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Hemoglobin Regulates Expression of an Activator of Mating-Type Locus α Genes in *Candida albicans*

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Phenotypic switching from the white to the opaque phase is a necessary step for mating in the pathogenic fungus *Candida albicans*. Suppressing switching during vascular dissemination of the organism may be advantageous, because opaque cells are more susceptible to host defenses. A repressor of white-opaque switching, *HBR1* (hemoglobin response gene 1), was identified based on its specific induction following growth in the presence of exogenous hemoglobin. Deletion of a single *HBR1* allele allowed opaque phase switching and mating competence, accompanied by a lack of detectable *MTL* α1 and α2 gene expression and enhanced *MTL*a*1* gene expression. Conversely, overexpression of Hbr1p or exposure to hemoglobin increased *MTL*α gene expression. The a1/α2 repressed target gene *CAG1* was derepressed in the same mutant in a hemoglobin-sensitive manner. Regulation of *CAG1* by hemoglobin required an intact *MTL*a*1* gene. Several additional Mtlp targets were perturbed in *HBR1* mutants in a manner consistent with commitment to an a mating phenotype, including *YEL007w*, *MF*α, *HST6*, and *RAM2*. Therefore, Hbr1 is part of a host factor-regulated signaling pathway that controls white-opaque switching and mating in the absence of allelic deletion at the *MTL* locus.

Candida albicans is both a commensal in the human gastrointestinal tract and a virulent pathogen that colonizes specific host organs and causes disseminated vascular infections (39). Adaptation to each host compartment may require recognition of spatial and temporal cues that induce reversible or heritable changes in phenotype. The latter are accomplished in *C. albicans*, despite its lacking a meiotic cycle, by nonmeiotic mating between diploid cells (12, 21, 35).

The *C. albicans* mating-type-like locus (*MTL*) has a genomic structure that is similar but not identical to that of the *Saccharomyces cerevisiae MAT* locus (20, 61). Both encode the transcriptional regulators **a**1, α1, and α2, but control of their mating regulatory circuits differs significantly. Mating in *C. albicans* is carried out between diploid mating partners, while in *S. cerevisiae* **a** and a cells are the products of meiosis. Functional **a** and α cells in *C. albicans* have been generated only through directed deletion of *MTL* genes or loss of an entire chromosome containing an *MTL* locus (21, 35). Thus, genomic rearrangements at the *MTL* locus are proposed to be the primary mechanism for generating mating-competent cells. This was supported by the deletion of *MTL* alleles in some isolates from mammals and clinical specimens. Second, *C. albicans* possesses a fourth gene in its mating locus, **a**2 (61). This gene product, as well as the α1 gene product, acts as a positive regulator of some genes required for their respective mating cell-type specificity. Third, a unique morphological change is required for *C. albicans* mating. Cells must convert from the typical yeast form to an elongated, opaque cell for high-efficiency mating. Opaque cells mate with a 10^6-fold-higher frequency than white cells (36).

White-opaque switching is one of several known processes that permit reversible changes in cellular morphology without detectable genomic rearrangements (51, 52, 56). In the white-opaque phase transition, cells switch between oval budding cells with smooth cell walls to opaque colonies of elongated cells with surface protrusions known as pimples (3). Opaque cells can be easily distinguished as red colonies on modified Lee's agar containing phloxine B (3). The opaque phenotype in the prototypical switching strain WO-1 (52) results from allelic loss of *MTL*a*1* (31). Consistent with the model of switching regulated through the **a**1/α2 dimer (22, 36, 55), disruption of either the *MTL*a*1* or the *MTL*α2 allele results in high-frequency white-opaque phase switching (ca. 10^{-3} per generation).

White-opaque switching alters phenotypic characteristics both in vivo and in vitro. Opaque cells have altered antigenic and adherence properties (2, 24), are more sensitive than white cells to destruction by neutrophils and oxidants (27), and differentially regulate metabolic genes (29). Switching occurs at some sites of *C. albicans* infection (28, 53), indicating that it is a normal component of the fungal life cycle within the host. Considering the vulnerability of opaque cells to host defenses, however, a mechanism to selectively suppress switching may offer a survival advantage when *C. albicans* enters the bloodstream.

The homeodomains of the **a**1 and α2 proteins are conserved in *C. albicans* and function similarly to the MAT **a**1 and α2 proteins in determining cell fate (20). In diploid *S. cerevisiae* cells, the **a**1/α2 dimer represses haploid-specific genes (*hsg*) (17). The α2 gene product acts as a corepressor in the regulation of **a** genes. The *C. albicans* **a**1/α2 dimer represses a subset of those genes repressed by MAT **a**1/α2 in *S. cerevisiae* (61). Intriguingly, *CAG1*, the *C. albicans* ortholog of the *hsg SCG1*,

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TABLE 1. *C. albicans* strains used in this study

Strain	Genotype or description	Reference
CAF2-1	*URA3/ura3*::λ *imm434*	14
CAI-4	*ura3*::λ *imm434/ura3*::λ *imm434*	14
CAI-8	As for CAI-4, except *ade2*::*hisGlade2*::*hisG*	14
BWP17	As for CAI-4, except *his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG*	63
CHY477	*ura3/ura3 mtla1*::*hisG/MTL*α*1*α*2 ade2*::*hisG3URA3hisG/ade2*::*hisG*	36
MM278	*ura3/ura3 MTL***a***1/mtl*α*1*::*hisG mtl*α*2*::*hisG ade2*::*hisGURA3 hisG/ade2*::*hisG*	36
CAMP R8	As for BWP17, except *HBR1/hbr1*::*ARG4*	This study
CAMP 43	As for CAMP-R8, except *RP10/rp10*::pCaExp-*URA3 his1*::*hisG/his1*::*pGEM-HIS1*	This study
CAMP 45	As for CAMP-R8, except *mtl*α*1*::*URA3 his1*::*hisG/his1*::*pGEM-HIS1*	This study
CAMP 47	As for CAI-4, except *mtl*α1::*URA3*	This study
CAMP 49	As for CAMP R8, except *mtl***a***1*::*URA3*	This study
CAMP 48	As for CAI-4, except *mtl***a**1::*URA3*	This study
CAMP 51	As for CAI-8, except *mtl***a**1::*URA3*	This study
CAMP 61	*hbr1*::*ARG4/hbr1*::*HIS1 RP10/rp10*::pExpCAT4-9-*URA3*	This study
CAMP 62	*hbr1*::*ARG4/hbr1*::*HIS1 RP10/rp10*::pExpCAT4-9-*URA3*	This study
CAMP 63	As for CAMP-R8, except *RP10/rp10*::pCaExp-*URA3 his1*::*hisG/his1*::*pGEM-HIS1*	This study
Red 3/6	As for WO1, except *ade2/ade2*	58

which encodes the α-subunit of the mating-specific G-protein complex, can functionally replace its yeast counterpart. The cell type-specific regulation of *CAG1* in *S. cerevisiae* is also consistent with its repression by the Mat **a**1/α2 dimer in diploid cells (48). Indeed, a transcriptional reporter using the predicted *hsg* operator sequences from the *CAG1* promoter demonstrated that *C. albicans* has an **a**1/α2 transcriptional repressor activity that requires the *MTL***a***1* gene (20).

We have now identified a suppressor of white-opaque switching that was isolated based on its specific induction following exposure of cells to hemoglobin. Hemoglobin is a host factor that regulates expression of cell surface receptors for fibronectin, laminin, and fibrinogen (64, 65) through a low-affinity, multivalent hemoglobin receptor (41). Hemoglobin induces increased adhesion to endothelial cells (64), and responsiveness to hemoglobin is conserved in other pathogenic species of the *Candida* genus (45). Therefore, hemoglobin may be an important environmental signal for pathogenesis of *C. albicans* in a mammalian host.

To define molecular mechanisms for these phenotypic alterations, we identified genes that are transcriptionally regulated in response to hemoglobin (40, 42). We show here that modulating the expression of one of these genes, *HBR1*, by disruption of a single allele leads to high-frequency white-opaque switching and mating in a wild-type **a**/α *MTL* background. We further show that Hbr1p suppresses phenotypic switching through stimulation of *MTL*α gene expression and regulation of known Mtl target genes, including *CAG1*.

MATERIALS AND METHODS

Cell culture conditions. *C. albicans* strains were routinely cultured in yeast nitrogen base (YNB) with ammonium sulfate, 2% glucose, low methionine, and appropriate supplements (50) with shaking at 250 rpm at 30°C. Bovine methemoglobin was added to cell cultures at 0.5 mg/ml and was prepared as previously described (41). Modified Lee's medium (8) containing 5 μg of phloxine B (Sigma, St. Louis, Mo.)/ml was used for identification of white and opaque switching cells. Switching frequencies were determined using phloxine B plates as described elsewhere (52).

General nucleic acid manipulations. Total yeast RNA was prepared using the hot acid phenol method (26). The 5′ end of the *HBR1* mRNA was mapped using primer PN10 and primer extension using 50 μg of total RNA from 44807 cells grown with 1 mg of hemoglobin/ml for 4 h (6). Genomic DNA was isolated as described previously (18). Genomic PCR analysis for the presence of *MTL* genes (47) and λ *imm434* (35) used the primers as referenced. All cloned PCR products were sequenced to ensure no errors had occurred during amplification (6). Yeast transformations were carried out by the lithium acetate technique modified for *C. albicans* (62). The *HBR1* DNA sequence from *C. albicans* strains CAI-4 and B311 was determined by standard methods (6). The *C. albicans* C9 genomic library (15) was obtained from the NIH AIDS Research and Reference Reagent Program.

Plasmid construction. A full-length *HBR1* gene fragment was synthesized by PCR amplification of the SY1 genomic clone (42) containing *CaHMX1* and *HBR1*, using primers P13 and PN28 and cloned into pCR-Blunt II TOPO (Invitrogen). The cloned fragment was then excised by BamH1-PstI digestion and inserted via identical sites into plasmid pCaDis to form pDisCat4 or into pCaExp to form pExpCat4-9. Plasmids pCaDis and pCaExp have been described previously (11). A transcriptional fusion of the *HBR1* promoter and the *Renilla* luciferase gene was created using *Pfx* polymerase (Invitrogen) amplification of a 502-bp *HBR1* promoter fragment, using primers P24 and P27 with the SY1 genomic clone as a template. This fragment was digested with SalI and cloned into SmaI-SalI-digested pUC19 to create pPT1-UC. The promoter region product was inserted as a PstI-KpnI fragment into PstI-KpnI-cleaved pCRW3 (58) to create pPT502.

Strain construction and gene disruption methods. Strains used in this study are listed in Table 1. *HBR1* and *MTL* gene disruptions were accomplished using the plasmid templates pRS-ARG4 ΔSpe1, pGEM-HIS1, and pGEM-URA3 (63) and the following primer sets: for *HBR1*, P20 and P21, PN198 and PN199; for *MTL***a***1*, PN108 and PN109; for *MTL*α1, PN110 and PN111 (Table 2). An *ARG4* cassette was used to disrupt the first allele of *HBR1* in strain BWP17, using primers P20 and P21. A total of 30 Arg^+ isolates were identified, and insertion into the *HBR1* gene was confirmed in four of these. Strain CAMP 63 was constructed by integration of plasmid pExpCAT4-9 digested at the unique NcoI site into the *RP10* gene in strain CAMP R8 and identified by Arg^+ Ura^+ selection (11). The correct integration site was determined by PCR using primers PN51 and PN202 to detect RP10 integration and primers PN51 and PN52 to detect the presence of the *HBR1-MET3* promoter fusion (data not shown). The second *HBR1* allele in strain CAMP 63 was targeted by a *HIS1* mutation cassette using primers PN198 and PN199, creating strain CAMP 61, or primers P20 and P21, creating CAMP 62. Disruption of the second *HBR1* allele was verified by PCR using primers P24 and PN203. *URA3* disruption cassettes were used to generate *MTL***a***1* and *MTL*α1 deletions and were confirmed by Southern analysis using gene-specific DNA probes (data not shown) and by reverse transcription-PCR (RT-PCR) analysis using MTL**a**1- and MTLα1-specific primers (see above; also see Fig. 4B, lane 1, below). Strain CAMP 35 was constructed using HindIII digestion of pPT502 to direct integration to an *ade2* allele of strain Red 3/6 (58).

Essential gene analysis. To test for the essentiality of *HBR1*, two isolates each of strains CAMP 63, CAMP 61, and CAMP 62 were grown for 3 days in nonselective medium (YPD with uridine) at 30°C. A total of 10^9 cells from each strain were plated on 5-fluoroorotic acid agar and incubated at 30°C for 3 days. The number of CAMP 63 URA^- colonies per number of cells plated was taken as the frequency of excision of the pExpCat4-9 plasmid occurring spontaneously

TABLE 2. Primers synthesized for this study

Primer	Sequence (5′ to 3′)	Use or reference
PN157	ATGGCCGTTCTTAGTTGGTGGAGT	Ribosomal RNA
PN158	GTAGTAGCGACGGGCGGTGTG	Pair with PN157
P24	CTTGCAACGTATCTCTTGGC	*HBR1* promoter cloning
P27	GGTTGTCGACGTGATGAGATGTGC	Pair with P24
P26	AACATTTGTGGTGAACAAGGATG	Actin ORF
PN91	TGATGGTGTTACTCACGTTGTTCC	Pair with P26
PN90	TGGCTAACTTCAATGTATCTGTTC	Intron, pair with PN91
PN36	GAGTTGCGTTCAGACACAGC	*HBR1* ORF internal
PN37	AAACTTTCGGGTACTTGGACATA	Pair with PN36
PN7	GAACGATTGTGTGGTCCAG	Phosphoglycerate kinase
PN8	GCAGATTTGACAGCAGCTACC	Pair with PN7
PN10	GTTCTGTTTGTTGGGAATTCAGC	*HBR1* 5′ transcript
P13	CGCGCGGATCCATGACAACCATGTCAAGAA	*HBR1* gene (5′)
PN28	GGTACCTGCAGATATCTATTGTGCAATATCTTC	*HBR1* gene (3′)
PN114	TATATGGGGTAAAGATGACGAT	*CAG1* ORF
PN115	AGATAGCCAAAACAAATAAAACAG	Pair with PN114
P20	TCATTCCCTGAGTTTAGTTTCTCAACTCAATCAAACTCTTGGTAAAGAGACGGTTTTCCCAGT CACGACGTT	*HBR1* disruption
P21	AGAGCTTATTCTATCAACGTTTTCATCCATCTCTTCAGCTGTGTCTGAACGCTGTGGAATTGTG AGCGGATA	Pair with P20
PN108	GTATGTCACCGTGTTTAGCTAATATGATCTTGAATAAAAGAAAACGAATAGTTTTCCCAGTCA CGACGTT	*MTL***a***1* disruption
PN109	GGCTAGGTTGAATTTGAACTTGATTTTGTTTCGTTTGGGTTCCTTCTGTGGAATTGTGAGCGG ATA	Pair with PN108
PN110	CGAGTACATTCTGGTCGCGATGCTCCAAGAAGAGACACAAGAGAAGTTCAAAAGTTTTCCCA GTCACGACGTT	*MTLα1* disruption
PN111	CCAGTCCACAAAATTCAATTTTGCATCAGGAAGAAGTAAATAATTATTGTGTGGAATTGTGAG CGGATA	Pair with PN111
PN198	CGAAGACAAATTGCTAGACTCGTTAGAGCCTGATTTGGAAAAGGGGGAGTTTTCCCAGTCAC GACGTT	*HBR1* disruption
PN199	CAGCTGTGTCTGAACGCAACTCAATTACTATGTCAGGAATATAGCTGTCCTGTGGAATTTGTA GCGGATA	Pair with PN 198
ACT1	AAGAATTGATTTGGCTGGTAGAGA, TGGCAGAAGATTGAGAAGAAGTTT	qPCR primer pair
MTLα1	AATTAGCGGGATGTTTGGACTCA, CTATCTGGGGCGTTGTATTATCA	qPCR primer pair
MTLα2	ATTATGTTGCAGCAGGATTCA, GATACGGATGGTTCTTGTGTTT	qPCR primer pair
CDC36	GAGCGTCCAGTATAAATCCACCAC, TCAAGACGGGCTCCACATTACTAT	qPCR primer pair
HST6	AAGCTACCGGATGGCGATTAC, AAAACACCGGACTTGATACACCTT	qPCR primer pair
CTG1	AAAAGGGAAGAATTAAGACTACTGG, ATTCTATTTACCCGTTCATCTTCA	qPCR primer pair
HBR1	TGAAATAGCAAAGGAAAGAGACTG, AATATCACAACAATGCCAATCAAC	qPCR primer pair
RAM2	TTTGGCCACCGATAATAC, TTTTTGCCAATGTCTCCA	qPCR primer pair
MFα	AGAATCTGCCGTTGAAGC, AGCATCGGCGTTAGCATC	qPCR primer pair
MTL**a**1	TAATAAAAGGGGAGGAAATAAA, TTGGGAAGGCTAACACC	qPCR primer pair
YEL007w	GAGGCGTGCTTATAGTTTCTGG, ACGCTTCTTTTTCTTCTTCTTGTC	qPCR primer pair
STE3	CGACGGGTATTCCCAAGAG, TGCATAACATCGCCAAACTG	qPCR primer pair

during unselected growth. The absence of Ura$^-$ colonies of strain CAMP 61 and CAMP 62 would indicate that the Arg-His mutations at the *HBR1* loci interfere with the essential function of this gene. The presence of colonies would indicate that the gene is not essential.

Quantitative mating analysis. Opaque cells of strains CAMP R8 (Ade$^+$ Ura$^-$) and CAMP 51 (Ade$^-$ Ura$^+$) were identified and isolated from phloxine B plates as described above. Mating between these two strains was carried out using cells grown to an optical density at 600 nm of 1.5 in YPAD medium at 25°C in a 4-h mating procedure as described previously (36). Mating frequency was determined in four independent experiments as follows: (number of Ura$^+$ Ade$^+$ conjugates/(number of Ura$^+$ Ade$^+$ conjugates + total limiting parent).

RT-PCR analysis. RT-PCR utilized 5 μg of total RNA, and first-strand cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen, Carlsbad, Calif.). Quantification of cDNA by PCR used the following primer sets: P26 and PN91 for *ACT1* (both contained within the distal exon); PN36 and PN37 for *HBR1*; and PN114 and PN115 for *CAG1* (Table 2). *MTLa1*, *MTL*α1, and *MTL*α2 (47) and phase-specific *WH11*, *SAP1*, and *OP4* (36) primers have been described elsewhere. To detect DNA contamination, a PCR primer within the *ACT1* intron sequence (P26) and the other within the distal exon (PN90) were used. Cycling parameters were as follows: initial denaturation, 94°C for 2 min; 15 to 26 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min. RT-PCR products were visualized by electrophoresis using 2% agarose ethidium bromide gels (E-Gel; Invitrogen) and digital photography (Kodak, Rochester, N.Y.).

Quantitative real-time RT-PCR (qPCR). Steady-state mRNA was quantified using real-time SYBR green fluorescence detection using a DNA Engine Opticon I continuous fluorescence detection system (MJ Research, Waltham, Mass.) and the Dynamo SYBR PCR kit (Finnzymes Oy). PCR primers were designed to amplify products of 100 to 180 bp using Primer Select software (DNASTAR, Madison, Wis.) with PCR settings of 95°C for 5 min, 40 cycles of 94°C for 15 s, 51 to 58°C for 15 s, and 72°C for 20 s. Melting curves were generated for each sample at the end of each run to serve as a validation of the purity of the amplified product. Each assay was performed in 96-well plates in duplicate using identical cDNA stocks for each primer set. Typically, for 5 μg of total mRNA reverse transcribed, the total volume of the reaction mixture was taken up to 100 μl with H_2O, and 4.5 μl of cDNA was used per well. Each cDNA preparation was normalized using the *CDC36* gene as an internal control, and each plate included

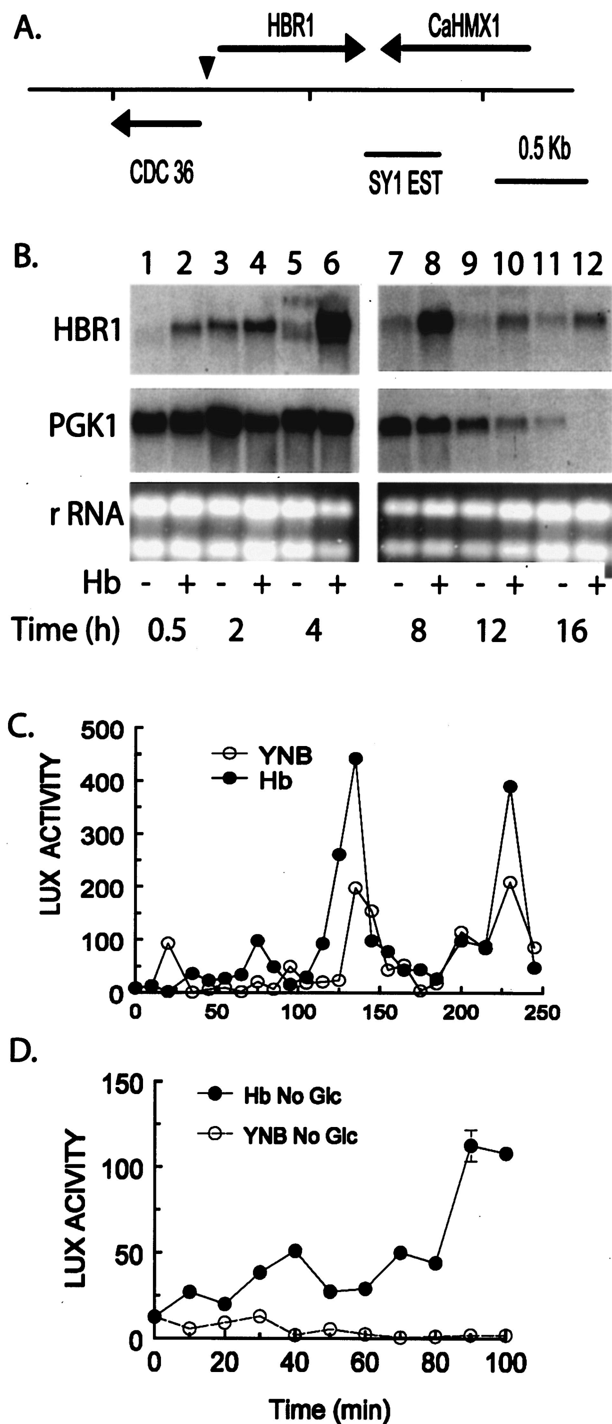

FIG. 1. Hemoglobin and growth signals control *HBR1* expression. (A) *HBR1-CDC36* region in *C. albicans*. The SY1 EST used to identify the *HBR1* genomic clone overlaps the 3′ end of *CaHMX1*, a predicted heme oxygenase (5, 49). The arrowhead indicates the mapped transcription start. This orientation and positioning of *CDC36* with *HBR1* is conserved with the ortholog *FAP7* in *S. cerevisiae*. (B) Hemoglobin increases *HBR1* basal expression. *C. albicans* 44807 cells were grown with and without 500 μg of hemoglobin/ml at 30°C, and RNA was isolated for Northern analysis. The same blot was sequentially hybridized with DNA probes of *HBR1* and *PGK1*. rRNA was used as a loading control (ethidium bromide-stained gel). RNA isolated from strain CAF2-1 gave similar results (data not shown). (C) Hemoglobin and glucose stimulate periodic *HBR1* transcriptional activity. CAMP 35 cells were transferred to YNB medium containing 2% glucose with (●) or without (○) 500 μg of hemoglobin/ml and sampled at the indicated times for luciferase activity. This assay was repeated four times with similar results each time. (D) Hemoglobin and glucose signaling to *HBR1* are separable. CAMP 35 cells were transferred to YNB medium lacking glucose with (●) or without (○) hemoglobin as indicated in panel C. Luciferase activity (LUX) is reported as light units per 5×10^4 cells.

this control to ensure plate-to-plate uniformity. Each RNA sample was derived from a cell culture grown for 4 h at 30°C and was prepared in an identical manner each time. Batch-to-batch variation of *CDC36* was tested for the three strains YJB6284, CAMP 43, and BWP17 grown under these standard conditions and was less than 5%. *Ct* values ± standard deviations (SD) for a test analysis were as follows (n = 16): 20.83 ± 0.27, 21.00 ± 0.30, and 20.42 ± 0.21. Primer sequences are listed in Table 2.

***Renilla* luciferase assays.** *C. albicans* cells were grown at 30°C in minimal YNB medium with ammonium sulfate and 2% glucose. Typically, 5×10^7 cells were harvested, and cell extracts were obtained by glass bead lysis (58). Luminescence was determined using a commercial substrate (Promega, Madison, Wis.), and luciferase activity is reported as light units per 5×10^4 cells.

Scanning electron microscopy. CAMP 43 opaque cells grown in modified Lee's medium overnight at 25°C were fixed in 4% formaldehyde–2% glutaraldehyde in phosphate-buffered saline at 4°C. Samples on carbon-coated coverslips were fixed with 1% OsO_4, dehydrated, and treated with tetramethylsilane (13). After coating with gold-palladium, the cells were observed with a Hitachi S-570 scanning electron microscope operated at 10 kV.

Protein sequence analysis. Proteins related to the human ortholog of Hbr1 (adrenal gland protein AD-004) were identified by BLINK analysis (http://www.ncbi.nlm.nih.gov) with a cutoff of 100. Sequences for the 19 unique gene products obtained were aligned using CLUSTAL W (60). The aligned sequences were analyzed using the PHYLIP phylogeny programs (J. Felsenstein, PHYLIP Phylogeny Inference package, version 3.5c.). The distance matrix data were analyzed using Fitch-Margoliash and least-squares methods (FITCH program). Sequence data for *C. albicans* were obtained from the Stanford Genome Technology Center website at http://www-sequence.stanford.edu/group/candida.

Nucleotide sequence accession number. The nucleotide sequence for *HBR1* was deposited in GenBank under accession number AF466197 (release date 1 April 2002).

RESULTS

Identification of HBR1 and its regulation by hemoglobin. We used random arbitrarily primed PCR to identify *C. albicans* genes that are transcriptionally altered in cells cultured with hemoglobin (42). One EST obtained from this analysis hybridized to a genomic DNA fragment containing two genes that were induced under these conditions (42) (Fig. 1A). One gene encodes a heme oxygenase (*CaHMX1*) that catalyzes conversion of heme to α-biliverdin (40). Immediately adjacent to *CaHMX1*, we identified a second open reading frame that was designated *HBR1* (*Hb*-regulated gene 1) (Fig. 1A).

Regulation of *HBR1* expression was characterized by Northern analysis of RNA extracted from *C. albicans* cells grown with and without hemoglobin to different stages of exponential and early stationary phase (Fig. 1B). Expression of *HBR1* was minimal in stationary-phase cells. Within 0.5 h after transferring cells from stationary phase into fresh medium, hemoglobin induced *HBR1* expression over basal levels, and this was further increased about threefold by 4 h. In separate experiments, steady-state *HBR1* mRNA levels determined by qPCR at 4 h following hemoglobin addition were increased twofold relative to cells at the same growth phase without hemoglobin, consistent with the Northern analysis (Table 3).

Expression levels of both basal and hemoglobin-induced

TABLE 3. Quantitative RT-PCR analysis of gene expression

Gene	*Ct* (± SD)[a] for strain	
	CAF2-1	CAMP 43
ACT1	12.9 ± 0.2	12.3 ± 0.33
HBR1	25.6 ± 0.16	26.4 ± 0.44
HBR1/Hb[b]	24.1 ± 0.7	25.2 ± 0.09
CDC36 (*n* = 16)	20.8 ± 0.27	20.4 ± 0.21
*MTL***a***1*	29.5 ± 0.1	27.5 ± 0.5
MTLα1	26.2 ± 0.16	>40
MTLα2	23.1 ± 0.24	>40

[a] *n* = 4 except where indicated.
[b] Bovine methemoglobin, 0.5 mg/ml (see Materials and Methods).

HBR1 mRNA were maximal during the early stages of exponential growth and declined at later times (Fig. 1B). *PGK1* mRNA levels were uniform during the period of maximal *HBR1* mRNA accumulation and declined to detection limits by 12 h (Fig. 1B) as the cell doubling time increased from 1.4 to 1.8 h (data not shown). Both changes indicated the transition to diauxic growth (1). *HBR1* basal mRNA levels also declined during this transition period, but *HBR1* steady-state mRNA was maintained by hemoglobin at detectable levels until 16 h (Fig. 1B). Thus, *HBR1* expression depends upon cell proliferation, and hemoglobin causes a two- to threefold increase in accumulation of steady-state mRNA levels that persists after basal levels have declined.

The major transcriptional start site of *HBR1* was mapped to a C residue at position −149 (data not shown). We analyzed *HBR1* transcription by fusing upstream regions with a *Renilla* luciferase (*Rlux*) reporter and integrating a single copy in *C. albicans* cells (58). An *Rlux* reporter construct containing 502 bp upstream from the predicted *HBR1* AUG start codon (strain CAMP 35) showed optimal responses to both proliferation and hemoglobin (data not shown). Following pregrowth for 48 h in glucose-containing medium, transfer of cells into new medium containing glucose but lacking hemoglobin resulted in a burst of *HBR1-Rlux* reporter activity within 20 min (Fig. 1C). Reporter activity rapidly returned to basal levels but was reproducibly followed by activity peaks at 130 and 230 min as the cells were allowed to grow (Fig. 1C). Transfer to medium lacking glucose did not result in activity above baseline, indicating that glucose is necessary for increased basal transcription (Fig. 1D). Hemoglobin addition increased the magnitude of these peaks 1.5- to 2-fold in the presence of glucose (Fig. 1C), and hemoglobin increased transcription in the presence or absence of glucose (Fig. 1D). These data indicate that *HBR1* transcription increases during cell proliferation and that signaling from exogenous hemoglobin (40) also regulates *HBR1* transcription.

The *HBR1* DNA sequence derived from our genomic clone exactly matched the Stanford genome database sequence (http://www-sequence.stanford.edu/group/candida). The predicted open reading frame consists of 747 bases, encoding a hypothetical protein of 248 amino acids. The protein sequence contains a predicted nucleotide-binding fold (P-loop) at the amino terminus and a consensus SUMO site, but no other functional motifs were identified (Fig. 2). Hbr1p has 68% amino acid identity with *S. cerevisiae* Fap7p and 62% DNA sequence identity. Although many genes are conserved between these two species, the relative positioning of genes on chromosomes is typically not conserved (25). However, both *HBR1* and *FAP7* share 5′ untranslated regions with the adjacent gene *CDC36* and are divergently transcribed from opposite strands (Fig. 1A). *FAP7* is an essential gene in *S. cerevisiae* (23).

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C.albicans           MTTMSRRYTP NIIITGTPGCGKSS HSSS LV SQLM qtlgkettih
S.cerevisiae         mea--RRYGP NIIVTGTPGCGKSS TCEF LK NKLK d---------
Consensus                     P NIIITGTPG GKS
Adenylate Kinase Consensus          GXPGXGKT
P-loop Consensus                    GXXXXGKS/T

45   PKHFNI SEIAKERDC I ESYDAKLDTS IVDEDKLLD S LEP DL EK GGV IVDWH VNDVF
     YKYYNI SDFAKDNDC F EGYDEGRKSH IVDEDKLLD M LEP LL RQ GNS IVDWH VNDNF
                KE        YDE       DEDKL  D   LEP       GG  VVDWH    CF
                R     F                RV                    I E

101  PERLIDLVVV LRTDNSNLFD RL KT RNYNDL KLQENLD CEI MEVILQEAKD SYIPDIVIEL
     PERLIDLVVV LRCDNSNLYS RL HA RGYHDS KIEENLD AEI MGVVKQDAVE SYEPHIVVEL
     PERLI   VV LRC    L   RL    RGY    KI  N    EI       EA   SY   IV EL

161  RSDT AEEMDE NVDEISSWVE T WI EDHPDGV SNElnkqynp DD SSDEGDDN
     QSDT KEDMVS NVSRIVAWEK M WL EQHPDGV TNE        PR SDDEDDED
           E M   NV  I  W     W

211  Sdseyeleedeqeeeeeereeydeetneemehtediaq
     SE------------------------------------
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FIG. 2. Conservation of the predicted amino acid sequences of *HBR1* and *FAP7*. The alignment of predicted protein sequences of Hbr1p and Fap7p is shown. A consensus sequence was generated using DIALIGN 2.2 (37; http://bibiserv.techfak.uni-bielefeld.de/dialign/) with the following eukaryotic orthologs of Hbr1p: *Schizosaccharomyces* (CAB52884), *Candida* (AF466197), *Saccharomyces* (CAA98740), *Caenorhabditis* (Q09527), *Drosophila* (AAF58491), *Oryctolagus* (AAF09498), *Homo sapiens* (Q9Y3D8), *Mus* (BAB29612), *Anopheles* (EAA06472), and *Arabidopsis* (BAB10972). All uppercase residues were aligned in the analysis. Consensus residues that were highly conserved are indicated below the aligned yeast sequences. Residues 65 to 68 are the SUMO consensus (43).

Hbr1p orthologs were identified in most archaeal and eukaryotic genomes but not in any eubacteria. Evolutionary distances between these apparent Hbr1p orthologs suggested a common ancestry for this protein between eukaryotes and archaea (30). A conserved consensus sequence extending from Leu(118) through Ala(148) was identified in all orthologs (Fig. 2), although no function has been defined for any of these hypothetical proteins apart from Fap7p (23, 43). Hbr1p contains a 56-amino-acid extension containing 54% acidic residues at its C terminus (Fig. 2A). The latter feature is lacking in most Hbr1p orthologs except for a short region in *S. cerevisiae* Fap7p (Fig. 2). The extension is not a sequencing artifact, because identical sequences were found in *C. albicans* strains CAI-4 and B311 (data not shown).

Disruption of a single *HBR1* allele enables white-opaque switching. The first allele of *HBR1* was mutated using an *ARG4* disruption cassette in strain BWP17 and was made prototrophic by the insertion of *HIS1* and *URA3* genes (see Materials and Methods). The prototrophic *HBR1*/*hbr1* strain CAMP 43, when plated on defined glucose-containing medium, generated colonies containing mixed populations of

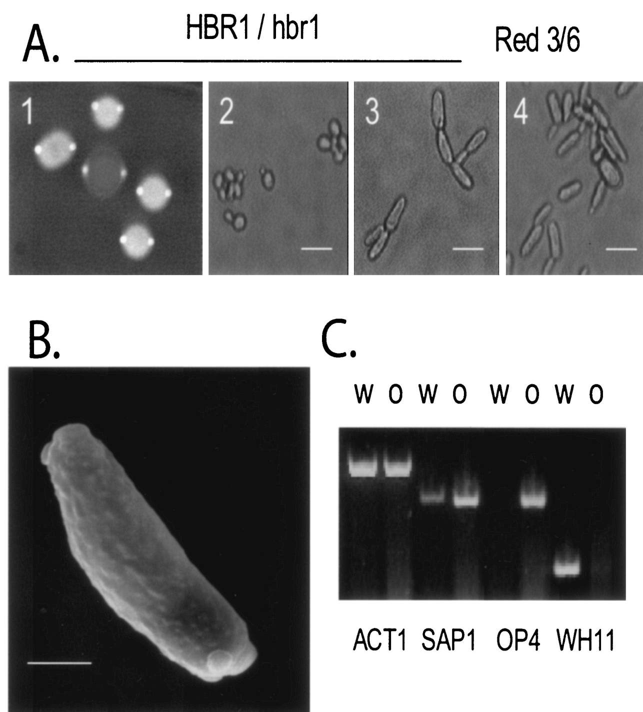

FIG. 3. *HBR1* deletion leads to high-frequency white-opaque phase switching. (A) Prototrophic $HBR1^{+/-}$ strain CAMP 43 growing on phloxine B agar plates (frame 1), showing an opaque colony (center) surrounded by white colonies, from microscopic examination of CAMP 43 (frame 2, opaque; frame 3, white), and Red 3/6 (frame 4, opaque) cells. Bar, 10 μm. (B) Scanning electron micrograph of a CAMP 43 opaque cell, illustrating the surface protrusions or pimples characteristic of opaque cells. Bar, 3 μm. (C) Regulation of phase-specific genes in the *HBR1* heterozygote was determined by RT-PCR analysis of RNA isolated from 4-h exponential-phase cultures of strain CAMP 43 grown at 30°C in YNB-glucose. W, white; O, opaque; *ACT1*, actin. Probes for phase-specific transcription were as follows: white, WH11 (59); opaque, OP4 and SAP1 (38).

white and elongated cells resembling opaque cells (3) (data not shown). Growth on phloxine B agar plates to detect switching revealed characteristic red and white colonies that contained opaque and white cells, respectively (Fig. 3A). As shown previously for the prototypical switching strain WO-1 (3), analysis of CAMP 43 opaque cells by scanning electron microscopy revealed the characteristic pimples of opaque cells (Fig. 3B). Thus, the *HBR1* heterozygote undergoes phenotypic switching that appears to yield an opaque cell.

Because *CDC36* and *HBR1* are divergently transcribed from a short common promoter region (Fig. 1A), we considered the possibility that the observed switching phenotype of the *HBR1* heterozygote could result from interruption of either *HBR1* or *CDC36* gene function. However, qPCR analysis indicated that *CDC36* mRNA levels were typically approximately 30-fold greater than *HBR1* levels in wild-type cells, and the *HBR1* mutation in CAMP 43 cells did not significantly alter the level of *CDC36* mRNA (Table 3). This result was highly reproducible in several *HBR1* mutants, indicating that disruption of an *HBR1* allele does not interfere with transcription of the adjacent *CDC36* gene.

The frequency of the white-opaque transition in CAMP 43

TABLE 4. White-opaque transition frequencies of *C. albicans* strains

Strain	Genotype				Frequency
	HBR1	*MTL*α*1*	*MTL*α*2*	*MTL***a***1*	
CAMP 43	−/+	+	+	+	3×10^{-3}
Red 3/6	+/+	+	+	−	5×10^{-3}
CAMP 63	−/+/+[a]	+	+	+	$<10^{-5}$
CAF2-1	+/+	+	+	+	$<10^{-5}$
CAMP 45	−/+	−	+	+	5×10^{-2}
CAMP 47	+/+	−	+	+	$<10^{-5}$
CAMP 48	+/+	+	+	−	1.2×10^{-3}

[a] pDisCat4 integrated as a single copy generating an HBR1 duplication.

cells was similar to that reported for the prototypical switching strain WO-1 (56), to those determined for an *mtl***a***1* mutant in the CAI-4 background (CAMP 48), and independently by us for the WO-1 derivative, Red 3/6 (Table 4). Consistent with a previous report (36), disruption of *MTL*α1 did not increase switching (Table 4, CAMP 47), but allelic deletion of *HBR1* in this background resulted in approximately 10-fold-higher switching than in a wild-type *MTL* background (Table 4, CAMP 43 and 45). Reintroduction of *HBR1* controlled by the *MET3* promoter under inducing conditions (CAMP 63) restored the white-opaque transition frequency to that of the parental CAF2-1 strain (Table 4). These data indicated that Hbr1p is a haplo-insufficient repressor of white-opaque phase switching.

***HBR1* is an essential gene in *C. albicans*.** The haplo-insufficient phenotype of the single allelic knockout of *HBR1* and the complementation results presented here indicated that *HBR1* gene dosage is crucial to proper regulation of the switching phenotype. Our inability to generate a knockout of the second *HBR1* allele by standard means (data not shown) prompted us to generate conditionally lethal *HBR1* double mutants. These strains (CAMP 61 and CAMP 62) contained deletions of both *HBR1* alleles with Hbr1p supplied in *trans* from a plasmid construct integrated into the neutral *RP10* locus (11). They differed only in the primers used to construct the *HIS1* mutagenic cassette (see Materials and Methods). The integrated plasmid pExpCAT4-9 in these strains contains *HBR1* linked to a *URA3* marker (11). Therefore, selection for URA^- cells on agar containing 5-fluoroorotic acid (46) should result in the absence of any colonies if *HBR1* were necessary for cell survival. A total of 10^9 cells from two isolates of each strain were tested and compared for the rate of marker excision from strain CAMP 63 lacking the second His insertion (Table 1). CAMP 63 cells produced Ura^- colonies at a frequency 2×10^{-7}, while the CAMP 61 and CAMP 62 strains produced no colonies. Therefore, the *HBR1* double deletion rendered the cells nonviable, indicating that *HBR1* is essential for cell survival.

Phase-specific genes confirm white-opaque switching. Changes in cellular morphology due to the white-opaque transition are accompanied by altered transcription of phase-specific genes, including white phase expression of *WH11* and opaque phase expression of *OP4* and *SAP1* (reviewed in reference 54). RNA was isolated from cultures comprising >95% opaque or white cells, respectively, and analyzed for phase-specific gene expression using RT-PCR. Expression of these

three genes in CAMP 43 cells followed the pattern expected for the respective cell types (Fig. 3C). Therefore, the molecular phenotype of the *HBR1* mutant is consistent with white and opaque phenotypes.

Hbr1p-regulated switching occurs in the absence of MTL deletions. White-opaque switching in *C. albicans* is negatively regulated via the *MTL* locus (36; reviewed in references 22 and 55). Therefore, switching in the *HBR1* heterozygous strain could arise either from rearrangements of this locus or from effects of Hbr1p on *MTL* gene expression. Genomic PCR analysis demonstrated that both white and opaque cells of strain CAMP 43 retained the full complement of *MTL* genes, whereas Red 3/6 was deleted for *MTL***a***1* (Fig. 4A, lanes 1 to 3), as previously reported (31). A clinical strain lacking both *MTL***a***1* and *MTL*α1 alleles was used as an additional control (Fig. 4A, lane 5). The presence of the genomic marker λ *imm434* (14) verified that strain CAMP 43 (Fig. 4A, lanes 1 and 2) was a derivative of the parental CAI-4 strain (Fig. 4A, lane 4). Thus, the *HBR1* heterozygote functions as a white-opaque switching strain in the presence of all three *MTL* genes.

Hbr1p regulates *MTL*α gene expression. The second possibility to explain switching in the *HBR1* heterozygote is that Hbr1p regulates *MTL* gene expression. We addressed this issue by measuring steady-state levels of *MTL* RNA by RT-PCR 4 h after transfer of stationary-phase cells to new medium. All three *MTL* alleles were expressed in the parental strain CAI-4 (Fig. 4B, lane 4), and disruption of the *MTL***a***1* gene did not produce a noticeable effect on *MTL*α gene expression (Fig. 4B, lane 1). However, *MTL*α expression could not be detected in either white or opaque $Hbr1^{+/-}$ cells, and *MTL***a***1* appeared to be expressed at a slightly elevated level (Fig. 4B, lanes 2 and 3). Measurement of mRNA levels from CAMP 43 and wild-type cells using qPCR indicated that *MTL*α gene mRNA expression in CAMP 43 cells was at least 16,000-fold less than in wild-type cells (Table 3). Therefore, Hbr1p is a positive regulator of both *MTL*α genes, and mutation of one *HBR1* allele prevents their expression.

Because Hbr1p appeared to be a limiting factor for *MTL*α expression in $Hbr1^{+/-}$ cells, we asked whether the same was true during the vegetative growth cycle in an $Hbr1^{+/+}$ strain. Both *MTL*α alleles were highly expressed during the exponential growth phase and decreased to basal levels by 24 h (Fig. 4C). This pattern paralleled that of *HBR1* expression (Fig. 1B and data not shown). If Hbr1p were limiting for *MTL*α expression in stationary-phase cells, maintaining high Hbr1p levels into stationary phase should sustain *MTL*α expression. To test this hypothesis, *HBR1* expression was increased directly by expression using a heterologous promoter or indirectly by the addition of hemoglobin to the cultures. As predicted, *HBR1* overexpression driven solely by the *C. albicans MET3* promoter in strain CAMP 63 sustained *MTL*α expression at high levels after 24 h, while expression decreased in the parental strain CAF2-1 (Fig. 4D) and was absent in the *HBR1* heterozygote (Fig. 4B, lanes 2 and 3 [24-h data not shown]). Addition of hemoglobin to strain CAF2-1, with an intact *HBR1* locus, caused only a modest increase in *MTL*α2 levels at 4 and 12 h (Fig. 4E, lanes 1 to 6). However, hemoglobin sustained *MTL*α2 expression up to 24 h (Fig. 4E, compare lanes 7 and 8). Therefore, increased *MTL*α expression in proliferating cells is mediated through Hbr1p, and exposure to hemoglobin sustains *MTL*α2 expression in early-stationary-phase cells through this pathway.

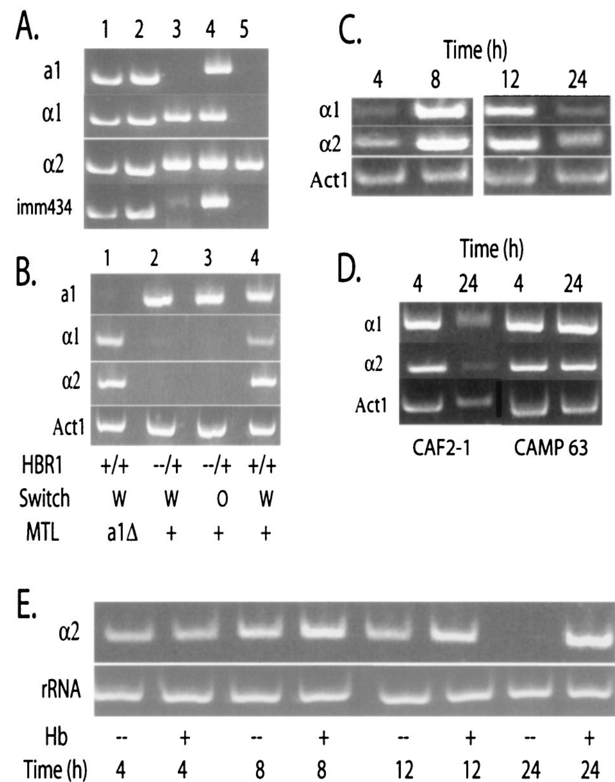

FIG. 4. Hbr1p is a positive regulator of *MTL*α expression. (A) *HBR1* heterozygosity does not lead to *MTL* gene deletions. Results of PCR analysis of genomic DNA using primers specific for *MTL* genes and for the λ*imm434* region used in construction of parental strain CAI-4 (14) are shown. Lanes 1 and 2, CAMP43, white and opaque, respectively; lane 3, Red 3/6, white; lane 4, CAI-4, white; lane 5, clinical isolate 156, white. (B) *MTL* α gene expression is not detectable in the *HBR1* heterozygote, as shown by an RT-PCR analysis of RNA isolated from 4-h exponential-phase cells cultured in YNB-glucose medium at 30°C. Gene-specific primers listed on the left were used in PCRs of 26 cycles (*MTL* genes) or 20 cycles (*ACT1*). Lane 1, strain CAMP 48; lanes 2 and 3, strain CAMP 43; lane 4, strain CAI-4. (C) *MTL*α genes are induced only during exponential growth. Results of the RT-PCR analysis of RNA isolated from CAF2-1 cells at the indicated times after transfer of stationary-phase cells to YNB-glucose medium are shown. (D) *HBR1* overexpression sustains *MTL*α expression into early stationary phase. Results of RT-PCR analysis of RNA harvested at 4 and 24 h after transfer of stationary-phase cells to low-methionine medium are shown. CAMP 63 contains an *HBR1* copy under the control of the *MET3* promoter. The *MET3* promoter maintained *HBR1* RNA at higher levels in the CAMP 63 strain than in strains containing only the native *HBR1* promoter (see Fig. 6B). (E) Hemoglobin can sustain MTLα gene expression into stationary phase. Strain CAF2-1 cells were cultivated in the presence or absence of 0.5 mg of hemoglobin/ml, and RNA was harvested at the indicated times. Primer sets for RT-PCR analysis are listed in the figure. *MTL*α, 26 cycles; rRNA, 15 cycles.

HBR1 regulates the a1/α2 repressor targets *CAG1* and *YEL007w*. To confirm that regulation of the *MTL*α genes by Hbr1 has functional significance, we measured mRNA levels of *CAG1*, an established direct target of the **a**1/α2 repressor complex in both *C. albicans* and *S. cerevisiae* (called *GPA1* in the

latter) (20, 48). We used qPCR to measure *CAG1* levels and normalized the values to those of an equivalent *MTL* wild-type strain (CAF-2) grown in YNB with glucose. The *CDC36* gene was chosen as an internal standard because its expression levels were comparable to those of the genes under study. In addition, *CDC36* expression did not significantly vary under any of the growth conditions or with any of the strains used in the experiments reported here (see Table 3 and Materials and Methods).

CAMP 43 white and opaque cells displayed 27- to 29-fold derepression of *CAG1* when compared with CAF-2 control cells grown under identical conditions (Fig. 5A). If this result were caused by **a**1/α2 dimer disruption through repression of *MTL*α genes, then increasing Hbr1p expression induced by hemoglobin should restore *MTL*α gene expression and repress *CAG1.* This was confirmed in both white and opaque CAMP 43 cells (Fig. 5A). The difference in the degree of inhibition between white and opaque cells was reproducible, but its basis is unknown. Nevertheless, the reversal of *CAG1* derepression was as predicted for increased α2 production as a result of increasing Hbr1 expression. This degree of inhibition is consistent with the twofold increase in *HBR1* mRNA levels in CAMP 43 and CAF-2 cells grown with hemoglobin (Table 3).

The role of **a**1/α2 in *HBR1*-dependent *CAG1* regulation was further supported by a comparison of *CAG1* mRNA levels from an *MTL***a***1* deletion strain (CHY477) grown with and without hemoglobin. Without hemoglobin, the 25-fold *CAG1* derepression was comparable to that of the CAMP 43 cells (Fig. 5A). Hemoglobin did not restore repression of *CAG1* in this strain or in a strain containing both *HBR1* and *MTL***a***1* mutations (CAMP 49) (Fig. 5A). These results were expected, since **a**1 is required for **a**1/α2 repressor function (36). Interestingly, CAMP 49 displayed *CAG1* mRNA levels somewhat higher than in either singly mutated strain (Fig. 5A), suggesting that some derepression of CAG1 in the HBR1 mutant may be **a**1/α2 independent. Nonetheless, these results confirm that hemoglobin- and Hbr1-dependent repression of *CAG1* occurs through **a**1/α2.

Modulation of **a**1/α2 repressor function by Hbr1 was also confirmed for a recently described gene of unknown function (*YEL007w*) that is expressed in **a** and α white cells but not in **a**/α cells (61). *YEL007w* mRNA expression was derepressed in the *HBR1* heterozygote (CAMP 43) and in *MTL***a***1* and *MTL*α*1,2* deletion strains, as expected for regulation by **a1**/α2 (Fig. 5B).

The *HBR1* mutation represses α cell and the a-cell molecular traits. The differential regulation of genes required for mating is a primary function of the *MTL*. Commitment to a specific mating type involves suppression of α-specific genes in **a** cells and vice versa (16). CAMP 43 cells lack *MTL*α gene expression and can undergo switching to the mating-competent opaque cell form. This indicated that *HBR1* mutant cells display some **a**-cell attributes, prompting us to further examine genes known to be differentially expressed between **a** and α cells.

The recently described α mating pheromone gene (*MF*α) (9, 32) and the **a** pheromone receptor (*STE3*) are expressed in functional α cells (61). We found that both of these genes were repressed in CAMP 43 but more strongly in opaque cells,

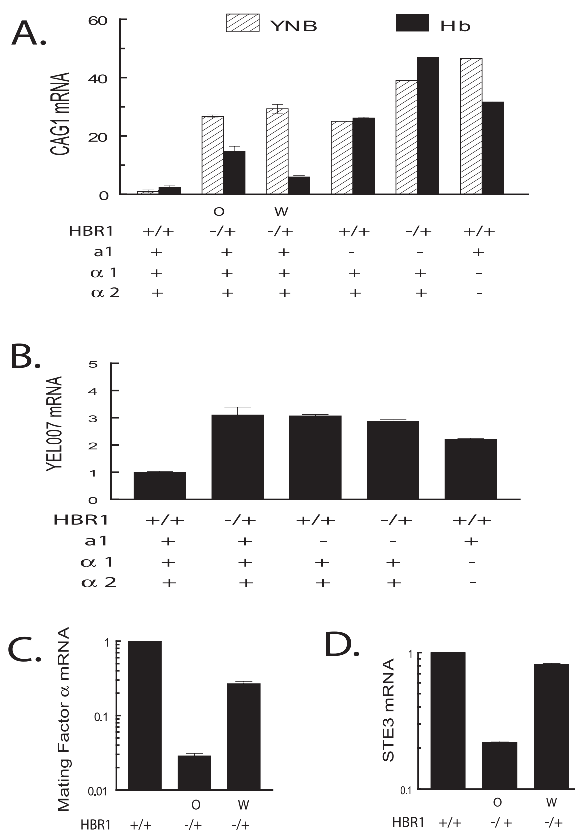

FIG. 5. Regulation of a1/α2 repression and α-cell gene targets through *HBR1.* (A) An **a**1/α2 target is derepressed in an *HBR1* heterozygote. Results of qPCR analysis of RNA isolated from 4-h log-phase cells with and without 0.5 mg of hemoglobin/ml are shown. *MTL* and *HBR1* genotypes as well as white (W) and opaque (O) phenotypes are indicated. *CAG1* expression from each strain was corrected for the level of the *CDC36* internal standard and then normalized to CAF2-1 levels (arbitrarily set at 1). Strain designations (from left to right): CAF2-1, CAMP 43, CAMP 43, CHY477, CAMP 49, and MM278. This figure represents two separate analyses. Levels are reported as ± the SD. (B). *YEL007w* expression is derepressed in *HBR1* heterozygous cells. qPCR analysis was carried out with the same cDNA preparations as described for panel A. Strain designations are as in panel A, except CAMP 43 opaque cells were not tested. Normalized levels are reported as ± the SD. (C and D) Two α-specific genes are down-regulated in CAMP 43 opaque cells. Mating factor α and STE3 mRNA levels were determined using qPCR with the same cDNA preparations as described for panel A. Levels are reported as ± the SD.

consistent with a transition to an **a**-cell phenotype (Fig. 5C and D).

We additionally analyzed the **a**-cell-specific genes *RAM*2 and *HST6*, both of which are required for processing and exporting **a**-factor. *RAM2* encodes the common α subunit of two prenyltransferases (57), and *HST6* is the ortholog of the ABC transporter *STE6* required for **a**-factor transport (44) and for **a** cells to mate (34). *RAM2* was induced sixfold in CAMP 43 cells, but not when *MTL***a***1* was deleted either alone or in an HBR1$^{+/-}$ background, nor in an *MTL*α*1,2* double deletion strain (Fig. 6A). These results could be rationalized if Hbr1p were required for **a**1 to act as a positive regulator of **a**-specific genes or if an **a**1 target is necessary for Hbr1p-dependent regulation of *RAM2.* The former hypothesis is supported by the observed

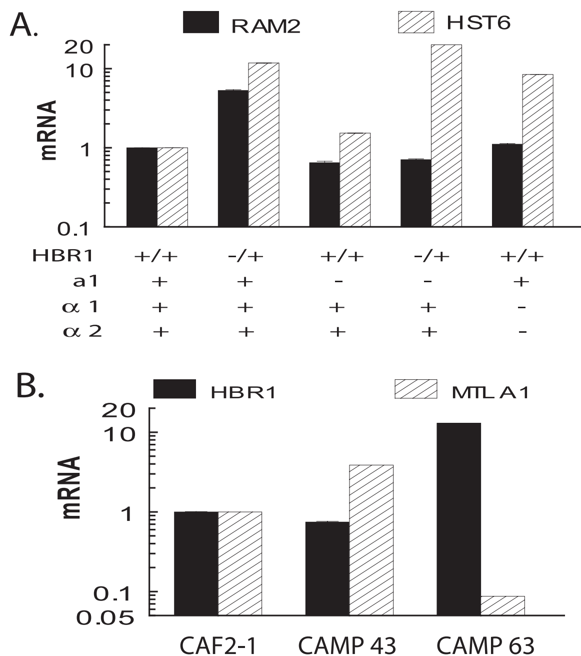

FIG. 6. *HBR1* regulates a-specific genes and is a negative regulator of MTL a. (A) Two **a**-specific genes are regulated through *HBR1*. qPCR analysis was performed using the same cDNA preparations as in Fig. 5. (B) Hbr1p expression represses *MTL***a***1* expression. Strain CAMP 43 contains only one *HBR1* allele, and CAMP 63 contains *HBR1* expressed at high levels under the control of the *MET3* promoter.

positive regulation of **a**1 in $HBR1^{+/-}$ cells (Table 3 and Fig. 6B).

Negative regulation of **a**1 by *HBR1* was confirmed by overexpressing Hbr1p (CAMP 63), which resulted in marked suppression of **a**1 levels (Fig. 6B). Therefore, Hbr1p may regulate some **a**-specific genes, such as *RAM2*, through its effects on **a**1 expression (Fig. 7).

HST6 expression was also substantially elevated in an $HBR1^{+/-}$ strain (Fig. 6A). Like *RAM2*, it was not induced in the **a**1 deletion, showing that elevated expression does not result from loss of **a**1 α2 repression. However, *HST6* differed from *RAM2* in that deleting one allele of *HBR1* in the strain lacking **a**1 elevated *HST6* expression to the same extent as in CAMP 43 (Fig. 6A). Therefore, **a**1 is not required for this response to Hbr1. Furthermore, deletion of α1 α2 was sufficient to elevate HST6 expression, a response not seen for RAM2 (Fig. 6A). This suggests that HST6 may be regulated by Hbr1 through its effects on α1 or α2. Although the mechanisms for regulation of MTL targets are clearly divergent, these results consistently show that loss of Hbr1 expression results in a gene expression profile typical of an **a** cell (Fig. 7).

HBR1 **heterozygotes function as mating-competent a cells.** Hbr1p regulation of *MTL*α genes and the targets mentioned above suggested that reducing Hbr1p dosage may permit opaque cells of this strain to mate as **a** cells. To test this, opaque *HBR1* heterozygotes (CAMP R8; Ura^- His^- Ade^+) were crossed with opaque *MTL***a** null cells (CAMP 51; Ade^- Ura^+) using the quantitative filter mating procedure as described previously (36) (see Table 1 for complete strain descriptions). Based on four independent crosses, Ura^+ Ade^+ colonies were isolated at frequencies of $(3.7 \pm 1.0) \times 10^{-3}$ on selective Arg^- Ura^- medium. Neither parental strain survived on this selective medium, indicating that spontaneous reversion to prototrophy was undetectable. This demonstrated that the *HBR1* heterozygote behaved like an **a** cell in an *MTL*a/*MTL*α *HBR1*/*hbr1* × *mtl***a**1/*MTL*α *HBR1*/*HBR1* strain cross. Therefore, mating is regulated by Hbr1 and can also occur in the absence of chromosomal deletions in the *MTL* locus.

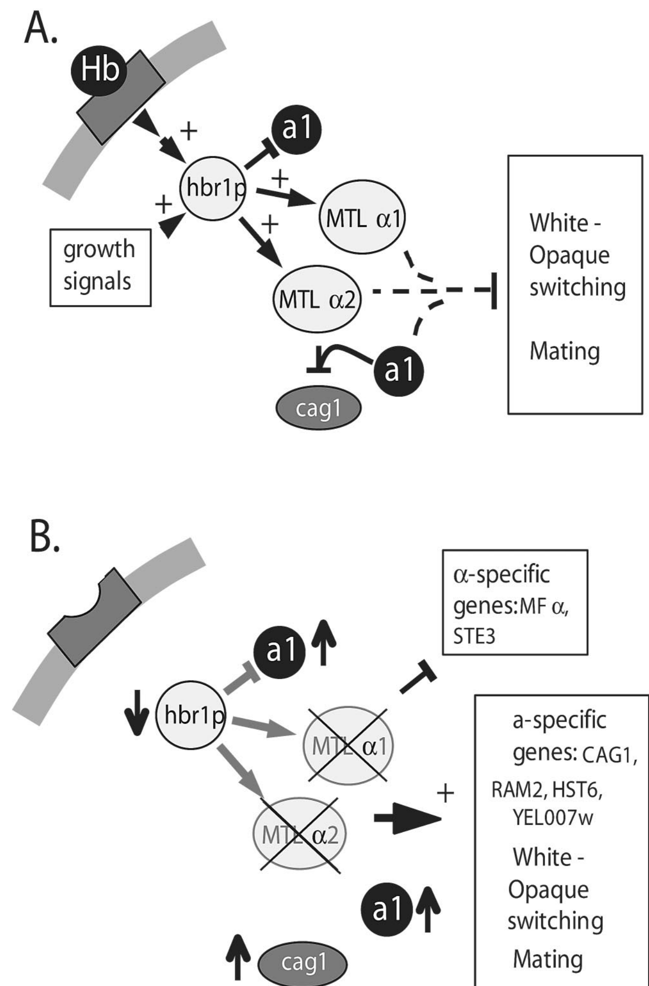

FIG. 7. Model for the role of *HBR1* in MTL gene regulation. (A) Hbr1p expression is stimulated by growth and by signals from the host factor hemoglobin. Normal cellular levels of Hbr1p support *MTL*α*1* and *MTL*α*2* expression and moderately repress *MTL*α*1*. Both alleles of *HBR1* are necessary to maintain *MTL*α gene expression and functional levels of the a1/α2 repressor. According to current models (36), this repressor limits expression of *CAG1*, white-opaque phase switching, and mating. (B) Limiting Hbr1p by removing stimuli controlling its expression or by allelic deletion represses *MTL*α*1* and *MTL*α*2* gene expression. These conditions derepress genes regulated by **a**1 α2, permit white-opaque phenotypic switching, increase expression of **a**-cell-specific genes, and decrease α-cell-specific genes. Through this process whereby *MTL*α*1* and *MTL*α*2* expression is down-regulated, the cells acquire **a** cell characteristics and are capable of mating. Thus, *HBR1* regulation of *MTL* genes is a mechanism that can allow mating without the deletion of *MTL* genes.

DISCUSSION

The *C. albicans MTL* locus (20) is a central regulator of phenotypic phase switching and mating in vitro (35, 36) and in vivo (21). However, mating competency in *C. albicans* has been previously achieved only through allelic deletion of *MTL***a***1* or *MTL*α2 (21, 35, 36). Our data demonstrate for the first time that phenotypic phase switching and mating can occur in cells containing an intact *MTL* locus. We show that the *MTL*α genes are positively regulated by Hbr1p and that loss of a single *HBR1* allele is sufficient to allow white-opaque switching and mating without *MTL* gene rearrangement. *HBR1* in turn is regulated by proliferation signals and exposure of cells to exogenous hemoglobin (Fig. 7). Therefore, both the availability of nutrients and hemoglobin in the local environment may be important regulators of mating for *C. albicans* in its mammalian host.

The haplo-insufficient phenotype of *HBR1* for repressing white-opaque switching suggests that Hbr1p plays a pivotal role in the control of *MTL*α gene expression. The white-to-opaque transition in *HBR1* heterozygotes occurred with a frequency comparable to that of *MTL***a***1* or *MTL*α*2* deletion strains (36, 54) (Table 4). Morphological characteristics and gene expression patterns in the opaque cells were indistinguishable from those generated by *MTL* deletion. *HBR1* mutants formed cells with the oblong shape and dimensions typical of opaque cells (52), possessed the characteristic cell surface pimples (3), and appropriately regulated phase-specific genes (38, 59) (Fig. 3). Reexpression of *HBR1* using the *MET3* promoter suppressed switching, confirming that the *HBR1* deletion caused the increased white-to-opaque switching frequency. The increased switching in *HBR1*/*hbr1* cells results from loss of positive regulation of *MTL*α gene expression by Hbr1p. Consistent with this model, we demonstrated loss of **a**1/α2 repressor activity for two known targets, *CAG1* and *YEL007w* (20, 48, 61), in an $HBR1^{+/-}$ strain.

Hbr1p positively regulates *MTL*α gene expression and is situated upstream of the switch event, but we do not know whether Hbr1p directly or indirectly induces *MTL*α expression. The two *MAT*α genes in *S. cerevisiae* are divergently transcribed from a common promoter region and are separated by less than 300 bp (4). The *C. albicans MTL*α genes are separated by more than 5 kbp (20), suggesting a divergence in regulatory mechanisms.

Hbr1p possesses a predicted P-loop but lacks known DNA-binding motifs (Fig. 2). The Hbr1p ortholog, Fap7p, was identified in a screen for mutants that failed to activate a *Gal1-LacZ* reporter by a Gal4p-Skn7p hybrid transcription factor in response to oxidative stress (23). This mutant contained a P-loop mutation (G19S), indicating that the P-loop is an essential structural feature for some Fap7p functions. In this mutant, interaction with the transcription factor Skn7p was disrupted, although the cells remained viable. Consistent with this result, Fap7p was found to interact with Skn7p in a two-hybrid assay and was nuclear localized (23). Skn7p has been shown to couple environmental inputs such as oxidative stress to intracellular signaling (reviewed in reference 19). However, H_2O_2 inhibited transcription from an *HBR1*-luciferase reporter construct (data not shown), which is inconsistent with an Skn7p interaction under oxidative stress. Nevertheless, this does not preclude interactions of Hbr1p with Skn7p under other metabolic circumstances.

The ability of Hbr1 to regulate mating is also not sufficient to explain why *HBR1* and the yeast ortholog *FAP7* are essential genes. Other critical functions may depend on these genes. *FAP7* was recently identified in a screen for mutants defective in noncoding RNA processing (43), suggesting that it has additional functions in *S. cerevisiae*.

HBR1 transcription is rapidly induced in response to hemoglobin exposure. The haplo-insufficiency of *HBR1* for *MTL*α expression and sensitivity of *CAG1* in the *HBR1* heterozygote to hemoglobin addition shows that the MTL signaling pathway is quite sensitive to regulation by this host factor. Hemoglobin also maintains *MTL*α2 levels into stationary phase in cells with an intact *HBR1* locus, demonstrating that this pathway allows hemoglobin to regulate the *MTL* locus in wild-type *C. albicans*. Hemoglobin $\alpha\beta$ dimers, released via erythrocyte lysis, signal to *HBR1* through an unidentified hemoglobin receptor (41). Although the acute phase protein haptoglobin can sequester hemoglobin $\alpha\beta$ dimers (7, 10), many pathogenic *Candida* species exhibit hemolytic activity that could release sufficient hemoglobin to overwhelm this protective protein (33). Thus, hemoglobin exposure may occur when the organism establishes a disseminated infection and is proximal to erythrocytes in a site with low-shear flow. Sensitivity of this pathway to hemoglobin may be modulated by cell proliferation signals that also regulate *HBR1* and, thereby, *MTL*α expression. Fungal cells in the exponential growth phase would express α genes and, therefore, the **a**1/α2 repressor independently of hemoglobin, but **a**1/α2 levels would decrease in the postexponential phase. In the latter case, *MTL*α expression would be more sensitive to hemoglobin stimulation and could restore repressor function to inhibit phase switching in stationary phase.

In summary, we show here that one of the genes regulated by hemoglobin suppresses the phenotypic switch required for mating in *C. albicans*. We previously demonstrated that hemoglobin alters the adhesive phenotype of *C. albicans* by inducing binding to several host matrix proteins (65), but this is the first direct evidence that hemoglobin regulates expression of genes known to be expressed during infection and mating. Importantly, we have identified *HBR1* as an essential gene that is regulated in response to a defined environmental cue in the vascular compartment of a mammalian host. In addition to regulating mating, *HBR1* presumably controls other important signaling pathways that may explain its requirement for vegetative growth.

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REFERENCES

1. **Alloush, H. M., J. L. Lopez-Ribot, B. J. Masten, and W. L. Chaffin.** 1997. 3-Phosphoglycerate kinase: a glycolytic enzyme protein present in the cell wall of Candida albicans. Microbiology **143:**321–330.
2. **Anderson, J., R. Mihalik, and D. R. Soll.** 1990. Ultrastructure and antigenicity of the unique cell wall pimple of the *Candida* opaque phenotype. J. Bacteriol. **172:**224–235.

3. **Anderson, J. M., and D. R. Soll.** 1987. Unique phenotype of opaque cells in the white-opaque transition of *Candida albicans*. J. Bacteriol. **169:**5579–5588.
4. **Astell, C. R., L. Ahlstrom-Jonasson, M. Smith, K. Tatchell, K. A. Nasmyth, and B. D. Hall.** 1981. The sequence of the DNAs coding for the mating-type loci of Saccharomyces cerevisiae. Cell **27:**15–23.
5. **Auclair, K., H. W. Huang, P. Moenne-Loccoz, and P. R. Ortiz de Montellano.** 2003. Cloning and expression of a heme binding protein from the genome of Saccharomyces cerevisiae. Protein Expr. Purif. **28:**340–349.
6. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1988. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
7. **Baumann, H., and J. Gauldie.** 1994. The acute phase response. Immunol. Today **15:**74–80.
8. **Bedell, G. W., and D. R. Soll.** 1979. Effects of low concentrations of zinc on the growth and dimorphism of *Candida albicans*: evidence for zinc-resistant and -sensitive pathways for mycelium formation. Infect. Immun. **26:**348–354.
9. **Bennett, R. J., M. A. Uhl, M. G. Miller, and A. D. Johnson.** 2003. Identification and characterization of a *Candida albicans* mating pheromone. Mol. Cell. Biol. **23:**8189–8201.
10. **Bowman, B. H.** 1993. Haptoglobin. *In* B. H. Bowman (ed.), Hepatic plasma proteins, vol. 149. Academic Press, San Diego, Calif.
11. **Care, R. S., J. Trevethick, K. M. Binley, and P. E. Sudbery.** 1999. The MET3 promoter: a new tool for Candida albicans molecular genetics. Mol. Microbiol. **34:**792–798.
12. **De Backer, M. D., P. T. Magee, and J. Pla.** 2000. Recent developments in molecular genetics of Candida albicans. Annu. Rev. Microbiol. **54:**463–498.
13. **Dey, S., T. S. Basu Baul, B. Roy, and D. Dey.** 1989. A new rapid method of air-drying for scanning electron microscopy using tetramethylsilane. J. Microsc. **156:**259–261.
14. **Fonzi, W. A., and M. Y. Irwin.** 1993. Isogenic strain construction and gene mapping in Candida albicans. Genetics **134:**717–728.
15. **Goshorn, A. K., and S. Scherer.** 1989. Genetic analysis of prototrophic natural variants of Candida albicans. Genetics **123:**667–673.
16. **Herskowitz, I.** 1987. A master regulatory locus that determines cell specialization in yeast. Harvey Lect. **81:**67–92.
17. **Herskowitz, I.** 1989. A regulatory hierarchy for cell specialization in yeast. Nature **342:**749–757.
18. **Hoffman, C. S., and F. Winston.** 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of Escherichia coli. Gene **57:**267–272.
19. **Hohmann, S.** 2002. Osmotic stress signaling and osmoadaptation in yeasts. Microbiol. Mol. Biol. Rev. **66:**300–372.
20. **Hull, C. M., and A. D. Johnson.** 1999. Identification of a mating type-like locus in the asexual pathogenic yeast Candida albicans. Science **285:**1271–1275.
21. **Hull, C. M., R. M. Raisner, and A. D. Johnson.** 2000. Evidence for mating of the "asexual" yeast Candida albicans in a mammalian host. Science **289:**307–310.
22. **Johnson, A.** 2003. The biology of mating in Candida albicans. Nat. Rev. Microbiol. **1:**106–116.
23. **Juhnke, H., C. Charizanis, F. Latifi, B. Krems, and K. D. Entian.** 2000. The essential protein fap7 is involved in the oxidative stress response of Saccharomyces cerevisiae. Mol. Microbiol. **35:**936–948.
24. **Kennedy, M. J., A. L. Rogers, L. R. Hanselmen, D. R. Soll, and R. J. Yancey, Jr.** 1988. Variation in adhesion and cell surface hydrophobicity in Candida albicans white and opaque phenotypes. Mycopathologia **102:**149–156.
25. **Keogh, R. S., C. Seoighe, and K. H. Wolfe.** 1998. Evolution of gene order and chromosome number in Saccharomyces, Kluyveromyces and related fungi. Yeast **14:**443–457.
26. **Köhrer, K., and H. Domdey.** 1991. Preparation of high molecular weight RNA, p. 398–405. *In* C. Guthrie and G. R. Fink (ed.), Yeast genetics and molecular biology, vol. 194. Academic Press, San Diego, Calif.
27. **Kolotila, M. P., and R. D. Diamond.** 1990. Effects of neutrophils and in vitro oxidants on survival and phenotypic switching of *Candida albicans* WO-1. Infect. Immun. **58:**1174–1179.
28. **Kvaal, C., S. A. Lachke, T. Srikantha, K. Daniels, J. McCoy, and D. R. Soll.** 1999. Misexpression of the opaque-phase-specific gene PEP1 (SAP1) in the white phase of *Candida albicans* confers increased virulence in a mouse model of cutaneous infection. Infect. Immun. **67:**6652–6662.
29. **Lan, C.-Y., G. Newport, L. A. Murillo, T. Jones, S. Scherer, R. W. Davis, and N. Agabian.** 2002. Metabolic specialization associated with phenotypic switching in Candida albicans. Proc. Natl. Acad. Sci. USA **99:**14907–14912.
30. **Leipe, D. D., E. V. Koonin, and L. Aravind.** 2003. Evolution and classification of P-loop kinases and related proteins. J. Mol. Biol. **333:**781–815.
31. **Lockhart, S. R., C. Pujol, K. J. Daniels, M. G. Miller, A. D. Johnson, M. A. Pfaller, and D. R. Soll.** 2002. In Candida albicans, white-opaque switchers are homozygous for mating type. Genetics **162:**737–745.
32. **Lockhart, S. R., R. Zhao, K. J. Daniels, and D. R. Soll.** 2003. Alpha-pheromone-induced "shmooing" and gene regulation require white-opaque switching during *Candida albicans* mating. Eukaryot. Cell **2:**847–855.
33. **Luo, G., L. P. Samaranayake, and J. Y. Yau.** 2001. *Candida* species exhibit differential in vitro hemolytic activities. J. Clin. Microbiol. **39:**2971–2974.
34. **Magee, B. B., M. Legrand, A. M. Alarco, M. Raymond, and P. T. Magee.** 2002. Many of the genes required for mating in Saccharomyces cerevisiae are also required for mating in Candida albicans. Mol. Microbiol. **46:**1345–1351.
35. **Magee, B. B., and P. T. Magee.** 2000. Induction of mating in Candida albicans by construction of MTLa and MTLα strains. Science **289:**310–313.
36. **Miller, M. G., and A. D. Johnson.** 2002. White-opaque switching in Candida albicans is controlled by mating-type locus homeodomain proteins and allows efficient mating. Cell **110:**293–302.
37. **Morgenstern, B.** 1999. DIALIGN 2: improvement of the segment-to-segment approach to multiple sequence alignment. Bioinformatics **15:**211–218.
38. **Morrow, B., T. Srikantha, J. Anderson, and D. R. Soll.** 1993. Coordinate regulation of two opaque-phase-specific genes during white-opaque switching in *Candida albicans*. Infect. Immun. **61:**1823–1828.
39. **Odds, F. C.** 1988. Candida and candidosis, 2nd ed. Bailliere Tindall, London, England.
40. **Pendrak, M. L., M. P. Chao, S. S. Yan, and D. D. Roberts.** 2004. Heme oxygenase in Candida albicans is regulated by hemoglobin and is necessary for metabolism of exogenous heme and hemoglobin to alpha-biliverdin. J. Biol. Chem. **279:**3426–3433.
41. **Pendrak, M. L., H. C. Krutzsch, and D. D. Roberts.** 2000. Structural requirements for hemoglobin to induce fibronectin receptor expression in Candida albicans. Biochemistry **39:**16110–16118.
42. **Pendrak, M. L., S. S. Yan, and D. D. Roberts.** Sensing the host environment: recognition of hemoglobin by the pathogenic yeast Candida albicans. Arch. Biochem. Biophys., in press.
43. **Peng, W. T., M. D. Robinson, S. Mnaimneh, N. J. Krogan, G. Cagney, Q. Morris, A. P. Davierwala, J. Grigull, X. Yang, W. Zhang, N. Mitsakakis, O. W. Ryan, N. Datta, V. Jojic, C. Pal, V. Canadien, D. Richards, B. Beattie, L. F. Wu, S. J. Altschuler, S. Roweis, B. J. Frey, A. Emili, J. F. Greenblatt, and T. R. Hughes.** 2003. A panoramic view of yeast noncoding RNA processing. Cell **113:**919–933.
44. **Raymond, M., D. Dignard, A. M. Alarco, N. Mainville, B. B. Magee, and D. Y. Thomas.** 1998. A Ste6p/P-glycoprotein homologue from the asexual yeast Candida albicans transports the a-factor mating pheromone in Saccharomyces cerevisiae. Mol. Microbiol. **27:**587–598.
45. **Rodrigues, R. G., S. Yan, T. J. Walsh, and D. D. Roberts.** 1998. Hemoglobin differentially induces binding of Candida, Trichosporon, and Saccharomyces species to fibronectin. J. Infect. Dis. **178:**497–502.
46. **Rothstein, R.** 1991. Targeting, disruption, replacement, and allele rescue: integrative transformation in yeast. Methods Enzymol. **194:**281–301.
47. **Rustad, T. R., D. A. Stevens, M. A. Pfaller, and T. C. White.** 2002. Homozygosity at the Candida albicans MTL locus associated with azole resistance. Microbiology **148:**1061–1072.
48. **Sadhu, C., D. Hoekstra, M. J. McEachern, S. I. Reed, and J. B. Hicks.** 1992. A G-protein alpha subunit from asexual *Candida albicans* functions in the mating signal transduction pathway of *Saccharomyces cerevisiae* and is regulated by the a1-alpha 2 repressor. Mol. Cell. Biol. **12:**1977–1985.
49. **Santos, R., N. Buisson, S. Knight, A. Dancis, J.-M. Camadro, and E. Lesuisse.** 2003. Haemin uptake and use as an iron source by Candida albicans: role of CaHMX1-encoded haem oxygenase. Microbiology **149:**579–588.
50. **Sherman, F.** 1991. Getting started with yeast. Methods Enzymol. **191:**3–21.
51. **Slutsky, B., J. Buffo, and D. R. Soll.** 1985. High-frequency switching of colony morphology in Candida albicans. Science **230:**666–669.
52. **Slutsky, B., M. Staebell, J. Anderson, L. Risen, M. Pfaller, and D. R. Soll.** 1987. "White-opaque transition": a second high-frequency switching system in *Candida albicans*. J. Bacteriol. **169:**189–197.
53. **Soll, D. R.** 1992. High-frequency switching in *Candida albicans*. Clin. Microbiol. Rev. **5:**183–203.
54. **Soll, D. R.** 2001. Phenotypic switching, p. 123–142. *In* R. A. Calderone (ed.), Candida and candidiasis. ASM Press, Washington, D.C.
55. **Soll, D. R., S. R. Lockhart, K. J. Daniels, R. Zhao, and D. Wessels.** 2004. Mating-type locus homozygosis, phenotypic switching and mating: a unique sequence of dependencies in Candida albicans. Bioessays **26:**10–20.
56. **Soll, D. R., B. Morrow, and T. Srikantha.** 1993. High-frequency phenotypic switching in Candida albicans. Trends Genet. **9:**61–65.
57. **Song, J. L., and T. C. White.** 2003. RAM2: an essential gene in the prenylation pathway of Candida albicans. Microbiology **149:**249–259.
58. **Srikantha, T., A. Klapach, W. W. Lorenz, L. K. Tsai, L. A. Laughlin, J. A. Gorman, and D. R. Soll.** 1996. The sea pansy *Renilla reniformis* luciferase serves as a sensitive bioluminescent reporter for differential gene expression in *Candida albicans*. J. Bacteriol. **178:**121–129.
59. **Srikantha, T., and D. R. Soll.** 1993. A white-specific gene in the white-opaque switching system of Candida albicans. Gene **131:**53–60.
60. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
61. **Tsong, A. E., M. G. Miller, R. M. Raisner, and A. D. Johnson.** 2003. Evolution of a combinatorial transcriptional circuit: a case study in yeasts. Cell **115:**389–399.

62. **Walther, A., and J. Wendland.** 2003. An improved transformation protocol for the human fungal pathogen Candida albicans. Curr. Genet. **42:**339–343.
63. **Wilson, R. B., D. Davis, and A. P. Mitchell.** 1999. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. J. Bacteriol. **181:**1868–1874.
64. **Yan, S., E. Negre, J. A. Cashel, N. Guo, C. A. Lyman, T. J. Walsh, and D. D. Roberts.** 1996. Specific induction of fibronectin binding activity by hemoglobin in *Candida albicans* grown in defined media. Infect. Immun. **64:**2930–2935.
65. **Yan, S., R. G. Rodrigues, D. Cahn-Hidalgo, T. J. Walsh, and D. D. Roberts.** 1998. Hemoglobin induces binding of several extracellular matrix proteins to Candida albicans. Identification of a common receptor for fibronectin, fibrinogen, and laminin. J. Biol. Chem. **273:**5638–5644.