Transcription Profiling of *Candida albicans* Cells Undergoing the Yeast-to-Hyphal Transition□D

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The ability of the pathogenic fungus *Candida albicans* to switch from a yeast to a hyphal morphology in response to external signals is implicated in its pathogenicity. We used glass DNA microarrays to investigate the transcription profiles of 6333 predicted ORFs in cells undergoing this transition and their responses to changes in temperature and culture medium. We have identified several genes whose transcriptional profiles are similar to those of known virulence factors that are modulated by the switch to hyphal growth caused by addition of serum and a 37°C growth temperature. Time course analysis of this transition identified transcripts that are induced before germ tube initiation and shut off later in the developmental process. A strain deleted for the Efg1p and Cph1p transcription factors is defective in hyphae formation, and its response to serum and increased temperature is almost identical to the response of a wild-type strain grown at 37°C in the absence of serum. Thus Efg1p and Cph1p are needed for the activation of the transcriptional program that is induced by the presence of serum.

INTRODUCTION

Candida albicans is an important pathogen, causing the majority of fungal infections in humans. These can range from relatively minor surface infections, such as thrush and vaginal yeast infections, to more serious and life-threatening systemic infections, particularly in immunocompromised individuals. Cancer chemotherapy, tissue transplantation, and HIV infection are generating a growing pool of individuals susceptible to such systemic infections (Corner and Magee, 1997).

Candida is usually a relatively benign commensal of humans, and the ability to become virulent is thus primarily determined by the immune state of the host (Lortholary and Dupont, 1997; Ashman, 1998). However, there are characteristics of *C. albicans* that contribute to its ability to cause disease in susceptible individuals. One of these is the ability to switch from a yeast form of growth to a filamentous form characterized either by pseudohyphae or true hyphae. Morphogenesis appears to be important for pathogenesis, because cells that are trapped in either the yeast (Lo *et al.*, 1997; Rocha *et al.*, 2001) or pseudohyphal states (Braun and Johnson, 1997) are less virulent in murine systemic infection models. Thus, the determinants of the morphological yeast-to-hyphal switch appear important for virulence.

By several approaches, genes have been identified that are expressed exclusively or primarily in the hyphal state. Signal transduction cascades, modulated by elements such as cAMP, mitogen-activated protein kinases, and pH-responsive modules, appear to regulate this yeast-to-hyphal transition (Whiteway, 2000). Transcription factors important in the ability to filament have been identified (for recent reviews, see Ernst, 2000; Liu, 2001). However, the draft sequence of the *C. albicans* genome (Tzung *et al.*, 2001; Scherer, 2002) has now also made the powerful technology of DNA microarrays available to investigate the transcriptional profiles of *C. albicans* cells. Initial efforts to apply this technology have used filters arrays containing 700 (Lane *et al.*, 2001a) or 2002 genes (Murad *et al.*, 2001a). Glass microarrays have also

been used by our group and others to study the response of *C. albicans* to antifungals (De Backer *et al.*, 2001; Cowen *et al.*, 2002). In the current study, we have investigated the behavior of over 6300 genes under a variety of conditions that include two different stimuli that induce a yeast-to-hyphal switch, the individual effects of serum or increased temperature, and the response of transcription factor mutants that are deficient in hyphal development.

MATERIALS AND METHODS

Strains

We used SC5314 (Gillum *et al.*, 1984) as a wild-type strain. Strains containing the $\Delta efg1$ (HLC52) or $\Delta efg1/\Delta cphI$ (HLC54) deletions have already been described (Lo *et al.*, 1997)

Construction of the C. albicans Microarrays

The *C. albicans* genome has been sequenced using a shotgun approach by the Stanford Genome Technology Center to a 7.5-fold redundancy (Version 4; http://www-sequence.stanford.edu/group/candida). We identified a total of 6580 potential open reading frames (ORFs) greater than 250 base pairs and produced a preliminary annotation. Until the publication of a unified nomenclature, we have used the following priority to name *Candida* genes: published genes in GenBank, mapping elements as reported by the University of Minnesota (http://alces.med.umn.edu/candida/), orf6.#### reference numbers as reported by the Stanford Genome Technology Center (http://www-sequence.stanford.edu/group/candida/), or our own Contig4-###_### nomenclature (see Supplementary Material). Some novel genes described in this report have been given a common name and deposited in GenBank. A more comprehensive annotation will be provided by a recently established international consortium. For details on the production of the microarrays, please see our web page (http://www.bri.nrc.ca/microarraylab) or the Supplementary Material. Version 5.2 of the array consists of 6333 amplicons printed in duplicates arranged in 48 subarrays (20×17 spots) including a row of exogenous control spots. Previous versions contained 55, 70, and 85% of the 6580 ORFs.

Growth Media and Conditions

Cultures were grown in Lee's medium (Lee *et al.*, 1975) or in 1% yeast extract, 2% peptone 2% dextrose (YPD)-based medium. Overnight cultures were inoculated from a fresh colony and were grown in YPD (pH 6.0–6.5) at 30°C. These overnight cultures were diluted to an OD_{600} of 0.05–0.1 in YPD or YPD + 10% FBS (fetal bovine serum) from Invitrogen (Carlsbad, CA), which has been previously incubated at 56°C for 30 min) and grown at 30 and 37°C, respectively, to an OD_{600} of 0.6–0.8 (~3 generations). Cultures grown in Lee's medium containing 10% glucose were started from an overnight culture grown in Lee's medium at 25°C. A 10-ml aliquot of this overnight culture was used to inoculate 1 liter of temperature-adjusted medium. Cultures were grown for ~3 generations at either 37°C to induce hyphae or 25°C to maintain yeast growth. For the time course analysis, cells were grown in YPD medium overnight to stationary phase, diluted to OD_{600} 0.05 in separate 500-ml flasks containing 250 ml of fresh YPD, and allowed to grow to OD_{600} 0.4 in a 30°C shaker. Half of the flasks were then inoculated with heat-inactivated FBS to a final concentration of 10% and incubated at 37°C for either 30 or 60 min. Cultures were harvested by filtration (0.45-μm filters, cat. no. schvu10re; Millipore, Bedford, MA) and were quick-frozen in an ethanol/dry-ice bath.

Isolation of RNA

Total RNA was extracted with the hot phenol protocol (Kohrer and Domdey, 1991) with the following minor modifications. The cells

from a 300-1000-ml culture ($OD_{600} = 0.8$) were processed separately in 50-ml tubes and were extracted three times for 10 min. For Lee's medium cultures, glass beads (425–600 μm; Sigma, St. Louis, MO, cat. no. G-8772) were added for the extraction. PolyA(+) mRNA was isolated with the MicroFastTrack 2.0 kit (Invitrogen, cat. no. K1520–03). Quantification was performed by fluorescence with the RiboGreen kit (Molecular Probes, Eugene, OR, cat. no. R-11490) on a CytoFluor 2300 (Millipore).

RNA Labeling

A mixture of 3 μg of polyA(+) mRNA, 1 μl control RNA (2 ng/μl; in vitro transcribed *Arabidopsis thaliana* G4 gene), 1.5 μl oligo(dT)$_{21}$ (100 pmol/μl), 3 μl dNTP-minus dCTP (6.67 mM each), 1 μl dCTP (2 mM), 4 μl DTT (100 mM), 8 μl 5× First Strand Buffer (Invitrogen) and water to a volume of 36 μl was denatured at 65°C for 10 min and cooled to room temperature for 5 min. The reverse transcription reaction was done at 42°C for 2 h after addition of 2 μl of cyanine 3-dCTP (1 mM) or cyanine 5-dCTP (1 mM; Perkin Elmer-Cetus/NEN, Boston MA, cat.. no. NEL999) and 2 μl of SuperScript II (Invitrogen, cat. no. 18064–014 : reverse transcriptase, DTT and 5× First Strand Buffer). The reaction was stopped and RNA degraded by addition of 5 μl EDTA (50 mM, pH 8.0), 2 μl NaOH (10 N) and incubation at 70°C for 10 min. The reaction was neutralized with 4 μl of acetic acid (5 M). Purification was done by isopropanol precipitation (1 volume) at −20°C for 1.5 h, followed by centrifugation (1 h at 12,000 rpm). The pellet was washed twice with cold 70% ethanol or, alternatively, with a Qiagen (Valencia, CA) column.

Hybridization

Solutions were made using standard saline-citrate buffer (1× SSC is 0.15 M NaCl and 0.015 M Na-citrate). Slides were prehybridized at 42°C for at least 1 h, with 50 μl of a solution containing 5× SSC, 0.1% SDS, 50× Denhardt's solution (1% Ficoll, 1% BSA, 1% PVP), and 1.5 μl tRNA (10 mg/ml; Baker's yeast, Roche Applied Science, http://biochem.roche.com, cat. no. 109517) and 1.5 μl of denatured genomic DNA (10 mg/ml; herring testes, Invitrogen, cat. no. s0277). The microarray slides were covered with a 24 × 60-mm glass coverslip (Fisher Scientific, Nepean, ON, Canada, cat. no. 12–545m) during all hybridization steps, and the hybridization chamber was kept at high humidity level with wet pieces of paper towels placed in the lower part of the chamber. Just before the hybridization, the DNA microarray slide was washed twice with 0.1× SSC at room temperature for ~2 min and centrifuged at 800 rpm for 3 min. The DNA microarray slide was kept dry for a minimal amount of time just before hybridization. The hybridization was as follows: the two cDNA targets were resuspended with 10 μl water, pooled together, and mixed with the hybridization buffer to a volume of 50 μl at a final concentration of 25% formamide, 5× SSC, 0.1% SDS containing 1.5 μl tRNA (10 mg/ml) and 1.5 μl denatured genomic DNA (10 mg/ml). This hybridization solution was heat denatured at 95°C for 3 min, cooled to room temperature, and applied onto the DNA microarray slide for overnight hybridization at 42°C. Afterward, slides were completely immersed in a large volume chamber (~250 ml buffer), and the coverslip was carefully removed before washing for 10 min at 42°C with 1× SSC, 0.2% SDS, and for two times 10 min at 37°C with 0.1× SSC, 0.2% SDS, and, finally, rinsing four times at room temperature in 0.1× SSC for ~3 min per rinse. Slides were spin-dried (800 rpm, 8 min) and stored protected from light until scanning.

Data Analysis

The DNA microarray slides were scanned with a ScanArray 5000 scanner (GSI Lumonics, then Packard BioScience, now Perkin Elmer-Cetus, Wellesley, CA; version 2.11) at a 10-μm resolution. The resulting 16-bit TIFF files were quantified with QuantArray software (Perkin Elmer-Cetus; versions 2.0 and 3.0). Quality control

Table 1. Number of replicates per experiment

Number	Cell line, treatment		Presence of elongated cells		No. of microarrays (no. of ORFs per array)			
	Experiment	Reference	Experiment	Reference	3609	4651	5668	6333
C	30°C	30°C	−	−	2	1	6	
1	FBS 37°C 30 min	30°C	−	−			2	4
2	FBS 37°C 60 min	30°C	+/−	−			3	4
3	FBS 37°C 6 h	30°C	+++	−	2	4	4	
4	FBS 37°C 6 h	25°C 6 h	+++	−		2	1	
5	FBS 37°C 6 h	FBS 30°C 6 h	+++	+		3	2	
6	FBS 37°C 6 h	FBS 25°C 6 h	+++	−		2	1	
7	FBS 37°C 6 h	HLC52 FBS 37°C 6 h	+++	+/−			3	2
8	FBS 37°C 6 h	HLC54 FBS 37°C 6 h	+++	−			3	1
9	FBS 37°C 6 h	37°C 6 h	+++	+/−		4	2	
10	Lee's 37°C 24 h	Lee's 25°C	++++	+		1	2	
11	FBS 25°C 6 h	25°C	−	−		2	1	
12	HLC52, 30°C	30°C	−	−			4	2
13	HLC54, 30°C	30°C	−	−			3	2
14	FBS 30°C 6 h	30°C	+	−			3	
15	HLC52 FBS 37°C	HLC52, 30°C	+/−	−			4	
16	HLC54 FBS 37°C	HLC54, 30°C	−	−			4	
17	37°C	25°C	+/−	−		1	2	
18	37°C	30°C	+/−	−		2	1	

Unless noted, experiments were performed on SC5314 (wt). Other strains used were HLC52 ($\Delta egf1$), and HLC54 ($\Delta efg1\Delta cph1$). Culture medium is YPD-based in all experiments except for No. 10. FBS, Addition of 10% fetal bovine serum.

and normalization of the data were performed in Microsoft Excel using standardized spreadsheets. To be included in the normalization and analysis, each spot had to satisfy three quality control criteria: (1) the signal intensity had to be significantly greater than local background (namely, in one of the two color channels, the signal intensity minus half of the SD had to be greater than the local background plus half of the SD); (2) the signal intensity had to be within the dynamic range of the photomultiplier tube as determined by the user with the help of a scatter plot of the $\log_{10}$ of background-substracted intensities; and (3) the raw intensities of the duplicate spots for each gene had to be within 50% of one another. For spots that met these criteria, the ratio of intensity of the two channels was normalized by the median ratio for the entire subarray consisting of 400 spots that had passed quality control. Finally, the $\log_2$ values of the ratios for each duplicate spot were averaged. Statistical analysis and visualization were performed with GeneSpring software (Silicon Genetics, Redwood City, CA). Using the available statistical tools (Student's *t* test of replicate samples showing a variation different from 1), we selected a list of 742 genes that showed a statistically significant ($p < 0.02$) variation of at least 1.5-fold under one of 18 studied conditions. Hierarchical clustering (Eisen *et al.*, 1998) of these 742 genes was done in GeneSpring using their standard conditions. K-means clustering (Calinski and Harabasz, 1974) of genes modulated after 30 min, 60 min, or 6 h of treatment with FBS/37°C was also done in GeneSpring but used the Pearson algorithm in which the shape of the expression profiles are more significant than their amplitude.

RESULTS

DNA Microarrays

The *C. albicans* genome sequence produced by the Stanford Genome Technology Center (release 4.0; http://www-sequence.stanford.edu/group/candida/) was used as the source for the ORFs required for the analysis. The available contigs were scanned by the Magpie sequence analysis software (Gaasterland and Sensen, 1996), and 6580 ORFs larger than 250 base pairs were selected for PCR amplification. Several of the reported experiments were performed while we were developing this technology; thus, hybridizations were performed on slides containing 55, 71, 86, or 91% (6333 ORFs) coverage (see Table 1). Details of the PCR amplification, quality control, and spotting procedures, as well as a preliminary genome annotation are available at our website (http://www.bri.nrc.ca/microarraylab), and figures and the complete dataset are located at http://www.cbr.nrc.ca/genetics/MBC2002/.

The analyzed data included a significant number of biological and technical replicates (n = 3–10, see Table 1). These include nine control hybridization experiments in which we compared the transcription profiles of independent cultures of Candida cells grown in YPD at 30°C. From 18 additional experimental regimes, we selected 742 ORFs that qualified as "significantly modulated" by passing both a statistical (*t* test, $p < 0.02$) and a fold-variation (1.5-fold up or down) cutoff. In the control experiments, only 7 ORFs (0.12%) would have been identified by such stringent criteria. As shown in Figure 1, these results were organized by two-dimensional hierarchical clustering (Eisen *et al.*, 1998). On the X axis, the 742 ORFs were clustered according to the similarity in their expression profiles, with each gene colored according to its change in transcript abundance (downregulated genes in green, upregulated genes in red). In the absence of reliable data, the genes are colored in gray. On the Y axis, the transcriptional responses observed in 18 different experimental regimes were clustered according to the similarities in the resulting transcriptional profiles. A detailed description of each experiment is shown in Table 1. The experiments include an evaluation of the transcriptional
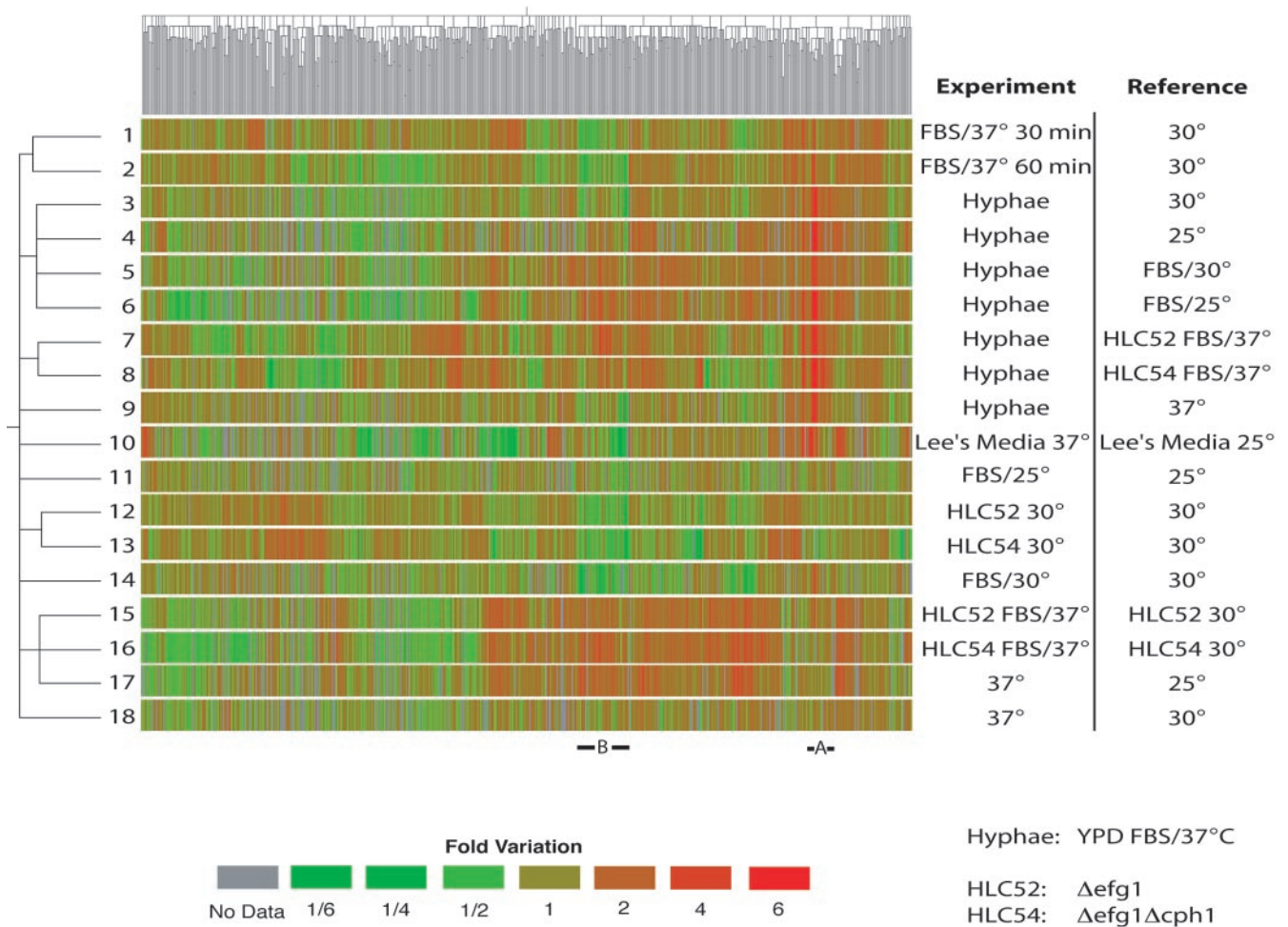
Figure 1. Two-dimensional clustering of gene expression data. The analysis was performed on 742 genes that showed a statistically significant variation in at least one of 18 experiments (see Table 1). Ratios of gene expression obtained by dividing the experimental by the reference samples are represented as a green-to-red color scale. Similarity between gene expression patterns is represented by the horizontal dendrogram. The vertical dendrogram represents the similarity between experiments. Bars and labels (A and B) represent subclusters examined in more detail in Figures 3 and 5.

changes during a time course of yeast-to-hyphae transition induced by serum and high temperature in YPD (lanes 1–3), an alternative hyphal induction model in Lee's Medium (lane 10), the individual effects of serum (lanes 13 and 14) or high temperature (lanes 17 and 18), a comparison of fully developed hyphae with yeast or elongated cells grown under partially inductive conditions (lanes 3–6 and 9), and the responses of cells missing one or two genes encoding transcription factors (lanes 7, 8, 11, 12, 15, and 16). Horizontal and vertical dendrograms are used to represent the similarity between the expression profiles of each gene and experiment, respectively. For example, in experiments 3 to 6, we compared cells with the hyphal morphology with cells with the yeast morphology. The resulting profiles are thus very similar, and the vertical dendrogram shows that these four experiments form a single subcluster. The same will be true

of groups of genes with similar responses to stimuli, two of which (subclusters A and B) will be examined in more detail.

Serum and Elevated Temperature Induction of the Yeast-to-Hyphal Switch

The yeast-to-hyphal transition is induced by transfer of *C. albicans* cells from growth in liquid YPD at 30°C to YPD + 10% serum at 37°C (compare Figure 2, a and d). The transcriptional profile of hyphae formed after 6 h of culturing in serum-containing medium at 37°C was compared with that of yeast control cells. The expression of 18 genes at least doubled upon the formation of hyphae, whereas the expression of an additional 56 genes reproducibly increased by ≥50%. (It should be noted that our use of experimental replicates tends to reduce the amplitude of fold-variations.) In addition, there were 46 genes whose expression was
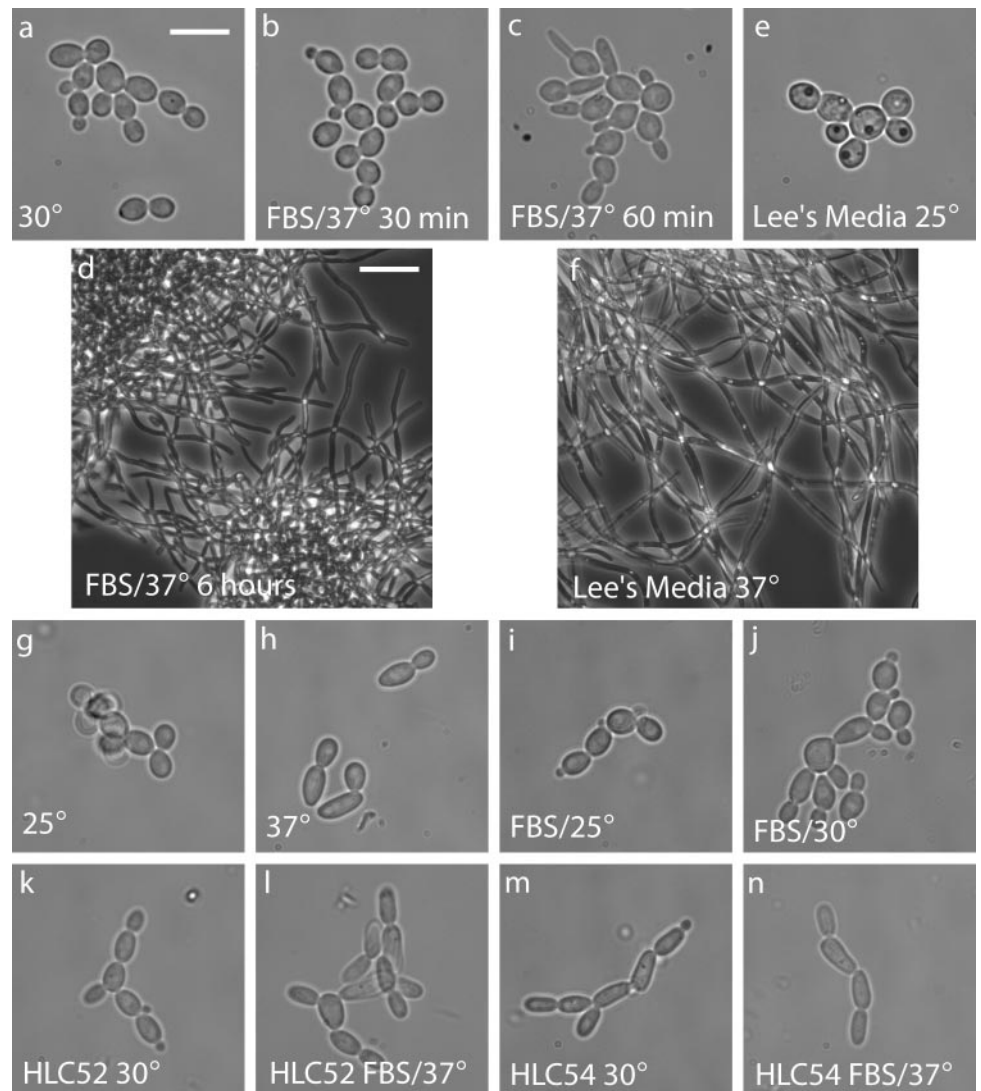
Figure 2. Morphology of *Candida albicans*. Yeast phenotype observed in YPD at 30°C (a). Hyphal development after growth at 37°C in YPD + 10% FBS for 30 min (b), 60 min (c), or 6 h (d). In Lee's medium, cells have a yeast-like morphology at 25°C (e) but develop into hyphae by 24 h after an increase in temperature to 37°C (f). Growth in YPD at 25°C (g) is indistinguishable from growth at 30°C, but an increase to 37°C induces cell elongation (h). Addition of 10% FBS in YPD has fewer effects at reduced temperatures of 25°C (i) or 30°C (j). Appearance of the hyphae-defective strains HLC52 (k and l) and HLC54 (m and n) grown in YPD at 30°C (k and m) or under hyphal-inducing conditions at 37°C in YPD + 10% FBS (l and n). The white bars represent a length of either 20 μm (d and f) or 8 μm (a–c, e, and g–n).

consistently reduced in the hyphal cells compared with the yeast cells. A partial list of these hyphae-modulated genes is presented in Table 2.

Subcluster A, illustrated in Figure 3, contains a large number of genes that were induced upon hyphal development. This section of the cluster is especially rich in genes that are unique to *Candida* (see Supplementary Data, Figure 1S). We identified several genes that had been previously recognized as hyphal-specific through independent analyses of differentially induced genes. *ECE1* (Birse *et al.*, 1993), *SAP4,5,6* (Monod *et al.*, 1994), *RBT1* (Braun *et al.*, 2000), and *HWP1* (Sharkey *et al.*, 1999) are induced fivefold or more in our microarray analysis. Other previously characterized hyphal-induced genes detected in this analysis include *DDR48* (Lane *et al.*, 2001a), *PHR1* (Porta *et al.*, 1999), and *RBT4* (Braun *et al.*, 2000). Results from some known hyphal-specific genes such as *HYR1* (Bailey *et al.*, 1996) and *ALS3* (Hoyer *et al.*, 1998) were omitted from the data analysis because of problems with PCR amplification, whereas others

(*PLD1, RFG1*; Hube *et al.*, 2001; Khalaf and Zitomer, 2001) were not detected in our initial search for ORFs in the version 4 assembly.

In addition to the expected genes, the transcription profiling revealed a number of genes whose expression had not previously been identified as being regulated by the yeast-to-hyphal transition. The upregulation of *PFY1* (orf6.6300), a *Candida* homolog of Profilin, as well as the expression of a homolog of the budding yeast *RDI1* inhibitor of Rho GTPases (orf6.6469), may reflect a need to modulate actin filament assembly during cell elongation (Pring *et al.*, 1992; DiNubile and Huang, 1997; Pollard *et al.*, 2000; Su *et al.*, 2001). A homolog of the *Saccharomyces cerevisiae YBL060W* gene (orf6.6814) contains a Sec7 domain and is a putative guanidine nucleotide exchange factor (Sata *et al.*, 1998). As in most other eukaryotes, the regulation of small GTPases is likely to play a role in *Candida* cell polarization. Other new genes include a previously undescribed superoxide dismutase that was named *SOD5* as well as orf6.8958, which

Table 2. Selected genes modulated during the yeast to hyphae transition induced by FBS/37°C

Gene	Ref. number	Fold	Function
Upregulated genes			
Secreted and cell surface proteins			
ECE1	orf6.2886	23.1	Unknown
SAP5	orf6.4427	9.9	GPI-anchored aspartic protease
HWP1	orf6.4883	6.5	Cell surface flocculin
SAP4	orf6.3803	5.7	GPI-anchored aspartic protease
SAP6	orf6.3624	4.8	GPI-anchored aspartic protease
SOD5[b]	orf6.7495	4.4	Cu, Zn superoxide dismutase
RBT1	orf6.2929	3.1	Unknown
DDR48	orf6.6854	2.9	Flocculent specific protein
PHR1	orf6.7524	2.2	Cell surface glycoprotein
RBT5-like	orf6.6914	1.8	Mycelial surface antigen precursor
Small GTPases and cytoskeletal modulators			
YBL060W[a]	orf6.6814	2.4	Homology to sec7 domain of GEF
YKE2	4-2790_0001	1.9	Non-native actin binding complex
RDI1[a]	orf6.9069	1.5	Rho GTPase inhibitor
PFY1	orf6.6300	1.5	Profilin
Other functions			
PTP3	orf.8958	3.2	Protein tyrosine phosphatase
GRE2	orf6.1740	2.0	Putative reductase
SNZ1[a]	orf6.6669	2.0	Snooze: stationary phase-induced gene family
HAL9[a]	orf6.8931	2.0	Putative zinc finger transcription factor
CHA1	orf6.6363	1.9	L-serine/L-threonine dehydratase
SEC24[a]	orf6.2185	1.5	ER to Golgi transport
Unknown functions			
IHD1[b]	orf6.6198	2.8	Unknown, putative transmembrane
YMR90	orf6.8783	2.3	Conserved hypothetical protein
RBT4	orf6.537	2.2	Similar to plant PR-1 class of pathogen related proteins
IHD2[b]	orf6.3925	2.0	Unknown
Downregulated genes			
DNA-binding proteins			
GIS2	orf6.4479	2.1	Cysteine-rich zinc finger motifs
CBF1	orf6.4385	1.7	Basic helix-loop-helix protein, binds to centromeres
YDR174	orf6.6542	1.7	HMG-class DNA-binding
TYE7	orf6.6049	1.6	Basic helix-loop-helix protein
CUP9	orf6.7646	1.6	Homeodomain
Lipid metabolism			
YER73	orf6.6640	2.0	Aldehyde dehydrogenase
YKR70	orf6.1739	1.9	Phosphatidyl synthase
DAK2	orf6.1906	1.8	Dihydroxyacetone kinase
SOU1	orf6.6126	1.7	Peroxisomal 2,4-dienoyl-CoA reductase
PLB2	orf6.795	1.6	Phospholipase
Cell surface proteins			
FLO1[a]	orf6.3288	3.8	Putative cell wall glycoprotein
CSP37	orf6.2388	2.8	Cell surface virulence factor
Other functions			
CHT2	orf6.2344	5.3	Endochitinase
YHB1	orf6.2156	2.1	Flavohemoglobin
RHR2	orf6.8673	1.8	DL-glycerol-3-phosphatase
HSP12	orf6.2761	1.8	12 kDa heat shock protein
YLR63	orf6.4669	1.8	Oligopeptide transporter
PKC1	orf6.9136	1.7	Protein kinase C
Unknown functions			
RHD1[b]	orf6.4552	5.0	Unknown
RHD2[b]	orf6.5566	2.7	Unknown, putative transmembrane
RHD3[b]	orf6.8294	2.4	Unknown
YER67	orf6.1218	2.1	Unknown

[a] Name of closest *S. cerevisiae* homolog.
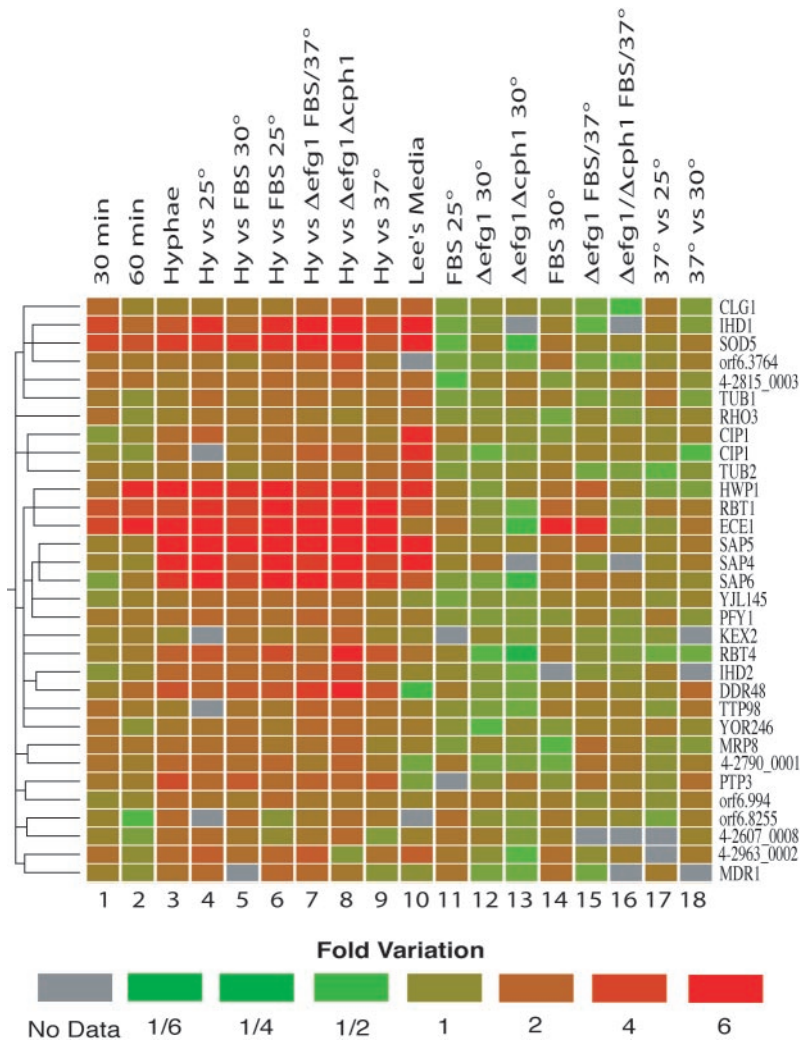
[b] New gene names.

Figure 3. Close-up representation of subcluster A (see Figure 1). This section of the two-dimensional clustering is especially enriched in genes that show a significant increase in expression in the hyphal phase (lane 3). Each of these genes is colored according to its change in expression. Downregulated genes are green, whereas upregulated genes are red. See Table 1 or Figure 1 for a detailed description of the experiments in the *x*-axis.

encodes a homolog of the *S. cerevisiae* Ptp3p tyrosine phosphatase (Wurgler-Murphy *et al.*, 1997; Zhan *et al.*, 1997). Increased expression of *SEC24*, an essential protein in budding yeast that is involved in vesicular transport may be necessary for the rearrangements of cell surface proteins (Pagano *et al.*, 1999). Finally, two uncharacterized ORFs (orf6.6198 and orf6.3925) show significant increases in expression but do not share significant homologies with any other proteins. These were renamed *IHD1* and *IHD2* (Induced during Hyphae Development). The 392-aa peptide encoded by *IHD1* is likely to be a trans-membrane protein because it contains hydrophobic domains at both its N- and C-terminal ends. The region next to the putative transmembrane domain is extremely rich in Ser/Gly residues. *IHD1* was recently identified by Murad *et al.* (2001b) as one of the genes coregulated by the Nrg1p and Tup1p repressors.

A previous attempt at using filter arrays to identify genes that are repressed in hyphae relative to yeast cells had only detected two *HSP12* homologues (Lane *et al.*, 2001a). In this study, we have identified 46 ORFs that show a significant reduction in expression. Most of these genes did not cluster

together in Figure 1 because they respond differently to individual environmental conditions. Generally, the extent of transcriptional repression was smaller than the levels of induction. In addition to *HSP12*, our analysis identified the previously reported hyphae-repressed gene *CHT2* (McCreath *et al.*, 1995). Csp37p, a cell surface protein whose absence leads to reduced virulence in a mouse model (Sentandreu *et al.*, 1997), has its expression levels consistently reduced about threefold in hyphae compared with yeast cells. Among the most highly repressed genes are three ORFs (orf6.4552, orf6.5566, and orf6.8294) that have no sequence similarity to currently identified genes. These genes were renamed *RHD1*, *RHD2,* and *RHD3* (Repressed during Hyphae Development). *RHD3*, like *IDH2*, is another putative membrane protein although it is rich in alanine and serine residues. Additional genes that are repressed upon induction of hyphal growth include Protein Kinase C (*PKC1*; orf6.9136; Paravicini *et al.*, 1996), a homolog of the budding yeast *FLO1* gene, which encodes a putative cell wall glycoprotein (Teunissen *et al.*, 1993; Watari *et al.*, 1994; Bidard *et al.*, 1995) and *RHR2*, a DL-glycerol-3-phosphatase that controls glycerol levels (Pahlman *et al.*, 2001). We also
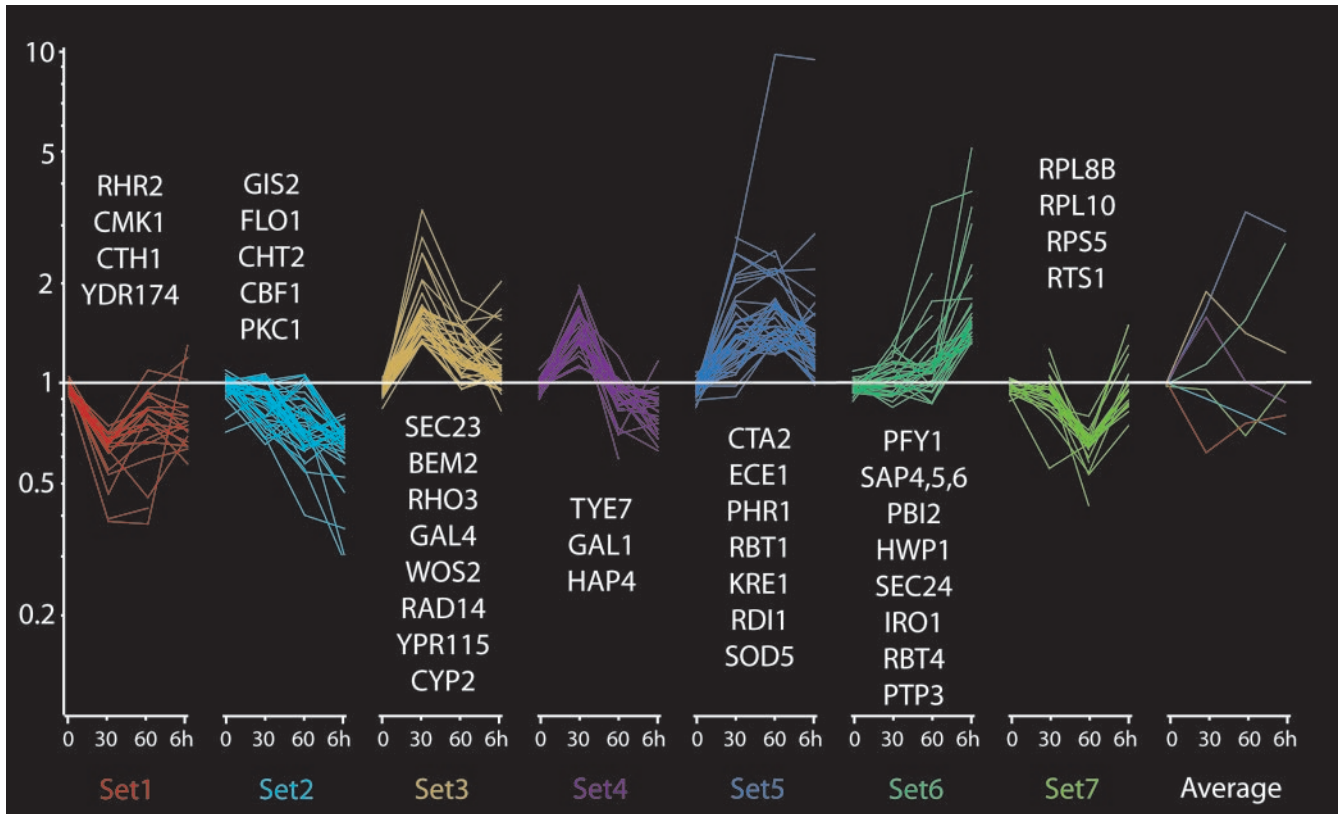
Figure 4. Changes in gene expression during a time course of the yeast-to-hyphae transition induced by FBS/37°C. The 232 genes that show a statistically significant modulation under the studied conditions were separated by K-means clustering according to their expression pattern at *t* = 30 min, 60 min, or 6 h. Each line represents one gene, and its change in expression, as defined by the *y*-axis, in the control arrays and the three times points. Lines are colored according to their K-means set. The rightmost graph shows the average change in gene expression for each of the seven K-means sets as defined by its color. Name of representative members of each sets are shown above or under each graph. For a complete list see the supplemental material.

noted that five of the repressed transcripts encode enzymes that are directly or indirectly related to lipid metabolism. The list of repressed genes includes several putative transcription factors. These include homologues of the yeast zinc-finger protein Gis2 (Balciunas and Ronne, 1999), the High Mobility Group Protein Ydr174p, the Cup9p homeoprotein (Knight *et al.*, 1994), and the bHLH proteins Tye7p (Nishi *et al.*, 1995) and Cbf1p (Eck *et al.*, 2001). In addition, our most recent arrays include *NRG1* (Murad *et al.*, 2001b), which is repressed three-fold in the hyphal cells.

Time Course of Gene Induction

We investigated the timing of the change in gene expression profiles associated with the switch in growth conditions as well as the identity of any genes specifically expressed before germ tube outgrowth and therefore associated with initiation of hyphal growth. After yeast cells were grown at 30°C in YPD and switched to growth at 37°C in the presence of 10% serum, the global transcriptional profile was determined at 30 and 60 min. At 30 min, germ tubes are either absent or initiating, whereas at 60 min a significant number of yeast cells contain germ tubes (Figure 2c). We used K-

means clustering to separate, into 7 distinct groups, 232 genes that show significant variation at the 30-min, 60-min, or 6-h time points. This clustering method separates genes according to the shape of their overall expression pattern and allows us to distinguish a variety of expression patterns during hyphal development (Figure 4). Many of the highly expressed genes at the 6-h time point are not strongly induced at the 30- and 60-min points; these include the *SAPs*, *RBT4*, *HWP1*, and *PTP3* genes (set 6). In contrast, some genes, such as *ECE1*, *RDI1*, *RBT1*, and the new hyphal genes *SOD5* and *IHD1*, are fully or almost fully induced by 60 min (set 5). Other genes show transient changes in expression. The transcripts of genes classified in sets 3 and 4 accumulate very rapidly but then decrease at the 60-min and 6-h points. These include a basic helix loop helix protein with homology to the *S. cerevisiae* transcription factor Tye7p and 4 chaperonins encoded by *WOS2*, *RAD14*, *YPN115*, and *CYP2*. Of note is the transient induction of orf6.7561, a homolog of *S. cerevisiae BEM2*, a Rho1-GAP protein involved in cell wall maintenance as well as the small GTPase Rho3p, a putative mediator of cell polarity (Wendland and Philippsen, 2001). *YDR174* is another gene worthy of note because it shows a constant ~40% reduction in all time points and encodes a

transcription factor of the HMG class. Finally, many of the genes grouped in set 7 are transiently repressed at the 60-min time point encode proteins involved in translation.

Alternate Hyphal Induction Conditions

Several other conditions have been identified that induce the yeast-to-hyphal transition. One of these include growth in Lee's medium followed by a switch from 25 to 37°C. We found that hyphal induction under these conditions generated a slightly different pattern of gene expression than that found with the serum plus 37°C treatment (Figures 1a and 6a). The majority of the genes strongly induced by the serum regime are also induced in the Lee's medium hyphae. However, the increase in *ECE1* transcripts is not as pronounced as in serum-treated cells because yeast cells grown in Lee's medium already express this gene to significant levels. In addition, other genes are induced under the serum regime that are not induced and are even repressed in Lee's medium induction; these include *DDR48* and the *PTP3* phosphatase. There are also significant differences in the reduction in transcript abundance of *RHR2* and *CHT2*. Finally, hyphae induced in Lee's medium show reduced expression in a set of conserved genes whose products are involved in protein translation possibly as a consequence of the reduced nutrient levels in Lee's medium and the longer growth period (24 vs. 6 h) necessary for hyphae development in this medium. A direct comparison between hyphae induced in YPD and Lee's medium was not done because the changes necessary for adaptation to completely different medium (synthetic vs. complex) are likely to obscure those changes that might be responsible for the differences between hyphal structures.

Separating Signals from Serum or Increased Temperature

Because the standard hyphal induction regime involves changes in two environmental parameters (temperature shift and the addition of serum), we characterized the transcription profile of cells undergoing either the addition of serum at lower temperatures of 25 or 30°C or the effects of 25–37°C and 30–37°C temperature shifts in the absence of serum. Treatment with FBS or incubation at 37°C alone are usually sufficient to induce some cell elongation (Figure 2, h–j), whereas serum at 25°C has no significant effects on morphology and gene expression (Figures 1, lane 11, and 2i). As shown in Figure 1, the increase in growth temperature from 30 to 37°C has only minor effects on gene expression, whereas the 25–37°C shift causes more pronounced changes (lanes 17 and 18). Although the upregulation of a few genes during the yeast-to-hyphal transition can be attributed to the increased temperature (see below), experiment clustering, and scatter plot analysis (Figures 1, lanes 3 and 17, and 6b) demonstrates that the transcriptional program induced by addition of FBS at 37°C is significantly different from the one initiated by adaptation to growth at 37°C. Two notable exceptions to this are the downregulation of *CHT2* and *RHD2*, both of which are repressed at 37°C in YPD medium. Growth temperature alone or the addition of serum at lower temperatures has very little effect on the induction of hyphal-specific genes with the notable exception of *ECE1* and *RBT1*, which respond well to serum alone (Figure 3). Thus,

these experiments allow us to separate changes in gene expression that are caused by environmental conditions from those specific to the yeast-to-hyphal switch. We also compared the expression profiles of FBS/37°C-induced hyphae with cells treated with partially inductive conditions. The profiles measured in these experiments clustered close to the standard FBS/37°C vs. YPD/30°C profile (Figure 1), and the only significant difference was in those genes that are especially sensitive to increased temperature (Figure 5). For example, genes encoding G protein subunits alpha (*CAG1*) and beta (Contig4–3039_0017) were up- and down-regulated, respectively, during the switch to the hyphal form. These changes were maintained when hyphae were compared with cells treated with FBS at 30°C but were lost when hyphae were compared with cells incubated at 37°C. Finally, a 25 to 37°C switch also reproduced this change in Galpha/Gbeta ratio, suggesting that these signaling proteins could be involved in an environmental response. Interestingly, a group of ~40 genes from subcluster B, illustrated in Figure 5, were repressed only at the 30- and 60-min time points (enriched in Figure 4, K-means sets 1 and 7) as well as with the addition of serum at low temperature. The expression of these genes then appears to increase during the later stages of hyphae development, potentially as a result of the long-term adaptation to growth at 37°C. This time-dependent response to two different environmental signals appears to be controlled by *EFG1* and *CPH1* as demonstrated below.

Role of Transcription Factors

Several transcription factors have been implicated in the yeast-to-hyphal transition. Efg1p, a bHLH transcription factor predicted to be the target of a cAMP-dependent kinase signaling pathway (Stoldt *et al.*, 1997; Bockmuhl and Ernst, 2001) has been shown to play a major role in the hyphal transition, because true hyphae fail to form in the absence of the gene, although pseudohyphal formation still occurs (Figure 2l). Deletion of a second transcription factor, *CPH1*, together with the *EFG1* disruption, creates cells that are totally defective in serum-induced hyphal formation (Lo *et al.*, 1997). To date, the consequences of deleting these transcription factors has only been studied on a few target genes.

We examined the transcriptional profiles of cells defective in *EFG1* or *EFG1/CPH1* under both yeast and hyphal formation conditions. A comparison of mutant and wild-type cells grown under yeast growth conditions showed that the absence of one or both of these transcription factors increases transcripts levels of 30 genes, whereas the expression of 44 genes appears to be reduced (see Supplementary Material). Many of these modulated genes encode proteins classified as transport modulators (*HGT1*, *CTR1*, a homolog of budding yeast *HST7*), whereas a significant number of the repressed genes encode ribosomal proteins or translation initiation factors. Some of the repressed genes (*RHR2*, *HSP12*, *GLK1*, *SNO1*, *ECM4*, and *GRE2*) have been shown, in *S. cerevisiae*, to be involved in stress response (Gasch *et al.*, 2000). The apparent repression of *IRO1* is probably due to the fact that the 3' end of this gene is missing from the deletion strains used in these studies. In contrast, the comparison of the wild-type and mutant cells under hyphal induction conditions (FBS/37°C), where the mutants produced only yeast cells and pseudohyphae, revealed a very
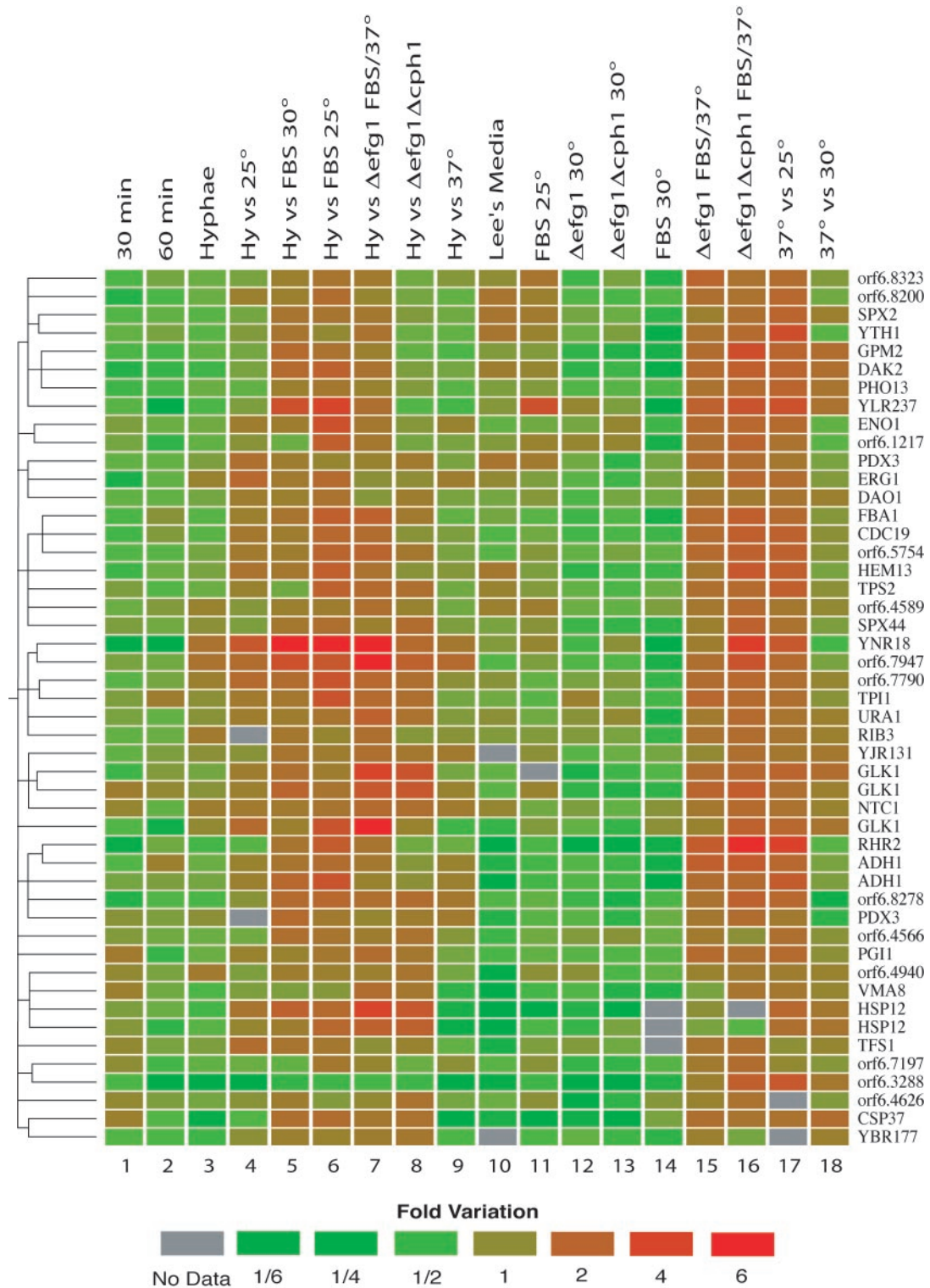
Figure 5. Close-up representation of subcluster B (see Figure 1). This section of the two-dimensional clustering is especially enriched in genes that are inversely modulated by the addition of serum or an increase in growth temperature. Each of these genes is colored according to its change in expression. Downregulated genes are green, whereas upregulated genes are red. See Table 1 for a detailed description of the experiments in the *x*-axis.
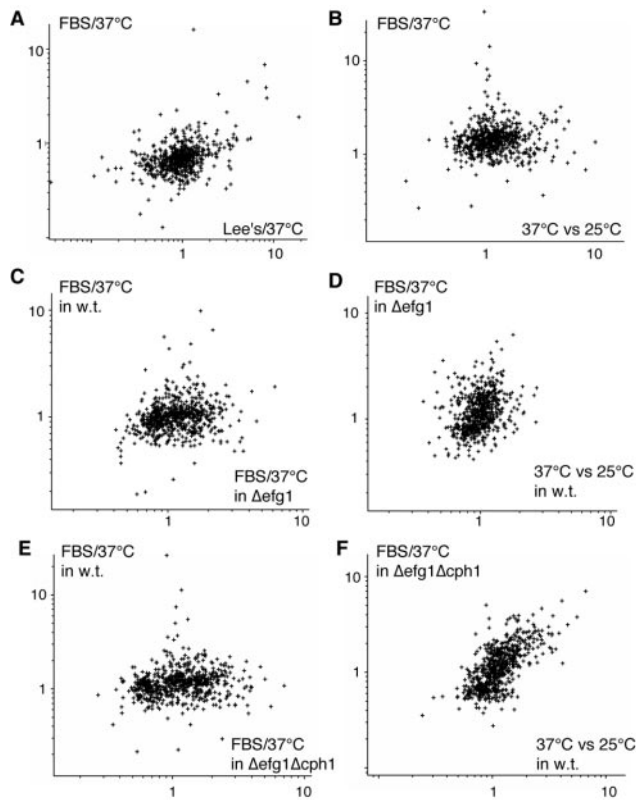
Figure 6. Comparison of the expression ratios of 742 significantly modulated genes. (A) Scatter plot of changes during the yeast-to-hyphae transition induced by treatment with FBS/37°C compared with a shift to 37°C in Lee's medium. (B) Scatter plot of changes during the yeast-to-hyphae transition induced by treatment with FBS/37°C compared with a 25–37°C temperature shift in YPD. (C) Scatter plot of the gene expression pattern during the response of wild-type or Δefg1 cells to a treatment to FBS/37°C. (D) Comparison of the changes in gene expression during the response of an efg1 mutant to FBS/37°C compared with the changes induced by a 25°C to 37°C shift in wild-type cells. (E) Scatter plot of the gene expression pattern during the response of wild-type or efg1Δcph1 cells to a treatment to FBS/37°C. (F) Comparison of the changes in gene expression during the response of an Δefg1Δcph1 mutant to FBS/37°C compared with the changes induced by a 25°C to 37°C shift in wild-type cells.

noticeable shift in the response to environmental cues. As seen in the experimental clustering of Figures 1, 3, and 5 and the scatter plots in Figure 6, c–f, most of the hyphal-modulated genes do not respond to FBS/37°C in the Δefg1 mutants, and none of them are activated in the double mutant. It might be assumed that the double knockout would fail to produce changes in gene expression patterns under hyphae-inducing conditions. Instead the transcriptional profiles suggest that Δefg1Δcph1 cells are unable to respond to the presence of serum and show instead a change in global gene expression patterns that mimics the response of wild-type cells that have adapted to growth at 37°C compared with cells grown at 25°C.

Finally, we compared the transcription profile of cells lacking both Cph1p and Efg1p to those lacking only Efg1p.

Under the three environmental conditions tested, the transcription factor double mutant had a very similar profile to the single *EFG1* disruption (Figure 1). Among hyphal-modulated genes, *CPH1* appears to be necessary for the FBS/37°C-dependent modulation of the secreted protein Ece1p, the transcription factor Tye7p, the cell surface protein Hwp1p, the flavohemoglobin Yhb1p and an unknown protein encoded by the orf6.8909 gene. These were fully or significantly modulated by FBS/37°C in the Δefg1 strain but not in the double mutant. Others, like *SAP5*, were only mildly induced by FBS/37°C in the Δefg1 strain, and the deletion of the *Cph1* alleles was necessary to completely abolish the response. There is little significant difference between both deletion strains when grown in YPD at 30°C, which suggests that *CPH1* does not have an *EFG1*-independent role in yeast morphology.

DISCUSSION

C. albicans is an important opportunistic human pathogen and can cause deadly systemic infections of immunocompromised individuals such as HIV-infected patients, tissue transplant recipients, and patients undergoing cancer chemotherapy. This organism has several cellular forms but is distinguished primarily by a dimorphic shift from a yeast-like growth pattern to a hyphal growth pattern. This morphogenesis appears important for the virulence of the organism (Lo *et al.*, 1997) and is regulated in part by transcription factors that are controlled by signaling pathways responding to a variety of extracellular conditions (Brown *et al.*, 2000; Ernst, 2000; Whiteway, 2000; Liu 2001). Molecular genetics can provide powerful tools for the analysis of pathogens. However the diploid nature of *C. albicans* and the absence of a natural sexual cycle have made classical genetics impossible. The detailed information generated by genome sequencing programs provides the opportunity to apply the tools of the postgenomic era to better understand virulence in this fungal pathogen.

We have examined the transcriptional profiles of ~6300 ORFs defined in *C. albicans* from the 4.0 release of the Stanford *Candida* genome sequencing project (Tzung *et al.*, 2001; Scherer, 2002). Out of 6580 identified *Candida* ORFs, 4821 (73%) had homologues in the genomes of the fungi *S. cerevisiae* or *Schizosaccharomyces pombe*, providing a strong measure of confidence in the ORF designation strategy. Because we used an early assembly of the *Candida* genome, some genes were spotted in multiple locations on the array. The fact that copies of the same gene typically clustered next to each other (see *CIP1* in Figure 3 and *GLK1, ADH1,* and *HSP12* in Figure 5) demonstrates that the large number of replicates used in this study has produced highly consistent data.

We initially chose to study hyphal induction by treatment with serum and a growth temperature of 37°C because these conditions most closely mimic those encountered during a systemic blood infection. We identified a number of genes whose expression is modulated during the switch to hyphal growth independently of the response to serum or temperature alone. Many of these genes were found in a single expression cluster along with previously characterized hyphal and virulence genes such as those encoding secreted aspartyl proteases (SAPs 4 through 6). Some genes in this

cluster have no known function and no obvious homologues in other organisms, whereas others encode proteins with predicted functions including a phosphatase, a superoxide dismutase homolog, and a Rho family GTPase inhibitor.

A common characteristic of signaling pathways is the need for their downregulation, and downregulating effectors are often induced by the signaling pathway they regulate (Burchett *et al.*, 1998; Garrison *et al.*, 1999). Induction of a PTPase and a Rho GTPase inhibitor therefore may implicate dual specificity kinases and Rho GTPases in the signaling pathways leading to hyphal induction. The induced superoxide dismutase is a member of a group of 3 Cu/Zn dismutases that are quite distinct from the *CaSOD1* gene identified through its strong homology with the *S. cerevisiae* Cu/Zn protein (Hwang *et al.*, 1999). Intriguingly, the induced gene is closely linked (2 kb) to a second family member that shows no change in transcript abundance in hyphal conditions. Thus there are 4 Cu/Zn superoxide dismutase proteins and 2 Mn proteins identified in *C. albicans*, and one of the Cu/Zn family members is strongly induced during the yeast-to-hyphal transition. Hyphal formation has been shown to be associated with increased generation of reactive oxygen species (Schmidt and Geschke, 1996).

The global transcriptional profiling approach has also permitted us to identify genes that were repressed in response to the hyphal switch. We observed a reduction in transcripts encoding protein kinase C, the endochitinase Cht2p, several DNA-binding proteins, and enzymes involved in lipid metabolism or glycerol biosynthesis. Deletions of the *C. albicans* *PKC1* genes are necessary for survival in hypo-osmotic medium but had no effect on dimorphism (Ernst, 2000), whereas the Cht2p endochitinase may be involved in cell wall reorganization or in the separation of daughter cells.

A time course of the yeast-to-hyphae transition induced by serum and high temperature has demonstrated that a large number of transcripts change in their abundance. These changes occur before cell elongation becomes apparent. The transient upregulation of homologues of the small GTPases Rho3p and the Rho-GAP protein Bem2p is of interest because these proteins have already been shown to play a role in the determination of cell polarity in fungi (Kim *et al.*, 1994; Cid *et al.*, 1998). We have also identified a large cluster of genes (Figure 5) that are coordinately downregulated in the early stages of the yeast-to-hyphal transition, possibly as part of an Efg1p/Cph1p-mediated response to the presence of serum. The levels of these transcripts then increase in response to an unknown pathway that appears to be modulated by temperature. Examination of the global gene expression profiles of Δefg1Δcph1 cells show them to be incapable of responding to serum, an observation that would have been difficult to substantiate from the study of individual target genes. These results are consistent with a report showing that an activated mutant of the *Candida Ras1* gene, which is proposed to regulate Efg1p, can bypass the requirement for serum in hyphal induction (Feng *et al.*, 1999). In serum-dependent hyphal development, the role of the Cph1p transcription appears to be relatively minor, but still significant. Lane *et al.* (2001b) have shown that Cph1p plays a much more significant role in hyphal development when cells are grown in SS Medium.

In addition to Efg1 and Cph1, many other transcription factors have been shown to modulate the yeast-to-hyphae

transition. These include Cph2p, Tec1p, Czf1p, Rim101p, and Tup1p, some of which exhibit changes in their transcript levels. It should be noted that the spot intensity for most of these genes was generally very weak, resulting in poor reproducibility. We have observed the Efg1-dependent repression of Cph2 and Tup1 transcription in hyphae. The gene for Tec1p, another transcription factor that was recently shown to be an effector of Efg1p and Cph2p (Schweizer *et al.*, 2000; lane *et al.*, 2001b), was only recently spotted on our microarray and thus lacks the high number of replicates necessary for statistical significance. We still observed a transient 2.5-fold increase in *TEC1* transcripts 30 min after treatment with FBS/37°C (p = 0.06). Another recent addition to our microarrays is the *NRG1* gene whose strong downregulation during hyphal development (Murad *et al.*, 2001b) has been confirmed in our very latest experiments. Our results have further identified several more significantly modulated transcription factor genes whose expression patterns may point toward a role in morphogenesis.

In conclusion, this study has revealed a significant number of genes whose transcriptional profiles are similar to those of known virulence factors and markers of the yeast-to-hyphae transition. These results provide new insights into the mechanisms for the initiation and maintenance of filamentous growth. We have also confirmed that the main function of the Efg1p and Cph1p transcription factors is to transmit signals induced by the presence of serum. The molecular roles of all of these genes can now be analyzed further through disruption analysis.

ACKNOWLEDGMENTS

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