium-phase growth. Dilution series were monitored for growth and morphology by light microscopy at between 7 and 14 days postplating.

**RESULTS**

**SRE1 is required for regulation of siderophore production.** To examine the role of SRE1, we generated an integrating RNAi construct to deplete its expression and introduced it into *H. capsulatum* by the use of *Agrobacterium*-mediated transformation. Quantitative RT-PCR (qRT-PCR) was used to determine that the *SRE1* transcript was depleted by at least 80% to 90% in knockdown strains (see Table S1 in the supplemental material for representative data). Three independent RNAi strains were monitored on high-iron medium, where they accumulated an orange pigment (Fig. 1A). Similar color changes, which are indicative of accumulation of iron-bound siderophores, have been observed in a number of fungi, including *Neurospora crassa*, *Ustilago maydis*, and *Blastomyces dermatitidis* upon disruption of genes encoding the Sre1 orthologs SRE, Urbs1, and SREB, respectively (8, 39, 42). Consistent with this finding, when control and SRE1 RNAi strains were examined for siderophore secretion into culture supernatants under low- and high-iron conditions, the SRE1 RNAi strains inappropriately secreted siderophores under high-iron conditions (Fig. 1B). Additionally, under low-iron conditions, the SRE1 RNAi strains accumulated a higher concentration of siderophores than control strains (Fig. 1B).

We attempted to use the color change of the SRE1-depleted strains as a phenotypic readout to identify insertion mutants in the *SRE1* gene that would fully disrupt SRE1 function. Of 200,000 insertion mutants generated by *Agrobacterium*-mediated transformation, only 2 showed orange pigmentation on high-iron medium (data not shown). In one mutant, the SRE1 locus was intact. The second mutant contained a 60-kb deletion that removed SRE1 and 28 neighboring genes (data not shown). Since this screen failed to yield a precise insertion at the SRE1 locus without other genomic perturbations, we used the aforementioned SRE1 RNAi strains for further analysis.

**Sre1 regulates the iron-dependent transcription of siderophore biosynthesis genes.** The simplest explanation for deregulated siderophore production in SRE1 RNAi strains is the effect of deregulated expression of siderophore biosynthesis and transport genes. We previously identified *H. capsulatum* siderophore biosynthesis and transport genes and showed that their expression was repressed under iron-replete conditions (18). These genes included L-ornithine monoxygenase (*SID1*), the enzyme catalyzing the first committed step in siderophore production; an acylase (*SID3*) homologous to *Aspergillus fumigatus* sidF, which is involved in triacetylflusarinine C synthesis; a nonribosomal peptide synthase (*NPS1*); an acid coenzyme A (CoA) ligase (*SID4*); an oxidoreductase (*OXR1*); a major facilitator superfamily (MFS) transporter (*MFS1*); and an ATP-binding cassette (ABC) transporter (*ABC1*). All of these genes except MFS1 are located adjacent
to each other in the genome, creating a genomic cluster of iron acquisition genes (18). To determine whether SRE1 was required to repress expression of these genes under high-iron conditions, we used qRT-PCR to compare the levels of expression of a subset of these genes under low- and high-iron conditions in control and SRE1 RNAi strains. Immediately after harvest at a 0-h time point, cultures were shifted into mRPMI medium (which does not contain exogenous iron), divided in half, and maintained in the absence or presence of 5 μM FeSO₄ (iron-replete conditions) for the next 9 h. In the control strain, expression of SID3, OXR1, and SID1 was strongly upregulated by 9 h after the shift to mRPMI medium lacking iron but remained repressed after the shift to mRPMI medium supplemented with iron (Fig. 2). In the SRE1 RNAi strains, SID3, OXR1, and SID1 failed to be repressed even under iron-replete conditions (Fig. 2), indicating that depletion of SRE1 eliminates iron-dependent transcriptional regulation of these genes. Additionally, to show that SRE1 did not regulate all genes in the vicinity of the siderophore genomic cluster, we examined expression of UBP1, which encodes an ubiquitin C-terminal hydrolase located approximately 6 kb upstream of this genomic region. In contrast to SID3, OXR1, and SID1, addition of iron and/or depletion of SRE1 did not affect expression of UBP1 (Fig. 2).

A GATA consensus site is required for iron-dependent transcriptional regulation. Fungal GATA transcriptional regulators, including most orthologs of Sre1, are known to recognize an HGATAR (5′-(T/A/C)GATA(A/G)-3′) motif (1, 11, 42). In Aspergillus fumigatus, an extended version of this motif [5′-ATC(T/A)GATAA-3′] was found to be enriched in the upstream regions of genes regulated by the Sre1 ortholog SreA (34). In our previous work, we identified a similar extended consensus site (5′-(G/A)ATC(T/A)GATAA-3′) that is present at least once in the upstream regions of all of the genes in the H. capsulatum siderophore biosynthetic cluster (18). Here we refer to this sequence as the Sre1 consensus sequence. Chao et al. used a gel-electrophoretic-mobility-shift assay to demonstrate that the Sre1 consensus sequence is bound in vitro by recombinant H. capsulatum Sre1 (4), suggesting that it might mediate Sre1-dependent regulation of gene expression in vivo. To determine the role of the Sre1 consensus site in vivo, we generated reporter constructs by fusing the green fluorescent protein gene (gfp) to the promoter regions of SID4 or OXR1. The SID4 promoter contains a single Sre1 consensus site, with an additional minimal HGATAR motif immediately upstream of the consensus sequence. We constructed a wild-type version of the promoter fusion with gfp, as well as versions containing a mutant consensus sequence and/or the upstream HGATAR motif (Fig. 3A). The reporter constructs in this set were integrated randomly into the genome of H. capsulatum by the use of Agrobacterium-mediated transformation, and
at least four independent isolates per construct were analyzed as follows. Immediately after harvest at a 0-h time point under iron-replete conditions, cultures were divided in half and maintained in the absence or presence of 5 μM FeSO₄ for the next 8 h. *gfp* expression and *SID4* expression (from its endogenous locus) were determined in each sample by the use of qRT-PCR and graphed relative to the corresponding expression level at the 0-h time point (Fig. 3A). If the reporter showed wild-type iron-dependent regulation, cells should derepress expression of the *gfp* reporter after 8 h of growth in iron-limiting medium, but not in iron-replete medium, as was the case for *SID4* expression in each strain (Fig. 3A). When the *gfp* reporter was driven by the wild-type promoter (pLH226), it was derepressed 13-fold after 8 h of growth in the absence of exogenous iron but only 3-fold in the presence of iron, indicating that its expression was iron regulated (Fig. 3A). Iron-regulated expression of the *gfp* reporter was not disrupted when only the upstream HGATAR motif was mutated (pLH228). However, mutation of the Sre1 consensus site either singly (pLH227) or in combination with the HGATAR mutation (pLH229) caused high basal expression of *gfp*, even under iron-replete conditions at the 0-h time point. Thus, there was no change in expression at either 8-h time point relative to the 0-h time point, indicating that the Sre1 consensus site was essential for wild-type iron-regulated transcription.

A number of genes in the siderophore biosynthetic cluster contain more than one Sre1 consensus site. To determine the importance of multiple consensus sites, we analyzed the *OXR1* promoter by the use of a *gfp* reporter. This promoter contains three Sre1 consensus sites as well as an upstream HGATAR motif (shown in blue in Fig. 3B) that overlaps with the full consensus sequence at the position furthest upstream. When *gfp* was placed under the control of the wild-type *OXR1* promoter, its expression was iron...
regulated (Fig. 3B [pED6]). In contrast, when all three Sre1 consensus sites were mutated, iron-regulated transcription was lost (pED32). Mutation of the 5′ Sre1 consensus sequence and the upstream HGATAR motif (pED29) did not perturb iron regulation. However, mutation of the two downstream Sre1 consensus sites was sufficient to eliminate iron regulation of gfp transcription (pED12). Therefore, only a subset of the consensus sites was required for iron-dependent regulation of expression. As expected, introduction of the reporter constructs did not perturb the iron-regulated transcription of the endogenous OXR1 gene (Fig. 3B).

Sre1 regulates a key subset of iron-dependent genes. To identify additional genes that are regulated by iron levels and/or by Sre1, we used whole-genome microarrays to examine the expression of genes from SRE1 RNAi and vector control strains under iron-replete and iron-limiting conditions. For gene expression analysis, we performed two independent time course experiments with two independent control and SRE1 RNAi strains. To achieve the largest dynamic range for these studies, we first studied the cells to iron starvation and then either maintained the cells under iron-depleted conditions or shifted them to iron-replete conditions. To obtain a stringent gene expression data set, we first identified the 3,810 genes that had adequate signal (see Materials and Methods) in at least 80% of the arrays from both time courses. These data were subjected to examination by Significance Analysis of Microarrays (SAM) (38) to identify those that were regulated by exogenous iron levels and/or by Sre1. We found that 327 genes were regulated by iron (95% confidence level) (Fig. 4A; also see Table S2 in the supplemental material). One-third of these genes were induced after the shift to iron-replete conditions, and two-thirds were repressed after the shift to iron-replete conditions. In addition to the genes described above, iron-regulated genes included those encoding a number of putative oxidoreductases, metabolic and mitochondrial enzymes, superoxide dismutase, and genes previously identified as induced during nitrosative stress (27) (see Table S2 in the supplemental material). SAM also revealed that 364 genes of various functions showed dependence on SRE1 for their normal transcriptional regulation (95% confidence level) (Fig. 4A; also see Table S3 in the supplemental material). Many of these genes were repressed by Sre1, although there were genes (described below) that required Sre1 for maximal expression. Additionally, a survey of the entire G217B genome revealed an exact match to the full Sre1 consensus sequence within 2 kb of their predicted transcription start site. A total of 3,810 genes were subjected to SAM; 364 were determined to be Sre1 dependent, 327 were determined to be iron dependent, and 115 contained an Sre1 consensus sequence. The numbers of genes that fell into each category are shown. (B) Heat map of 12 genes that are Sre1 dependent, iron dependent, and contain an Sre1 consensus sequence as described for panel A. Control and SRE1 RNAi strains were grown under iron-limiting (black numbers) and iron-replete (10 μM FeSO4, red numbers) conditions for 0, 0.5, 1, 4, and 8 h, as described in Materials and Methods. All time points were normalized to the control RNAI zero time point. Gene expression data from all time course experiments were subjected to complete linkage clustering; data from one representative time course are shown. MFS1 is the only gene depicted in panel B that does not contain the Sre1 consensus sequence. NIT1 is represented by two independent spots on the microarray. NIT22 and MRS3 contain the Sre1 consensus sequence but clustered separately from each other and from the other 10 genes. Red indicates upregulated genes, green indicates downregulated genes, black indicates genes that did not change in expression, and gray indicates that no data were available. The color bar at the bottom indicates the expression ratios on a log2 scale. (C) Heat map for CATB and CATP as described for panel B.

FIG 4 Expression of Sre1-dependent and iron-responsive genes. Control and SRE1 RNAi strains were grown under iron-limiting and iron-replete conditions as described in the text. (A) Venn diagram schematic of the genes that are Sre1 dependent, iron dependent, and/or contain an Sre1 consensus sequence within 2 kb of their predicted transcription start site. A total of 3,810 genes were subjected to SAM; 364 were determined to be Sre1 dependent, 327 were determined to be iron dependent, and 115 contained an Sre1 consensus sequence. The numbers of genes that fell into each category are shown. (B) Heat map of 12 genes that are Sre1 dependent, iron dependent, and contain an Sre1 consensus sequence as described for panel A. Control and SRE1 RNAi strains were grown under iron-limiting (black numbers) and iron-replete (10 μM FeSO4, red numbers) conditions for 0, 0.5, 1, 4, and 8 h, as described in Materials and Methods. All time points were normalized to the control RNAI zero time point. Gene expression data from all time course experiments were subjected to complete linkage clustering; data from one representative time course are shown. MFS1 is the only gene depicted in panel B that does not contain the Sre1 consensus sequence. NIT1 is represented by two independent spots on the microarray. NIT22 and MRS3 contain the Sre1 consensus sequence but clustered separately from each other and from the other 10 genes. Red indicates upregulated genes, green indicates downregulated genes, black indicates genes that did not change in expression, and gray indicates that no data were available. The color bar at the bottom indicates the expression ratios on a log2 scale. (C) Heat map for CATB and CATP as described for panel B.
gene was previously shown to be induced under conditions of nitrosative stress (27), and that gene encodes a protein that is homologous to ferric chelate reductase Fre2 from Aspergillus nidulans. Thus, these microarray data are consistent with previous work showing that both siderophore secretion and ferric reductase activity are induced in H. capsulatum under low-iron conditions (3, 16, 36, 37). The promoter region of the NIT1 gene contains two full Sre1 consensus sites, as well as an additional partial consensus sequence, suggesting that its transcriptional regulation may be mediated through these sites.

OMT1 is homologous to Aspergillus fumigatus ftmD, an O-methyl transferase involved in secondary metabolite production. Its coregulation with siderophore biosynthesis genes suggests that Omt1 could modify a H. capsulatum siderophore. Additionally, NIT21 and NIT3, which were also previously identified as H. capsulatum genes that are upregulated by nitrosative stress (27), are divergently transcribed from a regulatory region that contains a single consensus site. NIT21 encodes a second O-methyl transferase (in addition to OMT1) that is regulated by iron and Sre1. NIT3 encodes the ortholog of hapX/Hap43, a bZip transcription factor that is transcriptionally regulated by iron and Sre1 orthologs in Aspergillus nidulans, Aspergillus fumigatus, and Candida albicans (5, 15, 17, 21, 33). hapX/Hap43 is particularly interesting, because it is an important iron-responsive regulatory factor in other fungi that represses iron utilization genes under iron-limiting conditions and has been shown to be required for viability in a low-iron environment. Although NIT3 expression is repressed in control cells after 4 h in the presence of iron, we observed 40% derepression of NIT3 in the SRE1 RNAi strain under the same conditions, indicating that Sre1 is required for proper repression of the H. capsulatum hapX/Hap43 ortholog under iron-replete conditions.

The final two genes that contain the Sre1 consensus sequence and were repressed under iron-replete conditions in an Sre1-dependent manner are NIT22 and MRS3. NIT22 encodes a peroxisomal dehydratase whose ortholog in B. dermatitidis (Broad accession number BDBG_00052) was also shown to be regulated by the Sre1 ortholog SREB (8). MRS3 encodes a putative mitochondrial iron transporter whose ortholog in Saccharomyces cerevisiae can modulate cytosolic iron levels by promoting mitochondrial iron accumulation (24).

Twenty-four genes lacking an Sre1 consensus sequence, including the above-mentioned MFS1 (which is likely to be regulated by the consensus variant described above), were regulated by iron levels and by Sre1 (see Table S5 in the supplemental material). The other 23 transcripts included the catalases CATB and CATP (19). In many organisms, catalases are more highly expressed under iron-replete conditions, presumably to combat iron-related oxidative stress. In Aspergillus fumigatus, CATB (catalase B) expression is higher under iron-replete conditions than iron-limiting conditions in both wild-type and sreΔ strains (28). We found that the H. capsulatum CATB and CATP transcripts accumulated under iron-replete conditions and were depleted under iron-limiting conditions in control cells (Fig. 4C). Depletion of Sre1 by RNAi resulted in inappropriate expression of each catalase under iron-limiting conditions. In the case of CATB but not CATP, increased transcript accumulation under iron-replete conditions was dependent on Sre1.

Sixteen genes containing an Sre1 consensus sequence displayed iron dependence but were not regulated by Sre1 (Fig. 4A). It is possible that these genes could be regulated by Sre1 under environmental conditions that were not probed in our experiment or that potential regulation by Sre1 could be masked by residual Sre1 in the RNAi strains. Although many of these genes were of unknown function, one gene encoded a putative glutaredoxin whose ortholog in S. cerevisiae, Grx3, plays a role in iron homeostasis (12).

**Sre1 is a global regulator of gene expression.** Only 36 of the 364 Sre1-dependent genes were also affected by iron levels in our experiments, suggesting that Sre1 impacts other cellular processes in addition to the iron response (see Table S3 in the supplemental material). Most of the remaining 328 genes were repressed by Sre1 and, for the most part, represented a wide variety of genes that did not fall into a small number of easily discernible pathways. One exception was a group of amino acid biosynthesis genes, as well as a cluster of ribosomal protein genes that showed approximately 2-fold higher transcript accumulation when Sre1 was depleted in the RNAi strains (Fig. 5A). Only two of the genes depicted in Fig. 5A contain an obvious Sre1 consensus sequence, suggesting that Sre1-mediated repression of the majority of these ribosomal protein genes is likely to be indirect.

In contrast, some genes appeared to be positively regulated by Sre1 (Fig. 5B). The expression of these genes was significantly higher in control strains than in the SRE1 RNAi strains. These genes encoded a protein similar to human transcriptional coactivator SKIP, a hypothetical protein (represented by two independent oligonucleotides on the microarrays), a member of the GPR/FUN34 family, an endoglucanase, a methyltransferase, an MFS transporter (represented with two oligonucleotides on the microarrays), and a monocarboxylate transporter. The monocarboxylate transporter gene is the only one in this group that contains an Sre1 consensus site in its promoter. The promoters of all seven genes were analyzed by MEME (Multiple Em for Motif Elicitation) for novel consensus sites, but nothing of significance was found.

**Sre1 is required for optimal filamentous growth.** In similarity to the model suggested by our gene expression data, the role of Sre1 orthologs in other fungi is not limited to regulation of iron-responsive genes. For example, SREB, the Sre1 ortholog from the dimorphic fungus Blastomyces dermatitidis, has been shown to regulate filamentous growth at room temperature in addition to its traditional role in the control of iron response genes in yeast-phase cells (8). Since B. dermatitidis and H. capsulatum share a close phylogenetic relationship, we investigated the role of Sre1 in filamentous growth. Control and SRE1 RNAi strains were grown in the yeast phase at 37°C and then spotted onto different media and incubated at room temperature to promote filament formation (Fig. 6). On Sabouraud medium, which is highly stimulatory for filamentous growth, both control and SRE1 RNAi strains showed robust filamentation. However, on HMM medium, SRE1 RNAi strains showed delayed filamentation, as evidenced by both a smoother macroscopic appearance of the plated cells compared to controls (Fig. 6A) and a higher proportion of yeast cells as revealed microscopically (Fig. 6B). These data suggest that, under particular environmental conditions, the SRE1 RNAi strains showed a reduced propensity to filament at room temperature. The significance of the altered pigmentation of SRE1 RNAi strains grown at room temperature (Fig. 6A) is unknown but could reflect increased siderophore content analogous to the pigment change in Fig. 1A.
Iron acquisition and homeostasis are critical for optimal growth of \textit{H. capsulatum} in the environment and within the host (13, 14, 18). In this study, we used phenotypic analyses and whole-genome expression profiling to interrogate the regulon of Sre1, a conserved GATA family transcription factor whose orthologs repress the expression of iron acquisition genes in other fungi. We found that Sre1 is critical for the proper control of iron-regulated siderophore production in \textit{H. capsulatum}. Moreover, it is essential for iron-regulated expression of all of the siderophore biosynthetic and utilization genes we previously identified as well as a number of other genes, including \textit{NIT1}, which encodes a ferric chelate reductase, and \textit{NIT3}, which encodes the ortholog of the hapX/Hap43 transcriptional regulator of iron utilization genes. 

\textbf{FIG 5} Expression of iron-independent genes negatively and positively regulated by Sre1. SRE1-dependent clusters of genes that are (A) negatively and (B) positively regulated by Sre1 are shown. Control and SRE1 RNAi strains were grown under iron-limiting (black numbers) and iron-replete (10 \( \mu \text{M FeSO}_4 \), red numbers) conditions for 0, 0.5, 1, 4, and 8 h, as described in Materials and Methods. All time points were normalized to the control RNAi 0-h time point. Red indicates upregulated genes, green indicates downregulated genes, black indicates genes that did not change in expression, and gray indicates that no data were available. The color bar at the bottom indicates the expression ratios on a log2 scale.

\section*{DISCUSSION}

Iron acquisition and homeostasis are critical for optimal growth of \textit{H. capsulatum} in the environment and within the host (13, 14, 18). In this study, we used phenotypic analyses and whole-genome expression profiling to interrogate the regulon of Sre1, a conserved GATA family transcription factor whose orthologs repress the expression of iron acquisition genes in other fungi. We found that Sre1 is critical for the proper control of iron-regulated siderophore production in \textit{H. capsulatum}. Moreover, it is essential for iron-regulated expression of all of the siderophore biosynthetic and utilization genes we previously identified as well as a number of other genes, including \textit{NIT1}, which encodes a ferric chelate reductase, and \textit{NIT3}, which encodes the ortholog of the hapX/Hap43 transcriptional regulator of iron utilization genes. In vivo analysis of wild-type and mutant versions of an Sre1 binding site demonstrated that an intact Sre1 consensus sequence is critical for proper transcriptional regulation of siderophore biosynthesis genes.

Interestingly, the Sre1 regulon in \textit{H. capsulatum} seems to extend beyond iron homeostasis genes. A large number of Sre1-dependent genes in our microarray studies did not show significantly different levels of expression under iron-limiting and iron-replete conditions, suggesting that at least some of these genes affect processes that are distinct from the iron response. The SreA regulon in \textit{A. fumigatus}, in contrast, is thought to consist largely of genes involved in iron homeostasis, whereas the Cir1 regulon in \textit{C. neoformans} is not limited to iron response genes but also includes genes involved in virulence (20, 22, 34). The ability of Sre1...
orthologs to regulate cellular processes that appear to be iron independent has been established for B. dermatitidis SREB, which is required for cells to switch their morphology from the yeast form to the mold form on HMM medium in response to temperature (8). We found that H. capsulatum Sre1 is also required for the optimal development of filamentous cells in response to room temperature growth on HMM medium. Thus, we conclude that Sre1 mediates critical responses to environmental cues, including iron status and temperature.

Fungal GATA transcription regulators (including the orthologs of Sre1 in U. maydis [urbs1], Schizosaccharomyces pombe [srp1], and A. fumigatus [sreA]) (1, 11, 29, 34) are known to recognize an HGATAR sequence. In our previous work, we identified a consensus site that includes HGATAR in the promoters of the siderophore biosynthesis genes (18). Chao et al. showed that recombinant H. capsulatum Sre1 binds to this consensus sequence in vitro in an iron- and zinc-dependent fashion (4). In vivo, there are multiple occurrences of the consensus sequence in the promoter regions of siderophore biosynthesis genes and other Sre-dependent genes in both H. capsulatum and A. fumigatus (18, 34). This Sre1 binding site is also found in both orientations and at various distances from predicted start codons, but the significance of the orientation or location of the binding site for the activity of Sre1 is unknown. Analysis of promoter sequences (defined as within 2,000 bp upstream of a predicted transcription start site) in H. capsulatum strain G217B revealed that 340 promoters contain one or more versions of the consensus sequence. Among the 3,810 genes whose gene expression profiles were analyzed with SAM, 115 genes contained an upstream consensus sequence. The majority (74%) of these genes (Fig. 4A; see also Table S4 in the supplemental material) did not show regulation by Sre1 or by iron, suggesting that (i) the presence of the consensus sequence is not sufficient to recruit Sre1, (ii) transcriptional regulation of these genes by Sre1 might be observed under alternate environmental conditions, and/or (iii) there was residual Sre1 in the RNAi strains that obscured a potential phenotype. Conversely, the majority of Sre1-dependent genes from our microarray studies did not contain a consensus Sre1 binding site in their promoters, suggesting that they have a direct association with Sre1 via an alternate site (potentially a variant of the current consensus sequence) or that their regulation by Sre1 is indirect.

In our data set, SRE1 was not present in the list of significant iron-regulated genes due to missing microarray data for the SRE1 probe. Nonetheless, Sre1 expression in H. capsulatum was previously reported to be regulated by iron, showing a decrease in transcript levels under iron-limiting conditions (4). Interestingly, though the full Sre1 consensus sequence [5′−(G/A)ATC(T/A)GATTA−3′] is not present upstream of the SRE1 coding sequence, a closely related consensus sequence [5′−GATC(T/A)GATTA−3′], differing from the original consensus sequence only at position 9, is present in the SRE1 promoter of all annotated strains of Histoplasma as well as strains of the evolutionarily related Blastomyces and Paracoccidioides. This highly conserved sequence evokes the model that Sre1 regulates its own expression via a modified consensus sequence.

Our microarray data suggest that H. capsulatum Sre1 can both positively and negatively regulate gene expression, although we have not distinguished between direct and indirect control of transcription. In other fungi, orthologs of Sre1 play various roles. SreA, the A. fumigatus ortholog of Sre1, appears to act solely as a repressor (34); however, Cir1, the C. neoformans ortholog, acts as both an activator and a repressor of transcription (22). In this study, we identified a group of six genes that were positively regulated by Sre1 in an iron-independent manner. One of these genes, corresponding to the monocarboxyylate transporter, has an Sre1 consensus site in its promoter, but the other five genes do not. In terms of their function, both the monocarboxyylate transporter and the GPR/FUN34 family member protein have homologs in other fungi that are involved in acetate utilization (31), suggesting a possible link between Sre1 and regulation of other metabolic processes beyond iron acquisition. Interestingly, when we previously compared the transcriptomes of H. capsulatum filaments and yeast cells (26), we determined that both the endoglucanase and the GPR/FUN34 family members showed highly enriched expression in the filamentous form. Whether the role of Sre1 in filamentation is linked to expression of these genes is unknown and is to be the subject of future study.

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