The Histone Deacetylase Genes \textit{HDA1} and \textit{RPD3} Play Distinct Roles in Regulation of High-Frequency Phenotypic Switching in \textit{Candida albicans}

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Five histone deacetylase genes (\textit{HDA1}, \textit{RPD3}, \textit{HOS1}, \textit{HOS2}, and \textit{HOS3}) have been cloned from \textit{Candida albicans} and characterized. Sequence analysis and comparison with 17 additional deacetylases resulted in a phylogenetic tree composed of three major groups. Transcription of the deacetylases \textit{HDA1} and \textit{RPD3} is down-regulated in the opaque phase of the white-opaque transition in strain WO-1. \textit{HOS3} is selectively transcribed as a 2.5-kb transcript in the white phase and as a less-abundant 2.3-kb transcript in the opaque phase. \textit{HDA1} and \textit{RPD3} were independently deleted in strain WO-1, and both switching between the white and opaque phases and the downstream regulation of phase-specific genes were analyzed. Deletion of \textit{HDA1} resulted in an increase in the frequency of switching from the white phase to the opaque phase, but had no effect on the frequency of switching from the opaque phase to the white phase. Deletion of \textit{RPD3} resulted in an increase in the frequency of switching in both directions. Deletion of \textit{HDA1} resulted in reduced white-phase-specific expression of the \textit{EFG1} 3.2-kb transcript, but had no significant effect on white-phase-specific expression of \textit{WHI1} or opaque-phase-specific expression of \textit{OP4}, \textit{SAP1}, and \textit{SAP3}. Deletion of \textit{RPD3} resulted in reduced opaque-phase-specific expression of \textit{OP4}, \textit{SAP1}, and \textit{SAP3} and a slight reduction of white-phase-specific expression of \textit{WHI1} and 3.2-kb \textit{EFG1}. Deletion of neither \textit{HDA1} nor \textit{RPD3} affected the high level of white-phase expression and the low level of opaque-phase expression of the MADS box protein gene \textit{MCM1}, which has been implicated in the regulation of opaque-phase-specific gene expression. In addition, there was no effect on the phase-regulated levels of expression of the other deacetylase genes. These results demonstrate that the two deacetylase genes \textit{HDA1} and \textit{RPD3} play distinct roles in the suppression of switching, that the two play distinct and selective roles in the regulation of phase-specific genes, and that the deacetylases are in turn regulated by switching.

Most strains of \textit{Candida albicans} and related species switch, or can be induced to switch, between two or more general phenotypes distinguishable by colony morphology (14, 41, 42, 44, 48). Switching regulates a number of phenotypic characteristics, including putative virulence traits, and for that reason, it has been considered a higher-order virulence factor (42). Most strains of \textit{C. albicans} and related species switch, or can be induced to switch, between two or more general phenotypes distinguishable by colony morphology (14, 41, 42, 44, 48). Switching regulates a number of phenotypic characteristics, including putative virulence traits, and for that reason, it has been considered a higher-order virulence factor (42). Since switching occurs spontaneously and reversibly at relatively high frequencies (10^{-8} to >10^{-2}), it had been suggested (37, 44) that it either represents a spontaneous and reversible reorganization of DNA at a master switch locus, which occurs in yeast when genes are placed at sites adjacent to subtelomeric regions (17, 36). Because the “silent information regulator” gene \textit{SIR2} plays a role in gene repression in the latter mechanism, it was suggested that a \textit{C. albicans} homolog of \textit{SIR2} may play a role in the regulation of switching at a master switch locus in \textit{C. albicans} (37, 44). Perez-Martín et al. (35) subsequently demonstrated that deletion of a \textit{C. albicans} \textit{SIR2} homolog in strain CAI4 did in fact result in up-regulation of the switching system in that strain, which is analogous to the first switching system described in \textit{C. albicans} strain 3153A (41, 54). In addition to the “silent information regulator” (Sir) proteins, other classes of proteins are involved in the repres-
sion or silencing of developmentally regulated genes in eukaryotes. One such class of genes, the histone deacetylases, has been demonstrated to regulate chromatin structure through selective histone deacetylation, which in turn affects chromatin folding and interactions between DNA and DNA-binding proteins (2, 63). The role of the deacetylases in gene regulation has been demonstrated by generating mutants with loss of function or with dominant-negative effects in both Saccharomyces cerevisiae (25, 39, 58) and human cell lines (18, 20).

Recently, we tested whether the specific deacetylase inhibitor trichostatin A (10, 65) affected the white-opaque transition (26). We found that the inhibitor caused a selective increase in the frequency of switching in the white-to-opaque transition, but had no effect on the frequency of switching in the opaque-to-white transition, suggesting that deacetylation through a trichostatin-sensitive deacetylase selectively suppresses switching in one direction (26). Since the deacetylase Hda1p is highly sensitive to trichostatin A (7), we deleted the gene and obtained a mutant phenotype similar to that of trichostatin A-treated cells, supporting the conclusion that deacetylation through Hda1p suppresses switching selectively in the white-to-opaque direction (26). In addition to playing a role in switching, deacetylation may also play a role in the regulation of phase-specific gene transcription. We therefore cloned five of the major C. albicans histone deacetylase genes with homology to the genes that encode the five known histone deacetylases in S. cerevisiae (HDA1, RPD3, HOS1, HOS2, and HOS3) and analyzed their deduced protein sequences and expression patterns in the white-opaque transition. We have also generated, in addition to the HDA1 deletion mutant, a deletion mutant of RPD3. We present evidence that the expression of the deacetylase genes is affected by switching and that, while HDA1 plays a selective role in suppressing the basic switch from white to opaque, but not from opaque to white, RPD3 plays a role in suppressing the basic switch events in both directions. In addition, both HDA1 and RPD3 play indirect but distinct roles in regulating the levels of expression of select phase-specific genes.

MATERIALS AND METHODS

Maintenance of stock cultures. C. albicans strain WO-1 (42) was periodically cloned from a frozen stock culture on agar containing modified Lee’s medium (4). Strains TS3.3, a ura3 auxotroph (55), and TU17, a URA3 prototrophic derivative of TS3.3, were maintained on agar containing modified Lee’s medium with or without 0.01 mM uridine, respectively. Both the HDA1 and RPD3 homozygous mutant strains were maintained on agar containing modified Lee’s medium. The genotypes of all strains used in this study are described in Table 1.

Cloning the deacetylase genes HDA1 and RPD3. Based on short DNA sequence reports in the Stanford C. albicans genome database (www-sequence.stanford.edu), two sets of primers, DeACT5/DeACT5’ and RPD5/RPD5’ (Table 2), were designed to amplify by PCR DNA fragments containing HDA1 and RPD3 sequences, respectively, with genomic DNA of strain WO-1 as a template and with Taq polymerase. The PCR-derived fragments were then used to screen a YEMBL3A genomic library of C. albicans strain WO-1 (53). Of approximately 100,000 plaques screened for the HDA1 fragment or for the RPD3 fragment, 30 to 40 putative clones were identified. Ten putative clones of each gene were purified by a secondary screen and analyzed by Southern blot hybridization with the respective probe to assess purity. Based on these preliminary results, AD3.1 and ARP2.1 were chosen to represent CaHDA1 and CaRPD3, respectively.

To characterize HDA1, AD3.1 was digested with SalI, and a 2.1-kb fragment that hybridized with the respective probe was subcloned into pGEMZ to generate pDA14.1. The SaII DNA fragment was sequenced in both directions with an ABI model 373A automatic sequencing system and fluorescent Big Dye termination chemistry (PE-ABI Inc., Foster City, Calif.). In addition, approximately 1 kb of the 5’ upstream sequence and 0.5 kb of the 3’ downstream sequence of the SaII fragment were determined with AD3.1 as a template. To characterize RPD3, a 4.4-kb sequence of ARP2.1 was directly sequenced with the same protocol applied to PDA14.1. Then, by using the specific primer pair FANNRPS/ FANRPS’ (Table 2), the full-length RPD3 open reading frame (ORF) was amplified by PCR and subcloned into pGEMZ between end-repaired SalI sites to generate gRP615.

Cloning the deacetylase genes HOS1, HOS2, and HOS3. Homologs to S. cerevisiae HOS1, HOS2, and HOS3 were identified in the Stanford C. albicans genome database and then isolated by PCR with the 5’ and 3’ primers HOS1-5’/HOS1-3’, HOS2-5’/HOS2-3’, and HOS3-5’/HOS3-3’, respectively (Table 2), and with strain WO-1 DNA as a template. The HOS1, HOS2, and HOS3 DNA fragments encompassing the entire ORFs were subcloned into pGEM-T easy vector to derive pC55.1, pC56.2, and pC58.14, respectively. Approximately 500 to 600 bp of both the 5’ and 3’ ends of each of the three ORFs were initially analyzed to confirm the identity of each deacetylase gene.

Analysis of protein sequences and construction of a phylogenetic tree. Alignment of multiple protein sequences was performed with Clustal W/Jalview alignment editor, version 4.0 (60). Pairwise comparisons between protein sequences were performed with the PROTDIST program of the PHYLIP package, version 3.57C (http://evolution.genetics.washington.edu/Phylip.html). The unrooted dendrograms were generated by using the FITCH program of the PHYLIP package (the Fitch-Margoliash least-squares distance method) (11).

The data set was subjected to a bootstrap analysis (1,000 replicates) by sequential phylogeny inference, version 3.57C (http://evolution.genetics.washington.edu/Phylip.html). The unrooted dendrograms were generated by using the FITCH program of the PHYLIP package (the Fitch-Margoliash least-squares distance method) (11). The data set was subjected to a bootstrap analysis (1,000 replicates) by sequential use of the SECOBOOT, PROTDIST, and Consensus programs. Genetic distances were derived by the Dayhoff PAM matrix algorithm (8).

Construction of homologous HDA1 deletion mutants. Two different deletion cassettes were constructed, each spanning the essential deacetylation motifs. To generate the heterozygote, a chloramphenicol acetyltransferase (CAT)-URA3-CAT-based cassette (55) was constructed. The pDA14.1 plasmid containing the 2.1-kb SaII fragment of HDA1 (Fig. 1A) was digested with BstII, deleting 1,147 bp of the HDA1 ORF, end repaired with T4 DNA polymerase, and dephosphorylated with shrimp alkaline phosphatase (SAP). The linearized vector was purified by Triisoharose-EDTA (TBE)-sodium gel electrophoresis and ligated with the 3.5-kb CAT-URA3-CAT cassette from the plasmid pUC5 (55). The CAT-URA3-CAT cassette was isolated by digesting pCUC with BamHI, followed by end repair with T4 DNA polymerase. The resulting plasmid, pA48.1, contained the deleted version of HDA1 (Fig. 1A). pA48.1 was digested with SalI, and 25 μg was

### Table 1. Genotypes of strains used in this study

| Strain    | Parent | Relevant genotype | Source or reference |
|-----------|--------|-------------------|---------------------|
| WO-1      |        | Wild type         | 41                  |
| TS3.3     | Red3/6 | ade2::ade2, Δura3::ADE2/Δura3::ADE2 | 54                  |
| HDhe20    | TS3.3  | ade2::ade2, Δura3::ADE2/Δura3::Δade2, Δhda1::CAT-URA3-CAT/HDA1 | This study            |
| HDheF21   | HDhe20 | ade2::ade2, Δura3::ADE2/Δura3::Δade2, Δhda1::CAT/HDA1 | This study            |
| HDho15    | HDheF21| ade2::ade2, Δura3::ADE2/Δura3::Δade2, Δhda1::CAT::URA3::hisG | This study            |
| HDho19    | RDhe2  | ade2::ade2, Δura3::ADE2/Δura3::Δade2, Δhda1::CAT::URA3::hisG | This study            |
| RPh2      | TS3.3  | ade2::ade2, Δura3::ADE2/Δura3::Δade2, Δhda1::CAT::URA3::hisG | This study            |
| RPhF4     | RPh2   | ade2::ade2, Δura3::ADE2/Δura3::Δade2, Δhda1::CAT::URA3::hisG | This study            |
| RPho19    | RPhF4  | ade2::ade2, Δura3::ADE2/Δura3::Δade2, Δhda1::CAT::URA3::hisG | This study            |
| RPho3     | RPhF4  | ade2::ade2, Δura3::ADE2/Δura3::Δade2, Δhda1::CAT::URA3::hisG | This study            |
| TU17      | TS3.3  | ade2::ade2, URA3/Δura3::ADE2 | This study            |
used to transform the ura3− strain TS3.3 (Table 1). Recovered transformants were tested for heterozygosity by digestion of genomic DNA with Xhol and analyzed by Southern blot hybridization with the HD1A1 ORF. One confirmed heterozygous clone was subjected to 5-fluoroorotic acid (5-FOA) treatment in order to induce “pop-outs” of the URA3 gene. To generate the homozygote, a hisG-URA3-hisG-based cassette of the URA3 gene was constructed. The BglII deletion fragment of plasmid pDA1I was isolated, end repaired with T4 DNA polymerase, dephosphorylated with SAP, and subcloned at the EcoRV site of pGEM5Z to generate pC25.1 (Fig. 1A). pC25.1 was digested with EcoRV and BglII to delete 702 bp of DNA, end repaired with T4 DNA polymerase, dephosphorylated with SAP, and ligated with the hisG-URA3-hisG cassette from plasmid pMB8 (55). The hisG-URA3-hisG cassette was prepared by digesting pMB8 with SalI and BglII and end repaired with T4 DNA polymerase. The resultant disruption cassette, pC88.10 (Fig. 1A), was then digested with AplI and SacI. This DNA fragment could only target the functional HD1A1 allele. It was used to transform the selected heterozygous ura3− strain. Transformants, obtained on selection plates, were tested for homozygosity by Southern analysis as described earlier.

Construction of homozygous RPD3 deletion mutants. The hisG-URA3-hisG cassette was used to delete both alleles of RPD3. To construct the disruption cassette, pGRP16/5 was digested with SacI and BglII to delete 697 bp. The 4.0-kb hisG-URA3-hisG cassette was then inserted in its place to generate the plasmid pC7a.4 (Fig. 1B). The RPD3 disruption cassette was prepared by digesting pC7a.4 with AplI and Ncol and used in both the first and second rounds of transformation to obtain first the heterozygous strain and second the homozygous strain. Prior to the second round of transformation, the selected heterozygous clone was subjected to 5-FOA “pop-out” protocol to derive ura3 auxotroph. The heterozygous and homozygous strains were confirmed by probing Southern blots of PstI-digested DNA with the RPD3 ORF.

Northern and Southern blot analyses. Northern analyses were performed according to methods previously described (54, 55). To ensure that the growth conditions for the compared cell preparations were similar, cells of each cell type were removed from agar cultures and grown to the stationary phase in liquid culture. White-phase cells were then diluted to 106 and opaque-phase cells were diluted to 5 × 105 in fresh growth medium and grown to mid-log phase (9 × 106 and 5 × 105 cells per ml, respectively) prior to harvesting. Total RNA was extracted with the RNeasy mini kit according to manufacturer’s specifications (Qiagen, Inc., Santa Clarita, Calif.). The fold differences in transcript levels were measured according to methods recently described (55). The hybridization probes for WH1, SAP1, EF1G1, and OP1 contained the full-length ORFs, derived either by PCR with specific primers (Table 2) or by digesting them from the respective plasmids containing the cognate DNA inserts (32, 33, 53, 55). The probe for SAP3 was derived by PCR with the proximal activation sequence pAS3 as a template and the specific primers SAP3-5′ and SAP3-3′ (Table 2). Probes for HD1A1 encompassed either the full-length ORF or the 702-bp EcoRV-BglII deletion fragment from pC25.1, as described earlier. Probes for RPD3 encompassed either the full-length ORF or the 697-bp SacI-BglII deletion fragment from pGRP16/5, as described earlier. The probes for HOS1, HOS2, HOS3, HOS4, HOS5, HOS6, and TUP1 encompassed the full-length ORFs obtained by PCR with the primer pairs previously described (32, 52, 55). Specific restriction enzymes and probes are described in Results.

RESULTS

Cloning and characterization of the five histone deacetylase genes. A search of the C. albicans genome database for homology at the amino acid level initially revealed five genes homologous to each of the S. cerevisiae histone deacetylase genes HD1A1, RPD3, HOS1, HOS2, and HOS3. HD1A1 and RPD3 were cloned by probing a lambda genomic library of strain WO-1 (42) with PCR-generated DNA fragments based on nucleotide sequences reported in the Stanford C. albicans genome database. The three HOS genes were amplified from WO-1 genomic DNA by PCR with primers based on reported nucleotide sequences. The cloned HD1A1, RPD3, HOS1, HOS2, and HOS3 homologs contained ORFs encoding proteins with sizes of 653, 478, 392, 454, and 713 amino acids, respectively (Fig. 2).

To test whether the five C. albicans histone deacetylase proteins represented distinct members of the histone deacetylase superfamily, alignment of these proteins and 12 additional fungal histone deacetylases was performed with the Clustal W multiple alignment protocol described in Materials and Methods. The alignment of approximately 330 amino acids included the nine sequence blocks that identify histone deacetylase subtypes (29). The phylogenetic analysis (Fig. 3) revealed that the

**TABLE 2. Primers used in this study**

| Primer                          | Sequence                                      |
|--------------------------------|-----------------------------------------------|
| DeACTS5                        | 5′-GAT TGG ATC AGC AAT ATT TAC C-3′           |
| DeACTS3                        | 5′-GAT GTG ATT GGA GCT TGT CA-3′             |
| FANHDA5                        | 5′-GCG TCG GGA ATG TCG ACT GGT CAA GGA GAA-3′|
| FANHDA3                        | 5′-GCC CCG TAA GTA GCG ATT CCA GAT-3′        |
| RPD5                           | 5′-TCA TCA TAG AAC TCT CCA TCA-3′            |
| RPD3                           | 5′-AAG TGG TGA TCG ATT AGG ACC-3′            |
| FANRPDS5                       | 5′-ATT GGG CCC ATG TAT CAA GAA TTA CCA TTT G-3′ |
| FANRPD3                        | 5′-ATT GGG CCC CAT ATT ATT TAA TTT ATC-3′    |
| HOS4                           | 5′-CCT GCG CCC ATG GCA AAG AGA-3′            |
| HOS3                           | 5′-ATA GGG CCC TAG AGA CAA TAA ATG-3′        |
| HOS2                           | 5′-CCT GGG CCC ATG ACA CTG ATC A3′            |
| HOS1                           | 5′-ATA GGG CCC AGT CAT TAG TTC TCC-3′         |
| HOS3                           | 5′-CCT GGG CCC ATG TCG ACT TCA AAA-3′        |
| HOS3                           | 5′-ATA GGG CCC CAT ATT GGA TAC CAT CTT-3′    |
| SAP5                           | 5′-CCT TCT CTA AAA TTA TTA TGA AT-3′         |
| SAP3                           | 5′-TGA ATT TCA CCT TGG GGA CC-3′             |
| OPANDELIF                      | 5′-GG CAT ATG AAG TTT TCA CAA GCC-3′         |
| OPISPHER                       | 5′-GGT GGT TGC TCC TCC GCA CTA ATA AAG TTT TCT TTT-3′ |
| FANENFG15                      | 5′-GCG TCG CCA ATG TCA ACG TCT CCT CAT A3′   |
| FANENFG13                      | 5′-GCG CCG CCG TTT TCC TTC TTT GAC AGT CGT-3′ |
| RL3                           | 5′-TAG AAC CAT TGA CAG ACG-3′                |
| RL2                           | 5′-GGT GAG GAG TAT CAT CAT CCG-3′            |
| McMORF5                        | 5′-GAA TTA TCA GTA TTA GCA AGT ACT CAA GGT-3′|
| CMORF3                         | 5′-AAT GTG ATG AGC ATG ACC TGC TGC AGC ACC-3′|
| Tuppamf                        | 5′-TAA CGG ATC CCT ATG TCC ATG TAT CCA A3′   |
| Tuppstr                        | 5′-CAA CGG CAC ACA TAT ATA TAT ACT ACA CAC TTA-3′ |
five *C. albicans* deacetylases grouped into three distinct classes: the Hda1 class (*C. albicans* Hda1p), the Rpd3 class (*C. albicans* Rpd3p, HOS1p, and HOS2p), and the Hos3 class (*C. albicans* HOS3p). In every class, the *C. albicans* deacetylases were most similar to the corresponding *S. cerevisiae* deacetylase (Fig. 3).

Further comparison of the five *C. albicans* histone deacetylase proteins with *S. cerevisiae* Hda1p and ScRpd3p revealed that a 200-amino-acid stretch in the middle of all seven contained highly conserved amino acid residues (Fig. 2). In particular, a highly conserved functional deacetylation motif of 53 to 63 amino acid residues resided in the middle of this stretch (see box in Fig. 2). Pairwise comparison of the seven proteins revealed four highly conserved histidines (presented in boldface in Fig. 2) organized in pairs and separated by roughly the same number of amino acids (5, 10, 18, 26, 65). Comparison of the deacetylation motifs of the five *C. albicans* deacetylases and 17 additional deacetylases revealed 12 identical amino acid residues and overall a high level of conservation (data not shown).

Expression of the deacetylase genes in the white-opaque transition. Northern analysis revealed that expression of the deacetylases differed between white- and opaque-phase cells. The transcript levels of *HDA1* and *RPD3* were significantly lower in the opaque phase (Fig. 4). The transcript levels of *HOS1* and *HOS2* were slightly lower in the opaque phase (Fig. 4). In the case of *HOS3*, both the levels and the molecular size of the transcripts differed between the two phases. In the white phase, the molecular size of the transcript was 2.5 kb, and in the opaque phase, the molecular size of the less-abundant transcript was 2.3 kb. Northern analysis with two independent RNA samples revealed similar results.

Creating deletion mutants of *HDA1* and *RPD3*. To create deletion mutants of *HDA1* and *RPD3*, we employed the “ura-blast” gene knockout strategy (12) in strain WO-1 (42). To create the *HDA1* deletion mutant, the *ura3* strain TS3.3 was first transformed with the CAT-URA3-CAT-based *HDA1* disruption cassette from pA48.1 (Fig. 1A). Of 40 transformants, 3 proved to be heterozygous by Southern analysis with the full-
length HDA1 ORF and the EcoRV-BglII deletion fragment of the probe. The Southern blot of the ura3 derivative strain TS3.3 contained two bands at 9.8 and 3.6 kb, representing the "large" (L) and "small" (S) alleles of HDA1 (Fig. 5A). The ura3 heterozygote HdheF21 was transformed with the hisG-URA3-hisG-based HDA1 deletion cassette from pC88.10 (Fig. 1A). In one transformation experiment, 30 transformants were obtained, of which 1 proved to be homozygous, and in a second transformation experiment, 40 transformants were obtained, 7 of which proved to be homozygous.

FIG. 2. Sequence comparison of the five C. albicans histone deacetylases with S. cerevisiae Hda1p and Rpd3p. The sequences were aligned with the Clustal W multiple alignment editor (60). The rectangular unshaded box in the center represents the highly conserved deacetylation motif. The gray shaded area, including the unshaded rectangular box, contains amino acid residues conserved to various degrees in the deacetylases. The identical residues among all deacetylases are denoted by stars, while conservative replacements of amino acid residues are denoted by either two stacked solid circles (based on similar functional groups) or one solid circle (based on similar effects on secondary structure). Conserved histidines are presented in boldface. The accession numbers for HDA1 and RPD3 in GenBank are AF377894 and AF377895, respectively. Other accession numbers are as follows: ScHda1p, Saccharomyces cerevisiae Z71297; SCRpd3p, S. cerevisiae P32561. Ca, Candida albicans.
homozygous transformant HDho15 was selected from the first transformation, and HDho11 and HDho19 were selected from the second. When Southern blots were probed with the deleted HDA1 fragment, the parental strain TS3.3 exhibited the two allelic bands at 9.8 and 3.6 kb, and the homozygous strain HDho15 exhibited neither band, confirming that both alleles of HDA1 were deleted in HDho15 (Fig. 5A). A similar analysis proved that both alleles of HDA1 were deleted in HDho19 and HDho11 (data not shown).

To create the RPD3 deletion mutant, one ura3 cassette based on hisG-URA3-hisG was used to knock out both alleles. TS3.3 was transformed with the disruption cassette from pC7a.4 (Fig. 1B). Of 60 transformants, 44 (73%) proved to be heterozygous for RPD3. When Southern blots of PstI-digested TS3.3 DNA were probed with the RPD3 ORF, a single band was identified at 5.8 kb (Fig. 5B). When the URA4 heterozygote strain RPheF4 was retransformed with the pC7a.4 cassette, 40 transformants were obtained. Six proved to be homozygous disruptants. Two transformants, RPho19 and RPho3, were chosen for further analysis. The 5.8-kb band was missing in Southern blots of RPho19 (Fig. 5B) and RPho3 (data not shown) probed with the RPD3 ORF. Both contained the 6.2- and 9.1-kb molecular size bands. When probed with the deleted RPD3 fragment, the parent exhibited a 5.8-kb band (Fig. 5B), while RPho19 (Fig. 5B) and RPho3 (data not shown) exhibited no bands, confirming that both alleles of RPD3 were deleted in the latter strains.

Effects of HDA1 deletion on switching and phase-specific gene expression. We recently demonstrated that treatment with the deacetylase inhibitor trichostatin A (TSA) or deletion of the most TSA-sensitive gene, HDA1, had a selective effect on switching (26). While both TSA-treated cells and cells of the two independent mutant strains HDho15 and HDho19 switched from the opaque to the white phenotype at frequencies comparable to that of untreated wild-type cells (~10^{-3}), both TSA-treated and mutant cells switched from the white- to the opaque-phase phenotype at frequencies more than an order of magnitude greater than that of wild-type cells (~3 × 10^{-3}) (26). These results demonstrated that although the deletion of HDA1 selectively increased the frequency of switching in the white-to-opaque direction, it had no effect on the unique signature morphology of opaque-phase cells.

To assess the effects of deleting HDA1 on phase-specific gene expression, Northern blots of total cellular RNA of white- and opaque-phase cells of the parent strain TU17 and the

FIG. 3. Phylogenetic analysis of fungal histone deacetylases. The protein sequences of 15 different histone deacetylases from various fungi were aligned by using the Clustal W/Jalview multiple alignment editor, version 4, and subjected to phylogenetic analysis with PHYLIP software, version 3.57. The data set was bootstrapped, and the genetic distances were derived by the Dayhoff PAM matrix algorithm (8). The results of the bootstrap analysis (1,000 replicates) are shown either above or below the branches. All of the bootstrap values were above 77%, suggesting that the nodes are significant and reflect the correct phylogeny. Proteins from different fungal species are indicated by two-letter prefixes: Ca, Candida albicans; An, Aspergillus nidulans; Sc, Saccharomyces cerevisiae; and Sp, Schizosaccharomyces pombe. Accession numbers for the published sequences are as follows: ScHda1p, Z71297; Spcl3, AFO64207; ScHos1p, Z49219; SpPhd1p, BAA23598; AnHos2Ap, AF164342; ScHos2p, X91837; ScRpd3p, P32561; AnRpd3p, AF163862; Spchr6, AFO64206; ScHos3p, 1143503.

FIG. 4. Northern blot analysis of mRNA expression of the five cloned histone deacetylase genes of C. albicans strain WO-1 in the white (Wh) and opaque (Op) phases. Blots were probed with the full-length ORF of each of the five deacetylase genes. The ethidium bromide-stained 18S rRNA band is presented at the bottom of the hybridization patterns to assess loading. Molecular sizes of the bands are presented to the right of each blot. Note that HOS3 is expressed as an abundant 2.5-kb message in the white phase and a far-less-abundant 2.3-kb transcript in the opaque phase.
deletion strains HDho15 (Fig. 6A) and HDho19 (data not shown) were probed with the white-phase-specific genes WH11 (53) and EFG1 (55) and the opaque-phase-specific genes OP4 (32), SAP1 (33), and SAP3 (22, 62). Deletion of HDA1 had little effect on the basic developmental regulation of most of the phase-regulated genes tested (Fig. 6A). It also had no significant effect on the level of expression of WH11 in the white phase or on the level of expression of OP4, SAP1, or SAP3 in the opaque phase (Fig. 6A). Deletion did, however, reduce the level of the EFG1 transcript in the white phase fivefold. Similar results were obtained in a repeat experiment in which RNA was extracted from independent growth cultures of TU17 and both mutant strains HDho15 and HDho19.

When opaque-phase cell cultures are shifted from 25°C to 42°C, they switch en masse to the white phase (32, 38, 42, 53, 55). The average cell commits to the white-phase phenotype between 3 and 4 h, concomitant with the second cell doubling and a switch in phase-specific gene expression. At the time of phenotypic commitment, the expression of WH11 is activated and the capacity to induce OP4 and SAP1 expression by shifting cells from 37°C back to 25°C is lost (32, 42, 53, 55). To test whether HDA1 plays a role in this transition, opaque-phase cells of the control strain TU17 and the homozygous deletion mutant HDho15 were shifted from 25°C to 42°C. After 1 h (prior to phenotypic commitment) and 7 h (after phenotypic commitment), the samples were shifted back to 25°C. After 1 h at 42°C, both TU17 and HDho15 cells contained no detectable OP4 message. A shift to 25°C after 1 h at 42°C induced reexpression of OP4 in both TU17 and HDho15. After 7 h at 42°C, TU17 cells contained no OP4 message, and HDho15 cells contained a negligible level (data not shown). A subsequent shift to 25°C did not reactivate OP4 expression in either strain (data not shown), demonstrating that the normal switch in OP4 regulation had occurred. After 1 h at 42°C, both TU17 and HDho15 cells contained no detectable WH11 transcript, and a subsequent shift to 25°C did not up-regulate WH11 expression in either strain (data not shown). After 7 h at 42°C, both TU17 and HDho15 cells expressed a low level of WH11 transcript, and a shift to 25°C resulted in up-regulation of WH11 in both strains (data not shown), demonstrating that the switch in WH11 regulation had occurred in the mutant. These results demonstrate that deletion of HDA1 has no discernible effect on the basic activation and deactivation of phase-specific genes at the point of phenotypic commitment.

The effect of RPD3 deletion on switching and phase-specific gene expression. Deletion of RPD3 also had an effect on the frequency of switching, but the effect in this case was on switching in both directions. While the frequency of opaque-phase CFU in 5-day-old white-phase colonies of the parental strain TU17 was $4 \times 10^{-4}$, those of the homozygous deletion mutants
TABLE 3. Effects of deletion of putative deacetylase gene RPD3 on frequency of switching in the white-opaque transition

| Initial phenotype of strain | Genotype       | No. of colonies (frequency [CFU]) | Fold increase over TU17 | No. of original-phase colonies with opposite-phase sectors (frequency [CFU]) |
|-----------------------------|----------------|-----------------------------------|-------------------------|---------------------------------------------------------------------|
| White                       |                |                                   |                         |                                                                     |
| TU17                        | RPD3/RPD3      | 4,633                             | 2 (4 x 10^-4)           | 1 (2 x 10^-4)                                                      |
| RPho19                      | rpd3/rpd3      | 2,104                             | 31 (1 x 10^-2)          | 25                                                                  |
| RPho27                      | rpd3/rpd3      | 3,776                             | 56 (2 x 10^-2)          | 50                                                                  |
| RPho19+27                   | rpd3/rpd3      | 5,880                             | 87 (1 x 10^-2)          | 25                                                                  |
| Opaque                      |                |                                   |                         |                                                                     |
| TU17                        | RPD3/RPD3      | 3,466                             | 2 (6 x 10^-4)           | 0 (<3 x 10^-4)                                                     |
| RPho19                      | rpd3/rpd3      | 1,662                             | 139 (5 x 10^-2)         | 83                                                                  |
| RPho27                      | rpd3/rpd3      | 2,398                             | 24 (1 x 10^-2)          | 16                                                                  |
| RPho19+27                   | rpd3/rpd3      | 4,060                             | 163 (3 x 10^-2)         | 50                                                                  |

* Cells from white- or opaque-phase colonies were plated and incubated at 25°C for 5 days, and then the proportions of colony phenotypes (white, opaque, or sectored) were counted.

RPho19 and RPho3 were 1 x 10^-2 and 2 x 10^-2, respectively, representing 25- and 50-fold increases, respectively (Table 3). And while the frequency of white-phase CFU in 5-day-old opaque-phase colonies of the parental strain TU17 was 6 x 10^-4, those of the homozygous deletion mutants RPho19 and RPho3 were 5 x 10^-2 and 10^-2, representing 83- and 17-fold increases, respectively (Table 3). The increases in the frequencies of the transition from white to opaque and opaque to white in RPD3 deletion mutants are evident in cultures in which cells from white and opaque colonies are streaked across nutrient agar (Fig. 7). The same differences were also evident in the frequency of sectored colonies (Table 3). Scanning electron micrographs of white- and opaque-phase cells revealed that the RPD3 deletion had no effect on either the smooth-surfaced, round budding phenotype of white-phase cells or the pimpled, elongate phenotype of opaque-phase cells (data not shown).

To assess the effect of deleting RPD3 on phase-specific gene expression, Northern blots of total cellular RNA of white- and opaque-phase cells of the parent strain TU17 and the deletion mutant RPho19 were probed with the white-phase-specific genes WHI11 (53) and EFG1 (55) and the opaque-phase-specific genes OP4 (32), SAP1 (33), and SAP3 (22, 62). Deletion of RPD3 had no effect on the developmental regulation of any of the phase-specific genes (Fig. 6B). It had a small effect on the transcript levels of the white-phase-specific genes WHI11 and EFG1 and the opaque-phase-specific gene SAP1, all of which were approximately twofold lower (Fig. 6B). Deletion of RPD3 caused 10- to 15-fold decreases in the transcript levels of the two opaque-phase-specific genes OP4 and SAP3 (Fig. 6B). Similar results were obtained in a repeat experiment in which RNA was extracted from independent growth cultures of TU17 and two deletion strains, RPho19 and RPho3 (data not shown).

The expression of MCM1 and TUP1 in HDA1 and RPD3 deletion mutants. The opaque-phase-specific genes OP4 (30) and SAP3 (46; S. Lockhart and D. R. Soll, unpublished observations) contain MADS box protein consensus binding sites associated with the cis-acting activation sequences in their promoters, suggesting that a MADS box protein is involved in the regulation of these opaque-phase-specific genes. The MADS box binding sequences exhibited greatest homology to the MCM1 binding site in S. cerevisiae (30). We therefore cloned MCM1 by PCR using a sequence identified in the Stanford C. albicans database. Homology to the S. cerevisiae MCM1 gene was confirmed by partial sequencing. The cloned sequence was then used to probe Northern blots of the parental strain TU17, the HDA1 deletion mutants HDho15 and HDho19, and the RPD3 deletion mutants RPho3 and RPho19. We first found that the MCM1 transcript was high in white-phase cells and low in opaque-phase cells in parental strain TU17 (Fig. 8A). Neither deletion of HDA1 nor deletion of RPD3 affected the levels of expression in the white or opaque phase (Fig. 8A). The same blot was probed with TUP1, a transcription factor gene (6) expressed in white- and opaque-phase cells, but at slightly lower levels in the latter (R. Zhao, S. R. Lockhart, and D. R. Soll, unpublished data). Neither deletion of HDA1 nor deletion of RPD3 affected levels of expression in the white or opaque phase (Fig. 8A).

Expression of the deacetylases in the HDA1 and RPD3 deletion mutants. The deletion mutants HDA1 and RPD3 showed no hybridization bands with the respective probes, demonstrating that these strains were devoid of the respective deacetylase mRNAs (Fig. 8B). The deletion of HDA1 had no effect on the transcript levels of RPD3 and HOS3 (Fig. 8B) or HOS1 and HOS2 (data not shown) in either the white or opaque phase. Likewise, deletion of RPD3 had no effect on the transcript levels of HDA1 and HOS3 (Fig. 8B) or HOS1 and HOS2 (data not shown).

DISCUSSION

The molecular mechanisms involved in the downstream regulation of phase-specific genes in the process of high-frequency phenotypic switching have begun to emerge in recent years. In the white-opaque transition, which has been used as an experimental model system to study these mechanisms (46, 47), it has been demonstrated that phase-regulated genes are activated through the interaction of phase-specific trans-acting factors with specific cis-acting sequences in the promoters of these genes (30, 51, 55, 56). Because the cis-acting sequences in coordinately activated genes of the same phase differ, the simplest model of regulation involving a common trans-acting DNA-binding protein has been excluded (46), and alternative more complex models have been entertained (46). The emerging complexity of the circuitry that regulates the expression of
phase-specific genes in the white-opaque transition should have been expected, given the complexity of the molecular mechanisms regulating eukaryotic gene expression in general. Indeed, in the regulation of a gene, it has become clear that in addition to activators and repressors, chromatin-modulating machinery is recruited that is involved in establishing transcriptionally active or inactive states (57). Among the classes of proteins involved in chromatin modifications, the deacetylases have been demonstrated to function in the repression of gene loci through the selective deacetylation of histones H3 and H4 (2, 63). To test whether deacetylases also play a role in switching, we initially performed an experiment with the deacetylase inhibitor TSA and found that it caused a dramatic and selective increase in the frequency of switching in the white-to-opaque direction, but had no effect on switching in the opaque-to-white direction (26). Since TSA preferentially inhibits the major deacetylase Hda1p (7), we deleted the gene coding for this protein in C. albicans strain WO-1 and found that the mutant phenotype was similar to that of TSA-treated cells (26). Switching was selectively upregulated in the white-to-opaque direction only, suggesting that deacetylation through Hda1p functioned either at a “master switch locus” to suppress the event or at the site of an activator of the switch event (26). Here we have extended these studies by identifying the members of the deacetylase family in C. albicans, examining the effects of switching on expression, testing whether HDAl plays a role in phase-specific gene expression, and testing whether a second major deacetylase, RPD3, also plays a role in switching and phase-specific gene expression.

The histone deacetylases in C. albicans. We cloned five histone deacetylases from C. albicans with homology to the five deacetylases in S. cerevisiae. By generating phylogenetic trees based on homology comparisons between the amino acid sequences of the five cloned C. albicans deacetylase genes, the five S. cerevisiae deacetylase genes and deacetylase genes from additional fungi, three groups were identified. One contained C. albicans Hda1p; the second contained C. albicans Hos1p, Hos2p, and Rpd3p; and the third contained C. albicans Hos3p. In each group, the deacetylase with the highest homology to each C. albicans deacetylase was the S. cerevisiae homolog. A comparison of the deacetylation motif of 22 deacetylases that included the five C. albicans deacetylases revealed 12 identical amino acid residues and very high levels of homology overall. By using a more rigorous search of the protein database, a sixth C. albicans deacetylase was recently identified in the Stanford C. albicans genome database with homology to Rpd3p (data not shown). We are in the process of characterizing the role of
this new member of the histone deacetylase family by gene deletion and dominant-negative mutation strategies.

Both HDA1 and RPD3 play roles in the switching process. We had previously demonstrated the selective role of HDA1 in suppressing switching in the white-to-opaque direction (26), so we considered the possibility that a second deacetylase might selectively suppress switching in the opaque-to-white direction. We were therefore surprised to find that deletion of RPD3 resulted in an increase in the frequency of switching in both the white-to-opaque and opaque-to-white directions. The observation that deletion of either deacetylase results in an increase in switching in the white-to-opaque direction, but only deletion of RPD3 results in an increase in switching in the opaque-to-white direction, suggests the mechanisms for switching in the two directions are distinct. Other observations support this conclusion. First, an increase in temperature from 25 to >37°C leads to the mass conversion of opaque to white, but has no apparent effect on switching in the white-to-opaque direction (32, 38, 42, 53, 55). A decrease in temperature also selectively stimulates switching in the opaque-to-white direction only (42). Second, Kolotila and Diamond (27) demonstrated that leukocytes and oxidants selectively stimulated switching in the white-to-opaque direction, like TSA treatment and deletion of HDA1 (26). Third, misexpression of the white-phase-specific gene WH11 selectively stimulates switching in the opaque-to-white direction, but not the white-to-opaque direction (28). Finally, UV treatment stimulates switching in both the white-to-opaque direction and the opaque-to-white direction (32), as we have demonstrated here for RPD3 deletion. The effects of these different treatments on the frequencies of switching in the two directions are summarized in Fig. 9. It should be noted that although the characteristics of switching in the alternate directions differ, we cannot rule out the likely possibility that the reversible switch event occurs at a single locus.

Since deacetylases play a role in the repression of gene expression (20, 40, 59, 64), we must first entertain the possibility that Hda1p and Rpd3p suppress switching by removing acetyl groups from histone 3 and/or 4 at the actual site of the switch event. In this case, a switch event would be affected by increased or altered acetylation patterns through one of the many histone acetyltransferases in an activation complex at the
TABLE 4. Summary of the effects of the HDA1 and RPD3 deletions on gene expression during the white-opaque transition

| Gene type       | Expression level* | White phase | Opaque phase |
|-----------------|-------------------|-------------|--------------|
|                 | TU17 | HDho15 | RPho19 | TU17 | HDho15 | RPho19 |
| White-phase specific |     |       |        |       |        |       |
| WH11            | +++++ | +++++ | ++++ | --- | ++++ | ++++ |
| EFG1 (3.2 kb)   | +++++ | +++++ | ++ | - | -- | -- |
| HOS3 (2.5 kb)   | +++++ | +++++ | ++ | - | -- | -- |
| Opal-phase specific |     |       |        |       |        |       |
| OP4             | --- | --- | ++++ | ++++ | ++++ | ++++ |
| SAP1            | --- | --- | ++++ | ++++ | ++++ | ++++ |
| MCM1            | +++++ | +++++ | ++++ | ++ | + | + |
| HDA1            | +++++ | +++++ | --- | + | + | + |
| RPD3            | +++++ | +++++ | ::: | + | + | + |
| HOS1            | +++++ | +++++ | ++++ | ++ | + | + |
| HOS2            | +++++ | +++++ | ++++ | ++ | + | + |
| HOS3 (2.5 kb)   | +++++ | +++++ | ++++ | ++ | + | + |
| HOS3 (2.3 kb)   | --- | --- | ++++ | ++++ | ++++ | ++++ |
| Constitutive |     |       |        |       |        |       |
| TUP1            | +++++ | +++++ | ++++ | ++++ | ++++ | ++++ |

* +++++, full expression; ++++, slightly reduced expression; +++, very reduced expression; +, extremely reduced expression; --, no expression. A down arrow in parentheses represents significant down-regulation.

master switch locus. However, we must also entertain the alternate possibility that one or both histone deacetylases function to suppress one or more trans-activators of switching, which act directly upon the master switch locus. In this case, deletion of the deacetylase leads to up-regulation of the activator genes, and also to the activation of select phase-regulated genes, which act indirectly upon the master switch locus. In the latter case presumably through the down-regulation of silencing genes usually suppressed by the activity of the deacetylases. We assume that the down-regulation of select phase-specific genes in the deacetylase deletion mutants of C. albicans is effected by similar mechanisms.

**Conclusion.** We have presented evidence that the relationship between high-frequency phenotypic switching and the deacetylases is complex. First, both HDA1 and RPD3 play roles in the suppression of switching: the former in the selective suppression of switching in the white-to-opaque direction and the latter in both the white-to-opaque and opaque-to-white directions. The results do not distinguish in either case whether suppression is mediated by deacetylation directly at the site of the switch event or indirectly at the site of a gene that encodes an activator of the switch event. Second, switching affects the expression of deacetylases. For four of the five deacetylases, expression is higher in the white phase. Third, deletion of HDA1 or RPD3 in no case results in up-regulation of a phase-specific or phase-enriched gene, and in select cases, it results in down-regulation. Our results therefore demonstrate that the deacetylases play distinct roles not only in the suppression of switching, but also in the activation of select phase-regulated genes, in the latter case presumably through the down-regulation of suppressor genes.

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