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Sterol Regulatory Element Binding Protein Is a Principal Regulator of Anaerobic Gene Expression in Fission Yeast†

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Fission yeast sterol regulatory element binding protein (SREBP), called Sre1p, functions in an oxygen-sensing pathway to allow adaptation to fluctuating oxygen concentrations. The Sre1p-Scp1p complex responds to oxygen-dependent sterol synthesis as an indirect measure of oxygen availability. To examine the role of Sre1p in anaerobic gene expression in *Schizosaccharomyces pombe*, we performed transcriptional profiling experiments after a shift to anaerobic conditions for 1.5 h. Of the 4,940 genes analyzed, expression levels of 521 (10.5%) and 686 (13.9%) genes were significantly increased and decreased, respectively, under anaerobic conditions. Sre1p controlled 68% of genes induced ≥2-fold. Oxygen-requiring biosynthetic pathways for ergosterol, heme, sphingolipid, and ubiquinone were primary targets of Sre1p. Induction of glycolytic genes and repression of mitochondrial oxidative phosphorylation genes largely did not require Sre1p. Using chromatin immunoprecipitation, we demonstrated that Sre1p acts directly at target gene promoters and stimulates its own transcription under anaerobic conditions. *sre1*$^+$ promoter analysis identified two DNA elements that are both necessary and sufficient for oxygen-dependent, Sre1p-dependent transcription. Interestingly, these elements are homologous to sterol regulatory elements bound by mammalian SREBP, highlighting the evolutionary conservation between Sre1p and SREBP. We conclude that Sre1p is a principal activator of anaerobic gene expression, upregulating genes required for nonrespiratory oxygen consumption.

A family of membrane-bound transcription factors, called sterol regulatory element binding proteins (SREBPs), regulates lipid homeostasis in mammalian cells in response to cellular cholesterol levels. Two genes, *SREBF1* and *SREBF2*, code for three proteins: SREBP-1a, SREBP-1c, and SREBP-2. These basic helix-loop-helix transcription factors contain two transmembrane segments that anchor the cytosolic, N-terminal transcription factor domain to the membranes of the endoplasmic reticulum (12). SREBPs are activated by two proteolytic cleavage events in the Golgi apparatus that release the N terminus from the membrane, allowing it to enter the nucleus and activate gene transcription. Cholesterol controls the activation of SREBPs by regulating the endoplasmic reticulum-to-Golgi apparatus transport of these proteins and access to the Golgi apparatus-localized proteases (27). When sterol levels are high, SREBPs reside in the endoplasmic reticulum and are inactive. In sterol-depleted cells, SREBP cleavage-activating protein (SCAP) escorts SREBP to the Golgi apparatus for proteolysis (8).

Studies using tissue culture and animals have identified over 30 SREBP target genes required for lipoprotein uptake and synthesis of cholesterol, fatty acids, triglycerides, and phospholipids (12). A comprehensive expression-profiling study of mouse liver compared levels of gene expression from mice either overexpressing activated SREBPs or lacking SCAP (13). That study identified 33 SREBP target genes and confirmed previous results showing that SREBP-1a and SREBP-1c primarily activate the transcription of genes in monounsaturated and polyunsaturated fatty acid synthesis and that SREBP-2 activates the entire pathway for cholesterol synthesis and the low-density lipoprotein (LDL) receptor.

Our recent studies of *Schizosaccharomyces pombe* demonstrate that an orthologous sterol-regulated SREBP pathway exists in fission yeast (15). Yeast SREBP and SCAP, called Sre1p and Scp1p, function as an oxygen sensor by monitoring oxygen-dependent sterol synthesis as an indirect measure of oxygen supply. As sterol synthesis decreases under low oxygen, Sre1p is rapidly processed and stimulates the transcription of oxygen-requiring enzymes in ergosterol biosynthesis to maintain sterol homeostasis (15). Importantly, *sre1*$^+$ is required for anaerobic growth, indicating that Sre1p may control the expression of genes in multiple pathways essential for adaptation to low oxygen. Elucidating the transcriptional program controlled by Sre1p under anaerobic conditions will extend our understanding of hypoxic gene regulation in eukaryotes and permit a comparison of the transcriptional programs controlled by mammalian and yeast SREBPs.

In this study, we investigated the role of Sre1p in the transcriptional response to oxygen deprivation and identified 44 Sre1p target genes induced ≥2-fold anaerobically. Using genome-wide gene expression profiling, we first identified genes whose expression changed upon a shift to anaerobic conditions. The parallel analysis of gene expression in *sre1*Δ cells allowed the assignment of Sre1p-dependent target genes. Here, we report that Sre1p is a principal activator of anaerobic gene expression in *S. pombe*, controlling 68% of the genes induced ≥2-fold under anaerobic conditions. While Sre1p and SREBP bind to similar DNA sequences, the transcriptional targets of Sre1p are oxygen specific and show minimal overlap with those of SREBP. Sre1p directly activates genes required for nonrespiratory oxygen-consumptive pathways and controls

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TABLE 1. *S. pombe* anaerobically induced genes

Functional category and gene[d]	*S. cerevisiae* ortholog[a]	Description[b]	Fold change[c]	
			WT −/+ O_2	WT/*sre1*Δ
Amino acid metabolism				
cys11$^+$	*YGR012W*	Cysteine synthase	2.36	1.35
SPAC26F1.07	*YPR1*, *GCY1*	Aldo/keto reductase	2.07	1.35
SPCC584.01c	*MET10*	Sulfite reductase NADPH flavoprotein subunit	2.01	1.04
Carbohydrate metabolism				
gpd1$^+$	*GPD1*	Glycerol-3-phosphate dehydrogenase	2.32	0.88
tdh1$^+$	*TDH3*, *TDH1*, *TDH2*	Glyceraldehyde-3-phosphate dehydrogenase	2.08	0.73
SPACUNK4.16c	*TSL1*, *TPS3*	α,α-Trehalose-phosphate synthase	2.05	0.81
gpd3$^+$	*TDH1*, *TDH3*	Glyceraldehyde-3-phosphate dehydrogenase	2.03	0.87
SPAC750.08c	*MAE1*	NAD-dependent malic enzyme	2.02	0.81
mae2$^+$	*MAE1*	Malic enzyme	2.00	0.82
Electron transport				
SPAC17A2.05	*OSM1*, *YEL047C*	Fumarate reductase	3.04	4.21
SPBC23G7.10c		NADH-dependent flavin oxidoreductase	2.71	1.49
SPCC970.03	*CBR1*, *YML087C*, *YML125C*	Cytochrome b_5 reductase	2.24	1.93
SPAC26H5.09c	*YMR315W*	Oxidoreductase	2.09	0.73
Ergosterol metabolism				
SPAC1687.16c	*ERG3*	C-5 sterol desaturase	3.92	2.36
erg25$^+$	*ERG25*	C-4 methylsterol oxidase	3.14	3.05
erg11$^+$	*ERG11*	Cytochrome P450; C-14 lanosterol demethylase	2.94	1.29
erg5$^+$	*ERG5*	Cytochrome P450; C-22 sterol desaturase	2.76	1.34
SPAC25B8.01	*DAP1*	MAPR family steroid-binding protein	2.61	4.10
erg1$^+$	*ERG1*	Squalene monooxygenase	2.47	0.93
SPAC23H4.01c	*SWH1*, *OSH2*, *OSH3*	Oxysterol binding protein	2.32	0.84
SPCP1E11.05c	*ARE1*, *ARE2*	Sterol *O*-acyltransferase	2.22	1.65
erg6$^+$	*ERG6*	Δ-Sterol C-24 methyltransferase	2.20	1.77
Fatty acid metabolism				
cut6$^+$	*HFA1*, *ACC1*	Acetyl-CoA carboxylase	2.18	0.83
SPAC4H3.08	*FOX2*	Short-chain dehydrogenase	2.17	1.16
Heme biosynthesis				
hem13$^+$	*HEM13*	Coproporphyrinogen III oxidase	14.02	19.44
hem15$^+$	*HEM15*	Ferrochelatase	5.42	10.00
hem14$^+$	*HEM14*	Protoporphyrinogen oxidase	2.09	3.09
Lipid metabolism				
SPBP4H10.11c	*FAA1*, *FAA2*, *FAA3*	Long-chain fatty acid–CoA ligase	3.27	1.56
SPAC589.09	*CSR1*	*sec14* cytosolic factor family protein	2.67	1.97
SPAC8F11.08c		Esterase/lipase	2.10	1.74
SPBC16A3.10	*YOR175C*	Membrane-bound *O*-acyltransferase	2.09	1.71
lcf1$^+$	*FAA1*, *FAA3*, *FAA4*	Long-chain fatty acid–CoA ligase activity	2.05	1.01
Sphingolipid metabolism				
SPBC887.15c	*SUR2*	Sphingosine hydroxylase	5.51	4.22
SPBC32F12.01c	*ISC1*	Sphingomyelinase family protein	2.96	2.17
SPAC19G12.08	*SCS7*	Fatty acid hydroxylase	2.14	3.00
Transcription				
sre1$^+$		Membrane-tethered transcription factor	4.75	17.30
Other				
dam1$^+$	*DAM1*	DASH complex subunit	5.04	2.86
SPBC215.11c		Aldo/keto reductase	4.49	2.53
rga5$^+$	*SAC7*, *BAG7*	GTPase activating protein	4.23	4.18
mmd1$^+$	*LIA1*	Required for mitochondrial morphology and distribution	3.76	5.56
SPBC106.12c		RNA-binding protein	3.05	3.04
SPAC10F6.01c	*ECM17*	Sulfite reductase β-subunit	3.01	0.97
zym1$^+$	*CRS5*	Zinc metallothionein	2.54	2.78
SPCC965.06		Aldo/keto reductase	2.54	1.22
SPAC212.09c		Malic enzyme pseudogene	2.46	0.85
SPBC6B1.08c	*YER049W*	2 OG-Fe(II) oxygenase superfamily protein	2.43	1.59
SPCC1259.02c	*YBR074W*	M28 peptidase family protein	2.42	2.57
SPAP8A3.02c		2 OG-Fe(II) oxygenase superfamily protein	2.18	1.96
rds1$^+$		Protein involved in response to stress	2.09	0.76
Unknown				
SPAC22A12.06c	*FSH2*, *FSH3*		7.57	3.84
SPBPB7E8.01			4.03	2.29
SPAC18G6.01c	*FMP22*, *FMP34*		3.77	5.64
SPAC186.05c			3.21	2.75
SPBC21D10.06c			2.85	1.18

Continued on following page

TABLE 1—*Continued*

Functional category and gene[d]	*S. cerevisiae* ortholog[a]	Description[b]	Fold change[c]	
			WT −/+ O_2	WT/*sre1*Δ
SPAC19B12.10			2.78	2.30
SPAC1093.01			2.50	1.43
SPAC2E1P3.05c			2.41	3.01
SPAC23C11.06c	*YNL115C*		2.36	1.48
SPAC17A2.02c	*YJR116W*, *YPR114W*		2.32	1.28
SPAC1565.01	*YNR018W*		2.06	2.09
SPAC589.12	*CWH43*		2.06	1.41
SPCC1235.01			2.05	1.38
SPAC18G6.12c			2.03	2.63
SPAC637.03			2.02	1.34
SPAC13C5.06c			2.01	2.75

[a] Predicted *Saccharomyces cerevisiae* ortholog (37).
[b] Descriptions were obtained from the *S. pombe* GeneDB (http://www.genedb.org/genedb/pombe/index.jsp) with some additional hand editing.
[c] Data are presented as the average changes in expression of genes in WT samples without O_2 (WT − O_2) compared to their expression in WT + O_2 or *sre1*Δ − O_2 samples.
[d] Statistically significant Sre1p-dependent genes are in boldface type (see Materials and Methods). Genes whose anaerobic induction was confirmed by Northern analysis are underlined.

a transcriptional program that facilitates the adaptation to low-oxygen growth. Interestingly, induction of glycolytic enzymes and repression of mitochondrial genes did not require Sre1p, suggesting the presence of additional oxygen-dependent transcriptional regulators in fission yeast.

MATERIALS AND METHODS

Materials. We obtained Edinburgh minimal medium (EMM) and amino acids from QBioGene, yeast extract from Fisher, oligonucleotides from Integrated DNA Technologies, GelCode blue reagent from Pierce, alkaline phosphatase from Roche, horseradish peroxidase-conjugated affinity-purified donkey anti-rabbit immunoglobulin G (IgG) from Jackson ImmunoResearch, an In vivo$_2$ 400 Hypoxic Workstation from Biotrace, Inc., an *S. pombe* DNA microarray from Eurogentec, and prestained protein standards from Bio-Rad.

Strains and cell culture. Wild-type haploid *S. pombe*, KGY425 (*h*$^-$ *his3-D1 leu1-32 ura4-D18 ade6-M210*), and the *sre1*Δ strain (*h*$^-$ *his3-D1 leu1-32 ura4-D18 ade6-M210* Δ*sre1*::*kanMX6*) were grown to exponential phase at 30°C in EMM plus supplements (225 μg/ml each of histidine, leucine, adenine, lysine, and uracil) or in yeast extract plus supplements (YES) using standard techniques (15, 26). Anaerobic and hypoxic (0.2% ± 0.1% oxygen) growth conditions were maintained using an In vivo$_2$ 400 workstation (Biotrace, Inc.) as described previously (15). Briefly, 0.2% oxygen was maintained by mixing air and nitrogen. For anaerobic conditions, hydrogen gas and a palladium catalyst were used to convert trace oxygen to water in an environment of nitrogen gas. Yeast transformations and manipulations were performed using standard techniques (2).

Plasmids. Plasmids containing the upstream, noncoding promoter plus amino acids 1 to 3 of the product of *sre1*$^+$ fused to *lacZ* were generated by inserting genomic fragments generated by PCR into the *lacZ* reporter vector pSPE376 obtained from the Spanish Type Culture Collection (20). Genomic DNA was amplified by PCR (*Pfu* Ultra polymerase; Stratagene) using gene-specific primers (see Table S1 in the supplemental material), digested with SphI-SalI, and inserted between the SphI-SalI restriction sites in pSPE376. Plasmids containing the mutated SRE1, SRE2, or SRE3 sequence or a deletion of the region from positions −550 to −450 of the *sre1*$^+$ promoter were generated using splice overlap PCR (14). Briefly, the first round of PCRs used primer −850F with primer SRE1*R, SRE2*R, SRE3*R, SRE1*SRE2*R, or Δ-550-450R to generate the upstream region of the promoter. To generate the downstream region of the promoter, the *sre1* promoter reverse primer was used with primer SRE1*F, SRE2*F, SRE3*F, SRE1*SRE2*F, or Δ-550-450F. SRE2*SRE3* mutant plasmid was made as described above using the SRE2* plasmid as a template with the SRE3* mutant primers. Spliced PCR products were amplified with −850F and the *sre1* promoter reverse primer, digested with SphI-SalI, and inserted between the SphI and SalI restriction sites in pSPE376.

A plasmid containing the TATA box and the transcriptional start site sequence from the *nmt1*$^+$ gene fused to *lacZ* was created by inserting plasmid fragments from pREP3x generated by PCR into the *lacZ* reporter vector pSPE376 (25). *nmt1*$^+$ from TATA to the transcriptional start site was amplified from pREP3x by PCR using gene-specific primers (TATAF and TATAR), digested with SalI-SphI, and inserted between the SalI and SphI restriction sites in pSPE376 to make plasmid pES10. pES10 was used to generate plasmids containing either three SRE2 repeats or three SRE3 repeats. Complementary synthetic oligonucleotides containing either three SRE2 repeats or three SRE3 repeats with NheI (3SRE3F and 3SRE2F) or SpeI (3SRE3R and 3SRE2R) restriction site overhangs at their 5′ ends were phosphorylated at their 5′ ends using T4 polynucleotide kinase (NEB), annealed, and inserted between the NheI and SpeI sites of pES10.

The bacterial expression plasmid 6 × His-Sre1p(aa 256–366) was generated by inserting a restriction enzyme-digested PCR fragment into EcoRI-XhoI sites of pProEX-HTb (Life Technologies). The PCR insert was amplified from *sre1*$^+$ cDNA using the primers ES31 and SP317 (see Table S1 in the supplemental material) (15).

Northern blot and immunoblot analyses. Total RNA isolation and Northern blot analysis were performed as described previously (15). The *sre1*$^+$ probe was synthesized using a 1.1-kb XhoI DNA fragment from the *sre1*$^+$ cDNA. For other genes, PCR fragments used for Northern probe synthesis were generated using gene-specific primer pairs (see Table S1 in the supplemental material). Whole-cell yeast extract preparation, phosphatase treatment, and immunoblot analysis using anti-Sre1p IgG and horseradish peroxidase-conjugated anti-rabbit IgG were performed as described previously (15).

Microarray experiments. Data in Tables 1 and 2 represent the linear average changes in expression between anaerobically grown yeast and aerobically grown wild-type (WT) yeast or anaerobically grown WT yeast and anaerobically grown *sre1*Δ yeast. Log-phase WT and *sre1*Δ yeast cells were cultured aerobically or anaerobically in YES medium for 1.5 h. Cells (1×10^9) were harvested, total RNA was isolated, and RNA was purified using the RNeasy Midi kit (QIAGEN). Fluorescently labeled cDNA was generated using *S. pombe*-specific primers and Cy3- and Cy5-dCTP according to the manufacturer's instructions (Eurogentec). Labeled cDNA samples from strains were mixed and hybridized to an *S. pombe* DNA microarray slide (Eurogentec) containing 4,976 open reading frames (ORFs) spotted in duplicate. Slides were washed sequentially at room temperature for 5 min each with a solution containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.2% (wt/vol) sodium dodecyl sulfate (SDS), 2× SSC, and 0.2× SSC. Data were collected using a GenePix 4000B scanner, and normalizations and analysis were performed using GeneSpring 6 software (Silicon Genetics). For each comparison, two dye-reversal hybridizations (technical replicates) were performed, and each comparison was performed twice using cells cultured on different days (biological replicates). All statistically significant genes were identified by significance analysis of microarrays (SAM) using a median false discovery rate (FDR) of less than 0.1% (35). The SAM method assigns a value to each gene by analyzing the change in gene expression relative to the standard deviation of replicate samples. Genes with values greater than an adjustable threshold are designated statistically significant, and the percentage of genes potentially identified by chance is given as the median FDR. Only statistically significant genes were used for data analysis. To simplify presentation of

TABLE 2. *S. pombe* anaerobically repressed genes

Functional category and gene[d]	*S. cerevisiae* ortholog[a]	Description[b]	Fold change[c]	
			WT −/+ O_2	WT/*sre1*Δ
Mitochondrial electron transport and ATP synthesis				
cyc1$^+$	*CYC1*, *CYC7*	Cytochrome *c*	0.34	1.14
SPAC3A11.07	*NDE1*, *NDE2*, *NDI1*	NADH dehydrogenase	0.36	0.64
qcr8$^+$	*QCR8*	Ubiquinol-cytochrome *c* reductase complex subunit 7	0.39	1.00
SPAC22F3.07c	*ATP20*	ATP synthase subunit G	0.40	0.89
atp16$^+$	*ATP16*	F_1 ATPase delta subunit	0.41	0.86
atp14$^+$	*ATP14*	F_1 ATPase subunit H	0.43	1.02
atp5$^+$	*ATP5*	F_o ATPase delta subunit	0.43	1.08
cox6$^+$	*COX6*	Cytochrome *c* oxidase subunit VI	0.49	0.91
qcr10$^+$	*QCR10*	Ubiquinol-cytochrome *c* reductase complex subunit 11	0.50	1.16
Protein folding and modification				
SPBC1271.07c		*N*-Acetyltransferase	0.35	1.01
SPAC4H3.01	*CAJ1*, *DJP1*	Protein containing DNAJ domain	0.43	0.92
SPAC11D3.02c		Acetyltransferase, ELLA family protein	0.46	1.16
Other				
sod2$^+$	*NHA1*	CPA1 sodium ion/proton antiporter	0.30	1.01
grx5$^+$	*GRX3*, *GRX4*	Glutaredoxin	0.40	0.98
sod1$^+$	*SOD1*	Cu,Zn superoxide dismutase	0.42	0.97
SPCC4G3.17	*YBR242W*, *YGL101W*	Metal-dependent phosphohydrolase	0.45	0.71
SPAC1F12.10c	*YMR073C*	Heme binding protein	0.45	1.19
ebp2$^+$	*EBP2*	Member of the rRNA-processing EBP2 family	0.46	0.95
pmp20$^+$	*AHP1*	Peroxiredoxin	0.47	1.07
Unknown				
SPBC713.11c	*SNA1*, *SNA3*, *SNA4*, *PMP3*	Protein in UPF0057 family	0.41	0.85
SPAC6C3.02c	*YMR002W*	Protein containing CHCH domain	0.49	1.19
SPBC1271.08c		Sequence orphan	0.50	1.10

[a] Predicted *Saccharomyces cerevisiae* ortholog (37).
[b] Descriptions were obtained from the *S. pombe* GeneDB (http://www.genedb.org/genedb/pombe/index.jsp) with some additional hand editing.
[c] Data are presented as the average changes in expression of genes in WT samples without O_2 (WT − O_2) compared to their expression in WT + O_2 or *sre1*Δ − O_2 samples.
[d] Statistically significant Sre1p-dependent genes are in boldface type (see Materials and Methods). Genes whose anaerobic induction was confirmed by Northern analysis are underlined.

the results, Tables 1 and 2 list statistically significant genes identified by SAM that have average changes of greater than twofold (all statistically significant genes identified by SAM are given in Tables S4 and S5 in the supplemental material). Sre1p-dependent genes are those genes identified as statistically significant by SAM (FDR < 0.1%) from the anaerobic WT-versus-anaerobic *sre1*Δ array data. Data for the 13 Tf2 transposons were excluded from the analysis due to potential cross-hybridization resulting from the high sequence identity among transposons but are included in the complete list of array data (see Table S3 in the supplemental material).

Chromatin immunoprecipitation. Chromatin immunoprecipitation was performed using the ChIP assay kit (Upstate) according to the manufacturer's instructions. Log-phase wild-type and *sre1*Δ cells (2.5×10^7 cells/immunoprecipitation) were cultured under aerobic or anaerobic conditions at 30°C and fixed by the addition of formaldehyde (1% [wt/vol] final concentration) for 30 min. After the addition of 125 mM glycine, cells were washed three times with cold water. Cells were resuspended in 200 μl lysis buffer (1% [wt/vol] SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.0) containing protease inhibitors and lysed by vortexing with glass beads for 10 min at 4°C. The lysate was extracted with 5 volumes of dilution buffer (0.01% [wt/vol] SDS, 1.1% [vol/vol] Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl, pH 8.0, and 167 mM NaCl) and adjusted to 40 μl per 1×10^7 cells. DNA was sheared by sonication to an average length of 500 to 700 bp and cleared by centrifugation at 16,000 × *g* for 10 min at 4°C. Lysates were diluted 10-fold with dilution buffer and precleared by incubation with preblocked protein A/G beads (Upstate) at 4°C for 30 min. The beads were removed by centrifugation, and the lysate was divided into microcentrifuge tubes (1 ml/immunoprecipitation), reserving 40 μl of each sample as "input." The lysates were incubated with 2.5 μg/ml affinity-purified anti-Sre1p polyclonal antibody overnight at 4°C. Sre1p-DNA complexes were isolated and washed, and DNA was recovered according to the manufacturer's instructions.

Immunoprecipitated DNA was quantified by real-time PCR using Brilliant SYBR Green QPCR Master Mix (Stratagene) and a MyiQ single-color-detection thermal cycler (Bio-Rad). Each sample was analyzed in triplicate using gene-specific primers chosen by using Primer3 software that amplified a 100-bp fragment 200 to 500 bp upstream of the start codon (see Table S1 in the supplemental material) (31). A standard curve was constructed using twofold dilutions of genomic DNA, and input DNA was amplified alongside immunoprecipitated DNA as a reference for each primer pair. The percent input was calculated by dividing the average starting quantity for each sample by the corresponding starting quantity of input DNA. This procedure normalized for both the PCR efficiency of each primer pair and the efficiency of DNA purification. Recovery of bound DNA ranged from 0.1% to 0.4% input for the wild-type-plus-oxygen samples.

β-Galactosidase assays. Wild-type or *sre1*Δ cells carrying *sre1-lacZ* reporter plasmids were grown overnight in EMM minus histidine at 30°C to exponential phase. Cells were collected by centrifugation and cultured in YES at 30°C for 4 h. Cells were then collected and resuspended at 3×10^6 cells/ml in YES, YES equilibrated to 0.2% oxygen, or deoxygenated YES using an In vivo$_2$ 400 Workstation (Biotrace). Cells were grown for an additional 12 h at 30°C under aerobic, 0.2% oxygen, or anaerobic conditions to induce expression of Sre1p, and cell pellets were stored at −80°C. To assay *lacZ* expression, β-galactosidase activity was determined in permeabilized cells (1×10^7 cells) as previously described (6). Activity is expressed as Miller units $\{1{,}000 \times (A_{420})/[(t_{min})(V_{ml})(A_{600})]$, where t_{min} is the time in minutes and V_{ml} is the volume in milliliters$\}$.

Purification of recombinant Sre1p(aa 256–366). The recombinant Sre1p-DNA binding domain amino acids (aa) 256 to 366 [Sre1p(aa 256–366)] was purified under denaturing conditions in 8 M urea using Ni-nitrilotriacetic acid affinity chromatography from bacteria expressing 6 × His-Sre1p(aa 256 to 366) according to the manufacturer's instructions (QIAGEN). Ni-nitrilotriacetic acid-bound Sre1p(aa 256–366) was renatured by washing the column with a linear gradient of urea (8 M to 0 M) in refolding buffer (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl,

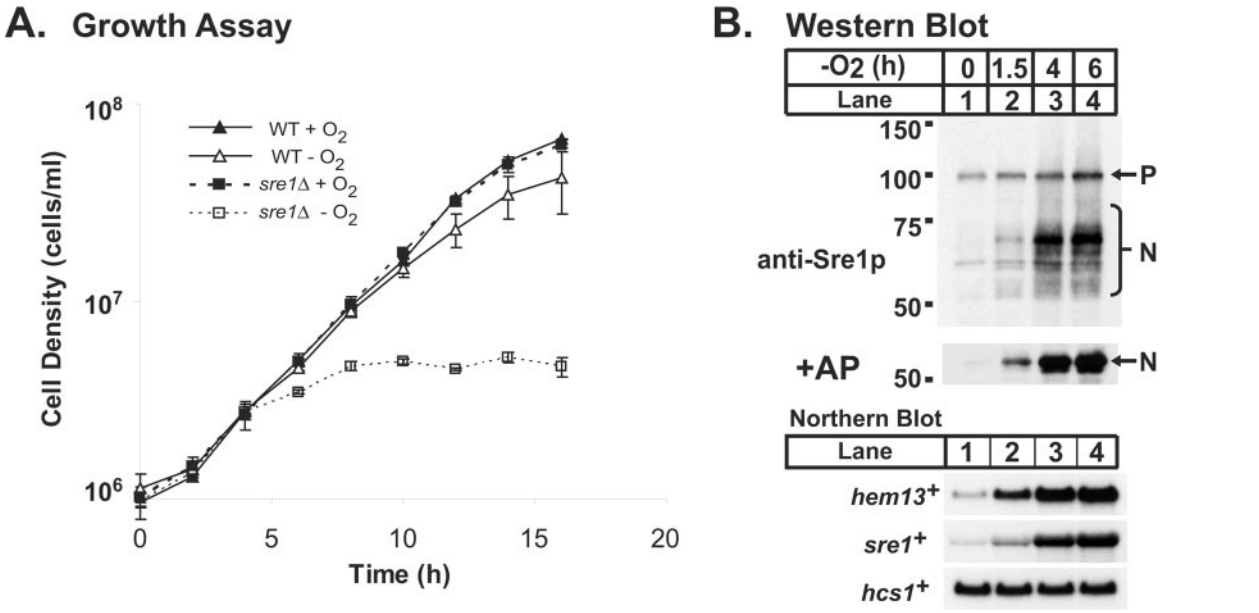

FIG. 1. Sre1p is required at early times for anaerobic growth. (A) Wild-type and *sre1*Δ yeast cells were cultured in rich medium under aerobic ($+O_2$) or anaerobic ($-O_2$) conditions for 16 h. Cell density was measured using a hemacytometer at the indicated time points. Data are the means from two replicates. Error bars equal 1 standard deviation. (B) Log-phase wild-type yeast cells were cultured in rich medium and shifted to anaerobic conditions at time zero. At the indicated time points, cell extracts and total RNA samples were prepared and analyzed. (Top) Cell extracts were subjected to immunoblot analysis using anti-Sre1p IgG before and after treatment with alkaline phosphatase (+AP). P and N denote the precursor and cleaved nuclear forms of Sre1p, respectively. (Bottom) Total RNA (5 μg) was subjected to Northern blot analysis with the indicated ^{32}P-labeled probes.

and 20% [vol/vol] glycerol) at 25°C. Purified, renatured protein was eluted from the column using refolding buffer containing 0.25 M imidazole. The typical yield was ~50 mg/liter of cell culture.

Electromobility shift assays. DNA binding reactions were performed in gel shift buffer (50 mM HEPES-KOH, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 40 μg/ml bovine serum albumin, 20% [vol/vol] glycerol, 1 mM dithiothreitol) as described previously (32). Reaction mixtures containing 80 nM Sre1p(aa 256–366), 2 nM of radiolabeled DNA probe, and 0.5 mg/ml poly(dI-dC) (GE Amersham) were incubated at 25°C for 30 min in siliconized tubes in the presence or absence of the indicated competitor DNA. DNA-protein complexes were resolved by 4% polyacrylamide gel electrophoresis and detected by autoradiography. ^{32}P-radiolabeled DNA probes were prepared by annealing two complementary oligonucleotides with 5′ overhangs (ATGGCCAT) that were filled in using Klenow (NEB) and [^{32}P]dCTP (Amersham). Probes for the human LDL receptor had 5′ overhangs of GATC. Cold competitor DNA was made by annealing two fully complementary oligonucleotides.

RESULTS

Sre1p is required at early times for anaerobic growth. The goal of this study was to identify genes regulated by Sre1p under anaerobic conditions. Our strategy was to use transcriptional profiling to identify oxygen-regulated genes and then to compare transcriptional profiles from wild-type and *sre1*Δ cells in the absence of oxygen to identify genes whose anaerobic transcription required Sre1p. Previous studies demonstrated that *sre1*$^+$ was essential for anaerobic growth (15). To determine when *sre1*$^+$ was first required, we performed a growth assay using wild-type and *sre1*Δ cells cultured in the presence and absence of oxygen. In the presence of oxygen, *sre1*Δ cells showed wild-type growth (Fig. 1A). In the absence of oxygen, wild-type cells displayed an aerobic growth rate but reached an average lower final cell density (4.2×10^7 cells/ml versus 6.6×10^7 cells/ml) after 16 h. In contrast, *sre1*Δ cell growth slowed significantly at 6 h and stopped at 8 to 10 h. Consistent with the essential requirement for Sre1p, viability of *sre1*Δ cells decreased dramatically after 8 to 10 h in the absence of oxygen, whereas wild-type cells showed no decrease in viability (data not shown). Low oxygen induced proteolytic activation of Sre1p at 1.5 h, and processing was maximal at 4 to 6 h (Fig. 1B, top panel) (15). In the middle panel of Fig. 1B, samples were treated with alkaline phosphatase to condense the phosphorylated nuclear form of Sre1p into a single species. Transcription of two Sre1p-dependent genes, *hem13*$^+$ and *sre1*$^+$, mirrored the activation of Sre1p (Fig. 1B, lower panel) (15). *hcs1*$^+$, coding for hydroxymethylglutaryl-coenzyme A (CoA) synthase, served as a loading control. These data indicate that *sre1*$^+$ is required at early times for anaerobic growth and viability.

Anaerobic gene expression: Sre1p-dependent upregulated genes. To examine genome-wide changes in gene expression during anaerobiosis, we performed transcription-profiling experiments using wild-type and *sre1*Δ cells at 1.5 h after cells were switched to anaerobic conditions. This early time point was chosen to avoid complications due to the absence of *sre1*$^+$ and to minimize indirect transcriptional effects. To identify genes upregulated under anaerobic conditions, we compared gene expression in wild-type cells grown in the absence of oxygen to that of wild-type cells grown in the presence of oxygen. A second comparison of wild-type and *sre1*Δ cells grown in the absence of oxygen allowed the assignment of Sre1p-dependent genes. Genes whose expression was significantly different in wild-type cells from that in *sre1*Δ cells in the absence of oxygen were designated Sre1p dependent. Using this procedure, genes that required Sre1p for complete up-

regulation or repression under anaerobic conditions were identified. For each comparison, we analyzed a total of eight data points from two independent experiments. Statistically significant genes were identified using SAM, with a mean false discovery rate of less than 0.1%, as described in Materials and Methods (35).

Of the 4,940 genes examined, expression of 521 genes (10.5%) showed a statistically significant increase after 1.5 h of anaerobic growth, and 115 (22%) of these genes required Sre1p for full anaerobic expression. Table 1 lists statistically significant genes whose expression increased at least twofold under anaerobic conditions and groups genes into functional pathways (a complete list of statistically significant anaerobically induced genes can be found in Table S4 of the supplemental material). Most genes whose expression changed code for metabolic enzymes rather than transcription factors, signaling proteins, or structural proteins.

Sre1p is a principal regulator of highly induced anaerobic genes. Of the statistically significant genes upregulated anaerobically by at least twofold, 44/65 (68%) were Sre1p dependent by microarray analysis, and we confirmed these results for many genes by Northern analysis (Table 1). Sre1p activated multiple genes required for nonrespiratory oxygen consumption pathways such as ergosterol, heme, and sphingolipid synthesis (Table 1) (30). Sre1p activated its own transcription, confirming previous results (15). In addition, many genes with uncharacterized functions were Sre1p dependent and highly upregulated anaerobically, including *dam1*$^+$ and *SPAC22A12.06c*.

Ergosterol synthesis from squalene is a highly oxygen-consumptive process. Synthesis of a single ergosterol molecule requires 12 molecules of O_2, and ergosterol synthesis has been estimated to account for 25% of nonrespiratory oxygen consumption in *Saccharomyces cerevisiae* (30). Under anaerobic conditions in *S. pombe*, enzymes in the oxygen-requiring late ergosterol synthesis pathway were upregulated (Fig. 2) (15). Sre1p was required for expression of every anaerobically upregulated enzyme downstream of lanosterol, including the oxygen-requiring enzymes *SPAC1687.16c*, *erg25*$^+$, *erg11*$^+$, and *erg5*$^+$. Importantly, *erg1*$^+$, the first oxygen-requiring step in the pathway, was induced anaerobically but was Sre1p independent. In addition to these biosynthetic enzymes, genes essential for the transfer of electrons from NADPH to enzymes of the sterol synthesis pathway (cytochrome b_5 reductase gene [*SPCC970.03*] and cytochrome b_5 gene [*oca8*$^+$]) were also upregulated and Sre1p dependent. Interestingly, the *S. pombe* homologs of *Saccharomyces cerevisiae ERG3*, *SPBC27B12.03c* and *SPAC1687.16c*, represented a gene pair that was differentially regulated under anaerobic conditions. *SPBC27B12.03c* transcription decreased under anaerobic conditions, while *SPAC1687.16c* increased. Only transcription of *SPAC1687.16c* required Sre1p, and we refer to this ORF as *erg3*$^+$ in our analyses.

Most genes required for oxygen-dependent heme biosynthesis were also upregulated anaerobically (Fig. 3). Indeed, all of the late enzymes were Sre1p dependent, including the oxygen-requiring enzymes *hem13*$^+$ and *hem14*$^+$. *hem13*$^+$ is a rate-limiting enzyme in heme biosynthesis under oxygen-limiting conditions and was the most highly upregulated gene (14-fold) in our analysis (Table 1) (19). Sre1p also activated anaerobic

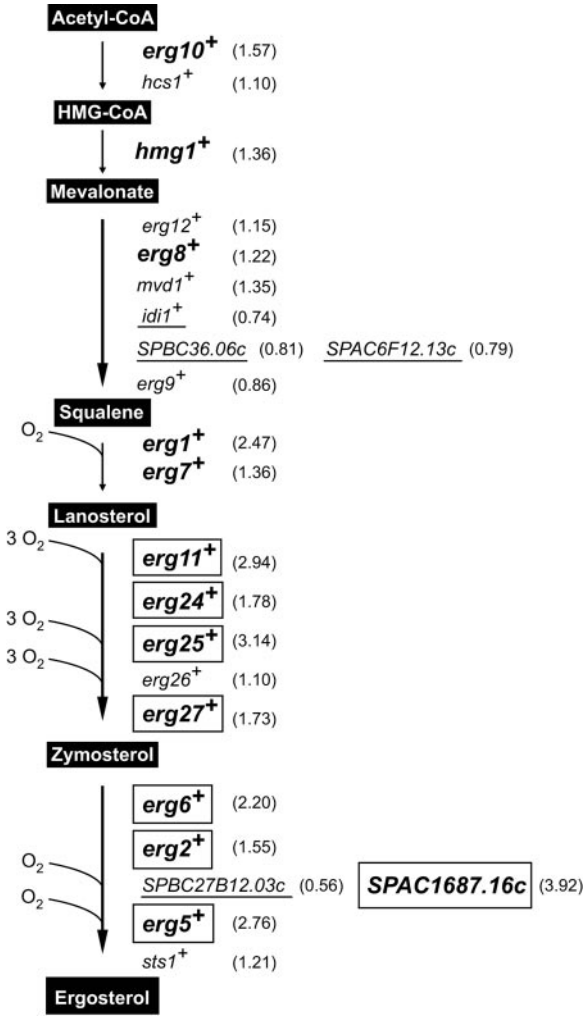

FIG. 2. Oxygen-regulated and Sre1p-dependent genes involved in ergosterol biosynthesis. Genes predicted to be involved in ergosterol biosynthesis are shown with key intermediates in black boxes. The average change (*n*-fold) in expression of each gene in wild-type cells in the absence of oxygen is given on the right. Significant anaerobically induced genes (boldface type), significant anaerobically repressed genes (underlined), and significant Sre1p-dependent genes (boxed) are shown. The *S. pombe* ergosterol synthesis pathway is modeled after *S. cerevisiae* using the *S. pombe* ortholog table and has not been determined experimentally (22, 37).

expression of multiple genes required for ceramide and sphingolipid synthesis, including the oxygen-requiring oxo-diiron enzymes *sur2*$^+$ and *scs7*$^+$ (Table 1; see Table S2 in the supplemental material). In addition, genes required for oxygen-dependent ubiquinone synthesis (*abc1*$^+$, *coq3*$^+$, *coq4*$^+$, *coq5*$^+$, and *coq6*$^+$) were induced and required Sre1p (see Table S2 in the supplemental material). These genes were upregulated less than twofold and thus were not included in Table 1. Interestingly, transcription of the Tf2 class of transposons was highly upregulated in the absence of oxygen in an Sre1p-dependent manner. However, due to sequence identity and potential cross-hybridization among the 13 individual transposons in the *S. pombe* genome, we excluded these elements from the analysis because it was unclear whether all or only one transposon

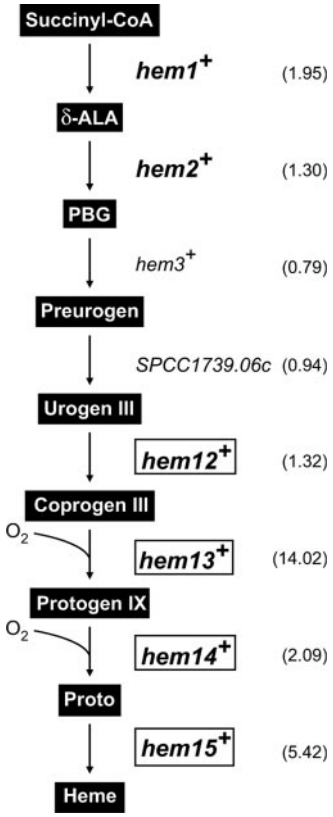

FIG. 3. Oxygen-regulated and Sre1p-dependent genes in heme biosynthesis. Genes predicted to be involved in heme biosynthesis are shown with intermediates in black boxes. The average change (*n*-fold) in expression of each gene in wild-type cells in the absence of oxygen is given on the right. Significant anaerobically induced genes (boldface type) and significant Sre1p-dependent genes (boxed) are shown. The *S. pombe* heme synthesis pathway is modeled after *S. cerevisiae* using the *S. pombe* ortholog table and has not been determined experimentally (30, 37). Abbreviations: δ-ALA, 5-aminolevulinate; PBG, porphobilinogen; Preurogen, hydroxymethylbilane; Urogen, uroporphyrinogen; Coprogen, coproporphryinogen; Protogen, protoporphyrinogen; Proto, protoporphyrin.

was upregulated. Finally, many anaerobically induced genes have not been assigned functions. Collectively, these expression-profiling data indicate that Sre1p is a major regulator of anaerobic gene expression and that Sre1p upregulates genes required for nonrespiratory oxygen-consumptive pathways under oxygen-limiting conditions.

Anaerobic gene expression: Sre1p-independent upregulated genes. As expected from previous studies of anaerobic gene expression in *S. cerevisiae*, genes required for glycolysis and fermentation were upregulated anaerobically as cells switched from respirofermentative to fermentative growth (Table 1; see Table S4 in the supplemental material) (18, 21, 28, 34). These glycolytic enzymes included hexokinase (*hxk2*$^+$), glucose-6-phosphate isomerase (*pgi1*$^+$), fructose bisphosphate aldolase (*fba1*$^+$), two isoforms of glyceraldehyde-3-phosphate dehydrogenase (*tdh1*$^+$ and *gpd3*$^+$), pyruvate kinase (*pyk1*$^+$), and alcohol dehydrogenase (*adh1*$^+$). Sre1p played a minor role in the induction of genes involved in carbohydrate metabolism insomuch as only 2 of the 19 anaerobically upregulated enzymes involved in carbohydrate metabolism required Sre1p, pyruvate decarboxylase (encoded by *SPAC13A11.06*) and lactate dehydrogenase (encoded by *SPAC186.08c*).

Other genes upregulated anaerobically that did not require Sre1p include the fatty acid synthesis genes encoding acetyl-CoA carboxylase (*cut6*$^+$), fatty acid desaturase (*SPCC1281.06c*), and the two subunits of fatty acid synthase (*fas1*$^+$ and *lsd1*$^+$). These data suggest that Sre1p-independent mechanisms that increase gene expression under anaerobic conditions exist in *S. pombe*.

Anaerobic gene expression: downregulated genes. Of the 4,940 genes examined, 686 (13.9%) showed decreased expression in cells grown for 1.5 h under anaerobic conditions, but only 37 (5%) of these downregulated genes required Sre1p for full repression of transcription. Table 2 lists 22 genes whose expression decreased at least twofold; only two of these genes required Sre1p (a complete list of statistically significant downregulated genes can be found in Table S5 of the supplemental material). As expected from previous studies of anaerobic gene expression in *S. cerevisiae*, the single largest group of genes repressed under anaerobic conditions was required for mitochondrial electron transport and oxidative ATP synthesis (18, 34). Downregulated genes included those encoding cytochrome *c* (*cyc1*$^+$), subunits of ubiquinol-cytochrome *c* reductase (*qcr8*$^+$, *qcr10*$^+$, and *rip1*$^+$), subunits of cytochrome *c* oxidase (*cox6*$^+$, *cox12*$^+$, *cox4*$^+$, and *cox8*$^+$), and subunits of the F_oF_1 ATPase (*SPAC22F3.07c*, *atp16*$^+$, *atp14*$^+$, *atp5*$^+$, and *tim11*$^+$). Other downregulated genes included those encoding two acetyltransferases (*SPBC1271.07c* and *SPAC11D3.02c*), a sodium/proton antiporter (*sod2*$^+$), and genes required for resistance to oxidative stress (*grx5*$^+$, *sod1*$^+$, and *pmp20*$^+$). Repression of these genes under anaerobic conditions did not require Sre1p.

Only two genes that were repressed at least twofold required Sre1p for full repression of transcription: *SPAC3A11.07*, encoding an NADH dehydrogenase required for respiration, and *SPCC4G3.17*, encoding an uncharacterized phosphohydrolase. However, unlike the anaerobically upregulated Sre1p-dependent genes, Northern analysis showed that Sre1p has a minimal effect on the repression of these two genes in the absence of oxygen (Fig. 4A). Collectively, these gene expression studies demonstrate that Sre1p is primarily an activator of anaerobic gene expression and does not play a major role in repression of genes under low-oxygen conditions.

Sre1p binds to target gene promoters. To test whether genes identified by expression profiling represented direct transcriptional targets of Sre1p, we assayed Sre1p binding to gene promoters using chromatin immunoprecipitation. Wild-type and *sre1*Δ cells were cultured in the absence and presence of oxygen for 4 h, and Sre1p-bound cross-linked DNA was isolated using polyclonal antibodies to Sre1p. Immunoprecipitated DNA was quantified using real-time PCR and primers directed to the promoter region of each gene. Sre1p bound to promoters of all four anaerobically upregulated Sre1p-dependent target genes tested (*sre1*$^+$, *hem13*$^+$, *erg3*$^+$, and *osm1*$^+$) (Fig. 4B). In each case, binding required the absence of oxygen and Sre1p. Northern blot analysis of the same cultures confirmed anaerobic induction of these genes and the requirement for Sre1p (Fig. 4A). Notably, Sre1p did not bind to the promoters of the two Sre1p-dependent anaerobically repressed genes, *SPAC3A11.07* and *SPCC4G3.17*, suggesting that the

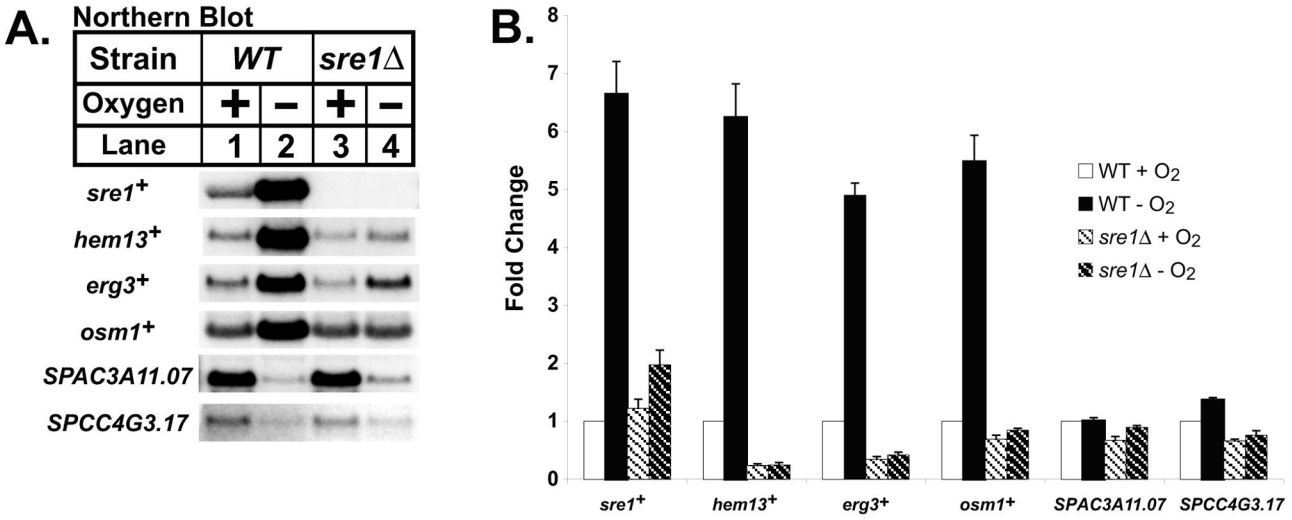

FIG. 4. Sre1p is recruited to target gene promoters in vivo. (A) Wild-type and *sre1*Δ yeast cells were cultured in rich medium under aerobic (lanes 1 and 3) (+) or anaerobic (lanes 2 and 4) (−) conditions for 4 h. Total RNA (5 μg) was subjected to Northern blot analysis with the indicated ^{32}P-labeled probes. (B) Chromatin immunoprecipitation was performed in parallel using formaldehyde-treated, sonicated cell extracts and anti-Sre1p IgG. Binding of Sre1p to upstream regions was assayed by real-time PCR, and bound DNA is expressed as the change normalized to the wild-type aerobic ($+O_2$) sample. Data are means of three replicates from one experiment, and error bars indicate 1 standard deviation. *erg3*$^+$ (*SPAC1687.16c*) and *osm1*$^+$ (*SPAC17A2.05*) were assigned names of their closest *S. cerevisiae* sequence homologs.

requirement for Sre1p in the repression of these genes is indirect. Together, these data demonstrate that Sre1p acts directly at the promoters of anaerobically upregulated target genes to activate gene expression in the absence of oxygen.

Identification of fission yeast SREs. Mammalian SREBPs bind a 10-bp DNA sequence in the promoters of target genes, called the sterol regulatory element (SRE) (33). While there is no strict consensus sequence for SREs (10), sequence selection experiments using SREBP-1 demonstrated that the SRE from human LDL receptor matched the optimal binding site for SREBP-1 (16). Next, we sought to identify a DNA binding site for Sre1p. Using computer-based DNA binding site searches, we failed to identify a common DNA sequence element in the promoters of a collection of highly upregulated Sre1p target genes. To characterize the DNA sequence recognized by Sre1p, we established a *lacZ* reporter system that allowed mapping of regions required for Sre1p-dependent gene expression. For this analysis, we chose to dissect the promoter of *sre1*$^+$. The 850-bp intergenic region upstream from *sre1*$^+$ was fused to *lacZ*, and the resulting plasmid was transformed into wild-type and *sre1*Δ yeast cells. To allow a comparison of wild-type and *sre1*Δ cells, Sre1p was activated by growth at 0.2% oxygen rather than under anaerobic conditions. *sre1*Δ cells showed no growth defect at 0.2% oxygen (data not shown). To test whether the reporter construct faithfully reported Sre1p activity, we assayed β-galactosidase activity in cells grown in the presence of oxygen or at 0.2% oxygen for 12 h. As expected, β-galactosidase activity increased under low-oxygen conditions and required Sre1p (Fig. 5A).

To identify elements required for oxygen-dependent transcription, we assayed β-galactosidase activity under conditions of 0.2% oxygen in yeast strains containing deletions of the promoter element in the reporter plasmid (Fig. 5B). Expression from reporter plasmids containing at least −550 bp required low oxygen and Sre1p. Deletion of the region at −550 to −450 bp reduced *lacZ* expression to that of cells lacking *sre1*$^+$. Deletion of the region at −450 to −350 bp further reduced *lacZ* expression. Consistent with these findings, using 5′ rapid amplification of cDNA ends, we determined that transcription of *sre1*$^+$ mRNA initiates at −434 bp (data not shown). These data suggest that elements required for Sre1p-dependent transcription reside in the region of the *sre1*$^+$ promoter at −550 to −450 bp.

To determine if yeast Sre1p could recognize the same sequence as mammalian SREBP, we examined whether Sre1p could bind the human LDL receptor SRE or a mutant SRE (Fig. 6A) (33). We expressed and purified the DNA binding domain of Sre1p(aa 256–366) from *Escherichia coli* (Fig. 6B) and assayed direct binding of this recombinant protein to DNA in vitro using an electromobility shift assay. Base pairs altered in the mutant probe are required for binding of mammalian SREBP in vitro (5). Sre1p(aa 256–366) bound to the wild-type probe but not the mutant probe (Fig. 6C, lanes 2 and 3). Binding was specific, as binding was blocked by incubation with increasing concentrations of cold, wild-type competitor probe but not mutant probe (Fig. 6C, lanes 4 to 11). These results indicate that yeast Sre1p can recognize and bind to the same sequence as mammalian SREBP.

Two sequence elements in the region of the *sre1*$^+$ promoter at −550 to −450 bp, denoted SRE1 and SRE2, closely resemble the SRE from human LDL receptor (Fig. 6A) (33). We next tested if Sre1p could bind directly to SRE1 or SRE2 sequences. Sre1p(aa 256–366) bound to a double-stranded DNA probe containing both SRE1 and SRE2 (Fig. 6D, lane 2). To determine whether Sre1p bound to both SRE sequences, we tested binding to probes with either or both of the mutated SRE sequences. Mutation of SRE1 had no effect on Sre1p(aa 256–366) binding, but mutation of either SRE2 or both sites

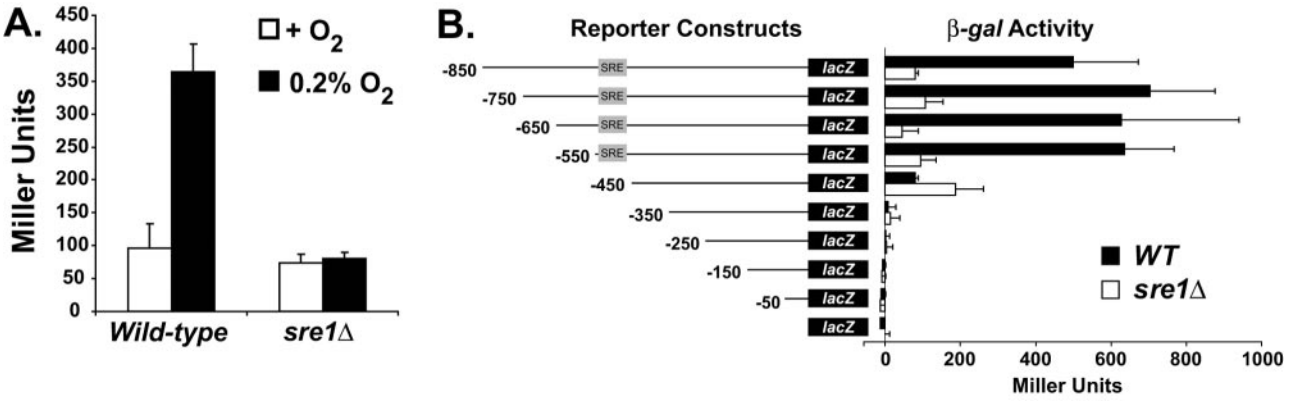

FIG. 5. The region −550 to −450 bp upstream of the *sre1*$^+$ start codon is required for Sre1p-dependent transcription. (A) Wild-type and *sre1*Δ cells carrying a reporter plasmid containing the 850 bp upstream of *sre1*$^+$ fused to *lacZ* were cultured in rich medium under aerobic conditions (+O_2) or conditions of 0.2% oxygen for 12 h. Cells were assayed for β-galactosidase activity as described in Materials and Methods. (B) Wild-type and *sre1*Δ yeast cells containing the indicated reporter plasmids were cultured in rich medium under conditions of 0.2% oxygen and assayed for β-galactosidase (β-gal) activity. Data are the means of six replicates from two independent experiments, and error bars indicate 1 standard deviation. The region required for Sre1p-dependent *lacZ* expression is denoted by SRE.

blocked binding (Fig. 6D, lanes 3 to 5). In vitro DNA binding was specific insomuch as binding was blocked by incubation with increasing concentrations of cold, wild-type competitor probe but not mutant probe (Fig. 6D, lanes 6 to 13). These data indicate that Sre1p binds directly to the SRE2 sequence in vitro.

To test if the SRE sequences were required for regulated expression from the *sre1*$^+$ promoter in vivo, we deleted the region at −550 to −450 bp and mutated SRE1, SRE2, or both sites in the −850 *lacZ* reporter construct. We transformed the resulting plasmids into wild-type cells and assayed β-galactosidase activity in cells grown in the presence or absence of oxygen for 12 h (Fig. 6E). Deletion of the region at −550 to −450 bp completely blocked anaerobic induction of the reporter plasmid, demonstrating that this region was required for Sre1p-dependent gene expression. Mutations in SRE1 had no effect and gave wild-type levels of β-galactosidase activity. However, mutation of SRE2 or both SRE1 and SRE2 reduced β-galactosidase activity to approximately half that of the wild-type reporter, indicating that SRE2 is required for full anaerobic expression from the *sre1*$^+$ promoter in vivo. These data mirror the in vitro binding results and suggest the presence of an additional element(s) in this region that is required for Sre1p-dependent anaerobic expression of *sre1*$^+$.

To identify these SRE sequences, we assayed binding of Sre1p to an overlapping series of 30-bp DNA probes that spanned the region at −570 to −430 bp of the *sre1*$^+$ promoter in 10-bp increments. Consistent with our previous results, Sre1p(aa 256–366) bound to probes 3, 4, and 5 (Fig. 7A) that each contain the SRE2 sequence. Additionally, Sre1p(aa 256–366) bound to probes 8 and 9 that were downstream of SRE2. On closer examination of the sequence overlap between probes 8 and 9, we identified a 10-bp sequence element (GT CAGTCCAC) that is similar to the mammalian fatty acid synthase SRE (GTCAGCCCAT), and we called this element SRE3 (Fig. 7A, bottom) (23).

To test if SRE3 is required for oxygen regulation of the *sre1*$^+$ promoter in vivo, we created −850 *lacZ* reporter constructs containing mutated SRE3 or mutated SRE2 and SRE3 sequences. As described above, β-galactosidase activity was assayed in cells 12 h after growth in the presence or absence of oxygen. Again, mutation of SRE2 reduced β-galactosidase activity in the absence of oxygen to approximately half that of the wild-type −850 *lacZ* reporter construct (Fig. 7B). Mutation of SRE3 or SRE2/SRE3 reduced β-galactosidase activity to basal aerobic levels. Collectively, these results indicate that Sre1p(aa 256–366) can bind to both SRE2 and SRE3 in vitro and that both SRE2 and SRE3 are required in vivo for full expression from the *sre1*$^+$ promoter under anaerobic conditions.

Given that SRE2 and SRE3 were necessary for anaerobic expression from the *sre1*$^+$ promoter, we next tested whether SRE2 or SRE3 alone was sufficient to confer oxygen regulation on a *lacZ* reporter. Plasmids containing three tandem copies of SRE2 or SRE3 upstream of the *nmt1*$^+$ minimal promoter driving *lacZ* were constructed. The resulting plasmids were transformed into wild-type yeast cells, and β-galactosidase activity was assayed 12 h after growth in the presence or absence of oxygen. Three tandem copies of either SRE2 or SRE3 were sufficient to direct oxygen-regulated *lacZ* expression equal to the −850 *lacZ* reporter construct (Fig. 7C). The minimal promoter construct without SRE sequences displayed basal activity. The experiments shown in Fig. 6 and 7 demonstrate that both SRE2 and SRE3 are necessary and sufficient for Sre1p-dependent anaerobic gene expression.

Next, we asked whether the promoters of other Sre1p-dependent genes also contained Sre1p binding sequences. Sequences identical to SRE2 or SRE3 were absent from the 1,000 bp upstream of the 18 Sre1p target genes that were most highly upregulated under anaerobic conditions. However, using the MEME program, which identifies DNA sequence motifs among a collection of sequences (3), we identified a DNA motif (A/G-C/T-C-A/G/T-N-N-C/T-C/T/G-A-C/T) that was present at least once upstream of these 18 genes and in SRE2 and SRE3. To assess the predictive value of this consensus, we searched for candidate Sre1p binding sites in the 1,000-bp upstream regions of the other three genes directly

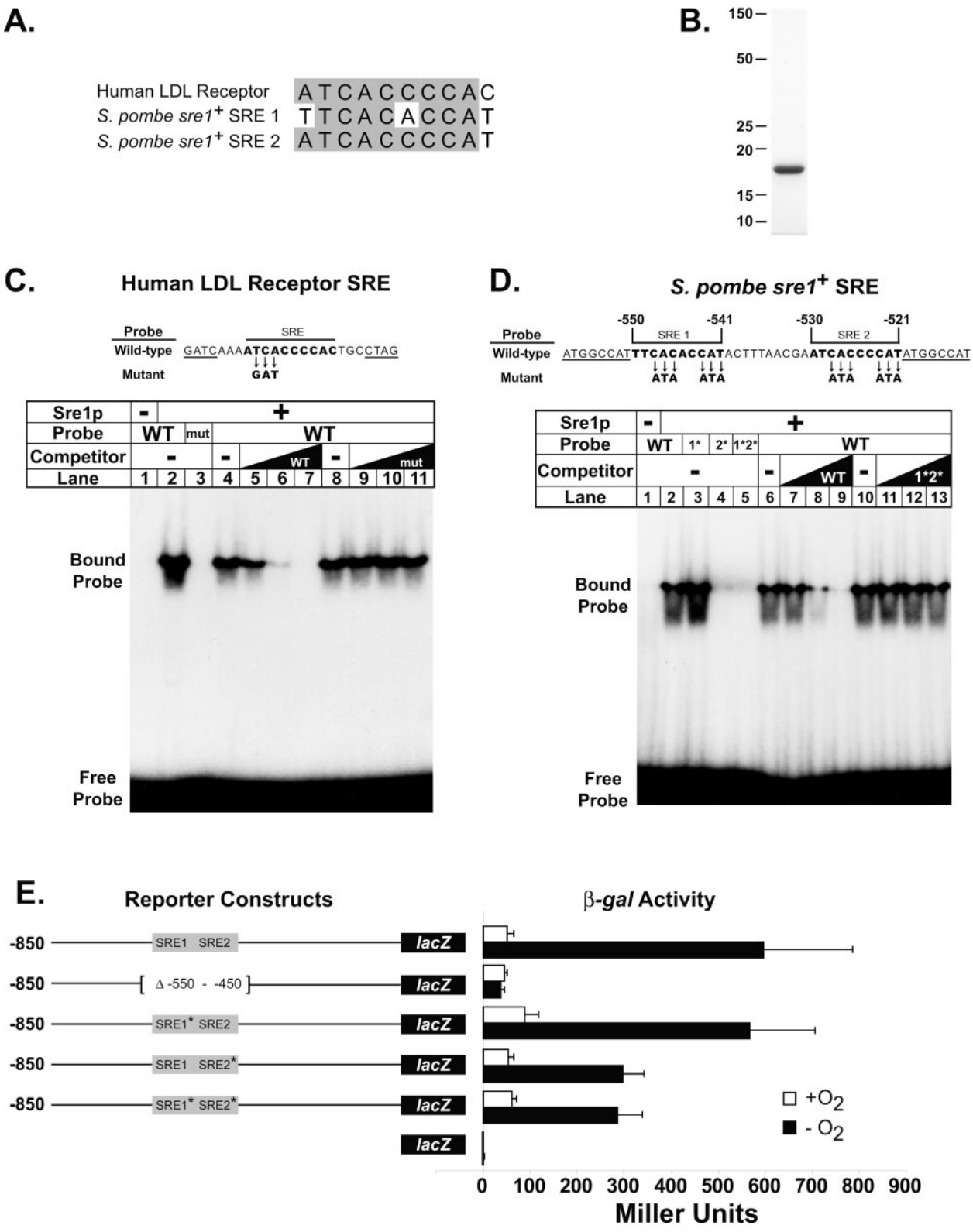

FIG. 6. Identification of a functionally conserved *S. pombe* SRE element. (A) Sequence alignments of the human LDL receptor SRE sequence and putative *sre1*$^+$ SRE sequences. Shaded boxes indicate sequence conservation with human LDL receptor SRE. (B) Purified, recombinant Sre1p(aa 256–366) (3 μg) was subjected to SDS-polyacrylamide gel electrophoresis, and the gel was stained with Gelcode blue (Pierce). (C) Purified Sre1p(aa 256–366) (lanes 2 to 11) was mixed with the indicated ^{32}P-labeled DNA probes in the presence (lanes 5 to 7 and 9 to 11) or absence (lanes 1 to 4 and 8) of unlabeled competitor DNA probes. The human LDL receptor wild-type probe sequence is shown with the SRE element in boldface type. The mutant (mut) probe contains the indicated mutations in the SRE element. Competitor probes

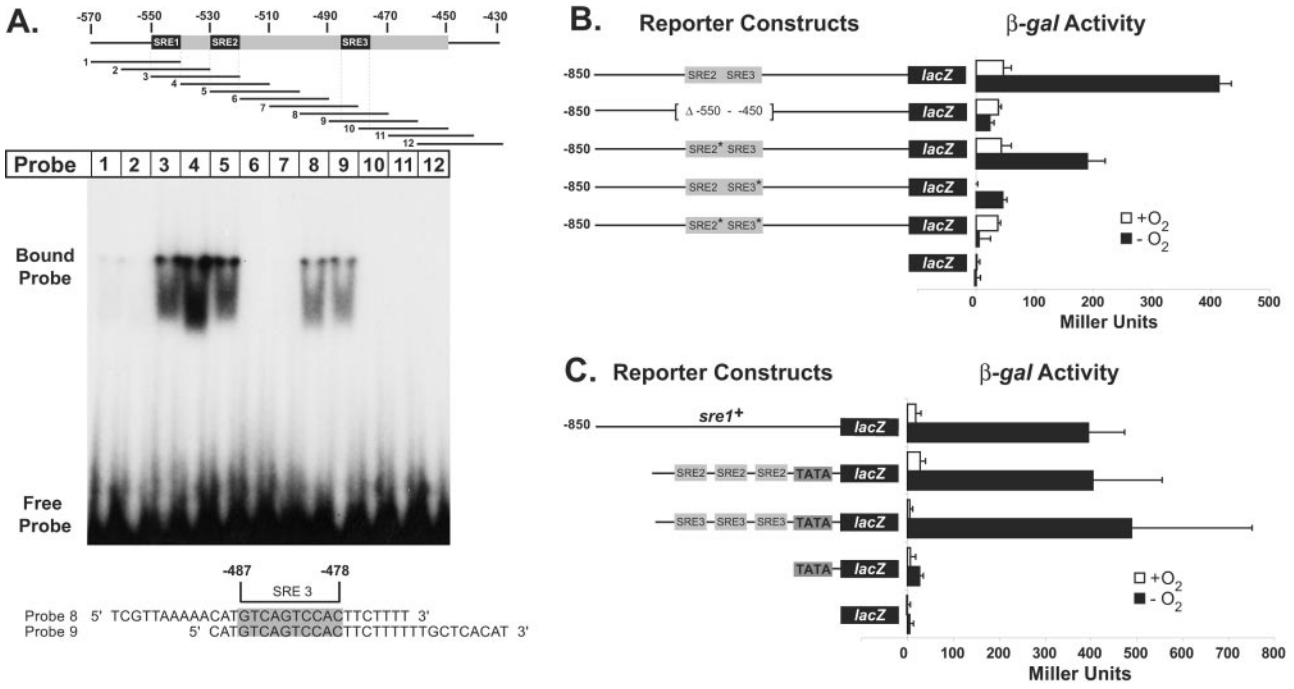

FIG. 7. SRE2 and SRE3 are each necessary and sufficient for anaerobic regulation of the *sre1*$^+$ promoter. (A) Identification of SRE3. Purified Sre1p(aa 256–366) was mixed with the indicated overlapping ^{32}P-labeled DNA probes that scan across the region at bp −570 to −430 of the *sre1*$^+$ promoter in 10-bp increments. Probes including SRE1, SRE2, and SRE3 are indicated. The SRE3 sequence identified by the sequence overlap between probes 8 and 9 is shown below. (B and C) Wild-type yeast cells containing the indicated reporter plasmids were cultured in rich medium under aerobic (+O_2) or anaerobic (−O_2) conditions for 12 h. Cells were assayed for β-galactosidase (β-gal) activity. (B) Mutations in SRE2 are the same as those shown in Fig. 6D. For SRE3, the same nucleotide positions were changed to ACT and ACA. Data are the means of three replicates, and error bars indicate 1 standard deviation. (C) Data are the means of six replicates from two independent experiments, and error bars indicate 1 standard deviation.

regulated by Sre1p in vivo (Fig. 4B). Candidate binding sites are listed in Table 3. We assayed Sre1p(aa 256–366) binding to each candidate SRE by electromobility shift assay (Table 3; see Fig. S1 in the supplemental material). Sre1p(aa 256–366) bound to two of five sequences upstream of *hem13*$^+$, one of two sequences upstream of *erg3*$^+$, and two of five sequences upstream of *osm1*$^+$, suggesting that these elements may be required for anaerobic expression in vivo (Table 3). However, sequences upstream of *osm1*$^+$ showed weak binding equal to that seen for SRE1, which is not required for expression in vivo (Fig. 6E). These results suggest that this consensus requires further refinement insomuch as the majority of the candidate Sre1p binding sequences failed to bind Sre1p in vitro.

Collectively, these data demonstrate that (i) Sre1p and SREBP are functionally conserved, (ii) Sre1p binds to SRE2 and SRE3 sequences that are necessary and sufficient for anaerobic gene expression in vivo, and (iii) similar to mammalian SREs, *S. pombe* SRE sequences do not conform to a strict consensus sequence that permits identification of Sre1p target genes by database searches.

DISCUSSION

This study presents the first genome-wide analysis of anaerobic gene expression in fission yeast and reveals that ~25% of the genome is differentially regulated 1.5 h after a shift to anaerobic conditions. Multiple lines of evidence indicate that Sre1p is a principal regulator of this early transcriptional response to low oxygen: (i) *sre1*$^+$ is essential for cell growth at early times after a shift to anaerobic conditions (Fig. 1A), (ii) Sre1p is required for anaerobic induction of 44/65 (68%) genes whose expression increases

were used at a 10-fold excess (lanes 5 and 9), a 100-fold excess (lanes 6 and 10), and a 1,000-fold excess (lanes 7 and 11). Underlined bases were included to label the probe. (D) Purified Sre1p(aa 256–366) (lanes 2 to 13) was mixed with the indicated ^{32}P-labeled DNA probes in the presence (lanes 7 to 9 and 11 to 13) or absence (lanes 1 to 6 and 10) of unlabeled competitor DNA probes. The wild-type probe sequence is shown, and putative SRE elements 1 and 2 are in boldface type. Mutant probe sequences are as follows: 1* indicates mutations in SRE1 with the wild-type SRE2 sequence, 2* indicates the wild-type SRE1 sequence with indicated mutations in SRE2, and 1*2* indicates mutations in both SRE1 and SRE2. Competitor probes were used at a 10-fold excess (lanes 7 and 11), a 100-fold excess (lanes 8 and 12), and a 1,000-fold excess (lanes 9 and 13). (E) Wild-type yeast containing the indicated reporter plasmids were cultured in rich medium under aerobic (+O_2) or anaerobic (−O_2) conditions for 12 h. Mutated SRE sequences are the same as those described above (D). Cells were assayed for β-galactosidase activity. Data are the means from six replicates of two independent experiments, and error bars indicate 1 standard deviation.

TABLE 3. Putative Sre1p binding sequences

Gene[a]	Positions	Strand	Sequence	Binding[b]
sre1$^+$	−550–−541 (SRE1)	Sense	TTCACACCAT	+
	−530–−521 (SRE2)	Sense	ATCACCCCAT	++++
	−487–−478 (SRE3)	Sense	GTCAGTCCAC	++
hem13$^+$	−758–−749	Sense	ATCGCGCTAT	++
	−646–−647	Antisense	ATCAACCTAT	−
	−359–−350	Sense	ATCGCACTAC	−
	−255–−246	Sense	ATCGTTCGAT	++++
	−96–−87	Sense	ACCAATTCAT	−
erg3$^+$	−122–−113	Antisense	ACCGAGTTAT	−
	−66–−57	Sense	ATCAGACCAT	++++
osm1$^+$	−826–−817	Antisense	ATCAAATCAT	+
	−721–−712	Antisense	ACCTTTTTAT	−
	−607–−598	Sense	ATCTAATCAT	−
	−537–−528	Sense	ATCATATTAT	−
	−183–−174	Sense	ATCTAACCAC	+

[a] *erg3*$^+$ (*SPAC1687.16c*) and *osm1*$^+$ (*SPAC17A2.05*) were named for their closest *Saccharomyces cerevisiae* orthologs.
[b] Binding of Sre1p(aa 256–366) to each sequence was ranked on a qualitative scale from −, indicating no binding, to ++++, indicating the strongest binding.

≥2-fold anaerobically in wild-type cells, (iii) Sre1p binds to anaerobically induced gene promoters in vivo (Fig. 4B), and (iv) Sre1p binds in vitro to two DNA elements required for oxygen-dependent transcription (Fig. 5 to 7; see Fig. S1 in the supplemental material). Collectively, these data suggest that Sre1p activates a broad program of gene expression essential for anaerobic growth.

Sre1p activates nonrespiratory oxygen-consumptive pathways. The primary goal of this study was to identify genes whose transcription required Sre1p under anaerobic conditions. To identify physiological Sre1p target genes, we examined gene expression in wild-type and *sre1*Δ cells after cells were shifted to anaerobic conditions for 1.5 h. This allowed us to study direct effects of Sre1p proteolytic activation that occurs by 30 min and to minimize transcriptional effects due to the requirement for *sre1*$^+$ in anaerobically grown cells (15). In support of this approach, preliminary experiments revealed that while the change of expression for many anaerobically induced genes is greater at 4 h than at 1.5 h, 39 environmental stress response genes are induced at 4 h but not at 1.5 h in *sre1*Δ cells (data not shown) (7). These data suggest that anaerobic *sre1*Δ cells activate a stress response prior to growth arrest. Thus, although the changes of gene expression are less dramatic at the earlier time point (1.5 h), the data are more accurate.

Using gene expression profiling and statistical analyses, we identified 115 anaerobically induced, Sre1p-dependent genes (see Table S6 in the supplemental material). Forty-four of these genes were upregulated ≥2-fold and are likely direct targets of Sre1p; 15/15 genes tested by Northern analysis showed Sre1p-dependent expression (Table 1), and Sre1p bound to the promoters of all four target genes tested in an oxygen-dependent manner (Fig. 4B). Like SREBP in mammals, Sre1p functions primarily as a transcriptional activator insomuch as Sre1p binds to promoters of genes that are upregulated, but not repressed, under anaerobic conditions (Fig. 4B).

Sre1p anaerobically activates the expression of genes in the nonrespiratory oxygen-requiring pathways of ergosterol, heme, sphingolipid, and ubiquinone biosynthesis (Table 1). Indeed, all of the known oxygen-requiring enzymes in these pathways (with the exception of *erg1*$^+$) are upregulated and are Sre1p target genes (Fig. 2 and 3; see Table S2 in the supplemental material). These data support the model that Sre1p upregulates enzymes in oxygen-requiring pathways to maintain flux through these pathways when oxygen is limiting. Previous studies demonstrated that Sre1p is activated within 1 h after wild-type cells were shifted to low oxygen (0.2%) and is required for ergosterol synthesis under these conditions (15). Our data suggest that Sre1p likely acts to maintain heme, sphingolipid, and ubiquinone levels during hypoxia in a similar fashion. Future studies are necessary to determine the importance of increasing ubiquinone biosynthetic enzyme levels under anaerobic conditions in the absence of a functioning respiratory chain. Given the role of ubiquinone as an antioxidant, increased ubiquinone levels may help protect the cell from reactive oxygen species generated upon reoxygenation.

Sre1p upregulates many genes not known to act in the biosynthetic pathways described above (Table 1). These uncharacterized Sre1p-dependent genes may code for enzymes that directly require oxygen, proteins required for oxygen-dependent processes, or potential regulators of adaptation to hypoxia. For example, Sre1p controls two putative aldo/keto reductases (encoded by *SPAC26F1.07* and *SPBC215.11c*) (Table 1) that may require oxygen. In addition, Sre1p induces the expression of two genes (*SPBC6B1.08c* and *SPAP8A3.02c*) that encode proteins belonging to the 2-oxoglutarate-Fe(II) family of dioxygenases, which includes the mammalian hypoxia-inducible factor 1α (HIF-1α) prolyl hydroxylases and asparaginyl hydroxylases (24). HIF-1α is the primary activator of hypoxic gene expression in mammalian cells, and HIF-1α hydroxylases control the oxygen-dependent stability and transcriptional activity of HIF-1α. *SPBC6B1.08c* and *SPAP8A3.02c* may act similarly to modulate low-oxygen gene expression in *S. pombe*.

Sre1p activates a program of gene expression different from that of SREBPs. Both mammalian SREBP and fission yeast Sre1p are proteolytically activated in response to sterol depletion (15, 29). In this study, we demonstrate that Sre1p binds to two functional SRE sequences in the promoter of *sre1*$^+$ and to the SRE of human LDL receptor. However, the transcriptional programs for these two transcription factors are distinct. SREBPs control lipid homeostasis in mammals by regulating transcription of all enzymes in cholesterol biosynthesis, enzymes in fatty acid biosynthesis, and genes that supply substrates to these pathways (13). Sre1p controls genes required for oxygen-dependent biosynthetic pathways and low-oxygen growth. In mammals, SREBP-1 activates fatty acid synthase and desaturase enzymes involved in monounsaturated and polyunsaturated fatty acid synthesis. In *S. pombe*, the oxygen-requiring fatty acid desaturase gene *SPCC1281.06c* (1.94-fold, *S. cerevisiae OLE1*) and genes encoding the two subunits of fatty acid synthase (*fas1*$^+$, 1.70-fold; and *lsd1*$^+$, 1.68-fold) are upregulated under anaerobic conditions (see Table S4 in the supplemental material). However, induction of these genes does not require Sre1p. Notably, *SPCC1281.06c* is the only member of the oxo-diiron family of enzyme genes (*OLE1*, *SCS7*, *SUR2*, *ERG3*, and *ERG25*) that is not regulated by Sre1p. Likewise, the SREBP target gene encoding malic enzyme is upregulated during anaerobiosis in *S. pombe* but does not require Sre1p.

Despite these differences, there is some overlap between these two data sets. Like SREBP-2 in mammals, Sre1p regulates genes required for sterol synthesis but only enzymes in the oxygen-requiring, late sterol biosynthetic pathway downstream of lanosterol. Thus, while both yeast Sre1p and mammalian SREBP respond to changes in sterol concentration, target gene specificity reflects differences in the physiological signals for SREBP and Sre1p activation. SREBP upregulates all enzymes in the cholesterol synthetic pathway, whereas under low-oxygen conditions, Sre1p activates only the oxygen-requiring portion of the sterol pathway.

The discovery that fission yeast SREBP functions in an oxygen-sensing pathway raised the question of whether SREBP performs a similar function in mammals (15). Genes involved in the oxygen-requiring pathways of heme, sphingolipid, and ubiquinone synthesis were not identified as mammalian SREBP target genes (13). These data suggest that if SREBP functions to regulate hypoxic gene expression in mammals, SREBP may act in concert with other transcription factors, such as HIF-1α, or require additional oxygen-dependent regulators.

***S. pombe* SRE sequences.** Using *lacZ* reporter plasmids and electromobility shift assays, we identified five Sre1p binding sequences that can potentially serve as SREs in *S. pombe*. Two SRE sequences that are both necessary and sufficient for anaerobic gene expression have been confirmed experimentally in the promoter of *sre1*$^+$. In addition, we identified three other sequence elements that bind to Sre1p(aa 256–366): two in the promoter of *hem13*$^+$ and one in the promoter of *erg3*$^+$. The failure to identify an Sre1p binding sequence in the 1,000 bp upstream of *osm1*$^+$ may indicate that this SRE sequence may be located more than 1,000 bp upstream of the *osm1*$^+$ ORF or that the consensus sequence used was too stringent to identify the SRE sequence in this region. Alternatively, Sre1p may not bind to the *osm1*$^+$ promoter directly but may be complexed to the *osm1*$^+$ promoter through another DNA binding protein. These studies indicate that our Sre1p binding site consensus should be used as a starting point for the identification of SRE sequences and that functional elements require experimental verification.

***S. pombe* anaerobic transcriptional response.** This initial analysis of anaerobic gene expression in fission yeast reveals that of the 4,940 genes analyzed, expression levels of 521 (10.5%) and 686 (13.9%) genes were significantly increased and decreased, respectively, after 1.5 h of anaerobic growth. Under anaerobic conditions, we observed a remodeling of the transcriptome to allow for changes in cellular energetics in the absence of the terminal electron acceptor, oxygen. As expected from previous studies of hypoxic gene expression in yeast (18, 28, 34), glycolytic enzymes were upregulated and components of the respiratory chain were concomitantly downregulated. However, these changes were largely independent of Sre1p. In addition, genes involved in regulating NADH/NAD$^+$ ratios were also increased anaerobically. In the presence of oxygen, excess NADH can be utilized by the electron transport chain to produce ATP. During anaerobiosis, alternative means are required to regenerate the NAD$^+$ required for glycolysis (4). *gpd1*$^+$, glycerol-3-phosphate dehydrogenase, and *adh1*$^+$, alcohol dehydrogenase, were upregulated anaerobically independently of Sre1p and have been suggested to be involved in the reoxidizing of NADH in *S. cerevisiae* under anaerobic conditions (4). Another proposed mechanism for regulating NADH/NAD$^+$ redox balance during anaerobiosis is through fumarate reductase (11). Interestingly, *osm1*$^+$ (*SPAC17A2.05*), encoding a predicted fumarate reductase, was highly upregulated by Sre1p during anaerobiosis (Table 1 and Fig. 4). Fumarate reductase converts fumarate to succinate and requires $FADH_2$ or $FMNH_2$ but not NADH. Thus, another enzyme likely oxidizes NADH while reducing the oxidized flavin generated from fumarate reductase to maintain the NADH/NAD$^+$ redox balance (11). Indeed, an uncharacterized Sre1p-dependent anaerobically upregulated gene, *SPBC23G7.10c*, contains an NADH-dependent flavin oxidoreductase domain coding sequence and could potentially serve this function.

Anaerobic gene regulation: *S. pombe* versus *S. cerevisiae*. While this study provides only a snapshot of changes in gene expression in *S. pombe* at 1.5 h after growth under anaerobic conditions, initial observations revealed that *S. pombe* and *S. cerevisiae* have developed different mechanisms to regulate anaerobic gene expression. In *S. cerevisiae*, the long-term transcriptional response to anaerobic conditions is controlled primarily by the Rox1p transcriptional repressor, with Rox1p regulating about one-third of the anaerobically expressed genes (18). During aerobic growth, *ROX1* transcription is upregulated by the heme-activated transcription factor Hap1p (17). Under anaerobic conditions, oxygen-dependent heme synthesis decreases, Hap1p activation of *ROX1* decreases, and Rox1p target genes are derepressed. One Rox1p target gene, the transcriptional activator Upc2p, is suspected to regulate 106 *S. cerevisiae* anaerobically induced genes, many of them in concert with Rox1p (18). Interestingly, Rox1p, Hap1p, and Upc2p homologs are not readily identifiable in *S. pombe*. Tye7p in *S. cerevisiae* is homologous to Sre1p in the DNA binding domain, but the role of Tye7p in oxygen-regulated or sterol-regulated gene expression is unclear (9).

Despite a lack of sequence or structural similarity between Sre1p and Rox1p or Sre1p and Upc2p, the gene expression programs of these transcription factors partially overlap. Sre1p upregulates 15/79 (19%) of the Rox1p target genes that have orthologs in *S. pombe*, including *erg6*$^+$, *SPAC17A2.05* (*OSM1*), *SPAC19G12.08* (*SCS7*), and *SPBC887.15c* (*SUR2*) (18, 37). Of the 37 Upc2p target genes that have *S. pombe* orthologs (36), 10 genes, including *erg24*$^+$, *erg25*$^+$, *SPCP1E11.05c* (*ARE1*), and *oca8*$^+$ (*CYB5*), showed Sre1p-dependent anaerobic induction. Furthermore, anaerobically induced *hem13*$^+$, *hem14*$^+$, *erg11*$^+$, and *erg3*$^+$ are predicted targets of Rox1p, Upc2p, and Sre1p (18, 34, 36).

One striking difference in anaerobic gene expression between *S. cerevisiae* and *S. pombe* is the complete absence of the anaerobically induced, Upc2p-regulated *DAN/PAU* cell wall genes and the sterol transporters *AUS1* and *PDR11* in the *S. pombe* genome (1, 38). While anaerobic *S. cerevisiae* imports sterols and unsaturated fatty acids, whose synthesis requires oxygen (30), *S. pombe* does not import cholesterol, and the addition of ergosterol and unsaturated fatty acids has no effect on the anaerobic growth of *S. pombe* (data not shown). These data suggest that long-term anaerobic growth of *S. pombe* may be limited by stores of ergosterol esters that consist of one sterol molecule and one unsaturated fatty acid. Thus, *S. pombe*

and *S. cerevisiae* have evolved distinct mechanisms for regulating anaerobic gene expression. Interestingly, both organisms measure oxygen by responding to changes in oxygen-dependent products: heme in *S. cerevisiae* and sterols in *S. pombe*. It remains to be tested whether *S. pombe* also possesses a heme-sensing mechanism for regulating anaerobic gene expression.

In this report, we identify the oxygen-regulated transcription factor Sre1p as a principal regulator of anaerobic gene expression in *S. pombe*, controlling 68% of the genes induced ≥2-fold under anaerobic conditions. Sre1p represents a new type of hypoxic transcription factor in that Sre1p is a membrane-bound transcription factor that must be proteolytically activated to upregulate gene expression. Sre1p controls genes in the nonrespiratory oxygen-consumptive biosynthetic pathways of sterol, heme, sphingolipid, and ubiquinone biosynthesis. Future studies will investigate the function of uncharacterized Sre1p target genes and examine how widespread this mechanism of anaerobic gene control by SREBP is in other organisms.

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