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Identifying Phase-specific Genes in the Fungal Pathogen *Histoplasma capsulatum* Using a Genomic Shotgun Microarray[D]

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A fundamental feature of the fungal pathogen *Histoplasma capsulatum* is its ability to shift from a mycelial phase in the soil to a yeast phase in its human host. Each form plays a critical role in infection and disease, but little is understood about how these two morphologic phases are established and maintained. To identify phase-regulated genes of *H. capsulatum*, we carried out expression analyses by using a genomic shotgun microarray representing approximately one-third of the genome, and identified 500 clones that were differentially expressed. Genes induced in the mycelial phase included several involved in conidiation, cell polarity, and melanin production in other organisms. Genes induced in the yeast phase included several involved in sulfur metabolism, extending previous observations that sulfur metabolism influences morphology in *H. capsulatum*. Other genes with increased expression in the yeast phase were implicated in nutrient acquisition and cell cycle regulation. Unexpectedly, differential regulation of the site of transcript initiation was also observed in the two phases. These findings identify genes that may determine some of the major characteristics of the mycelial and yeast phases.

INTRODUCTION

Fungal infections are a growing medical threat, particularly for immunocompromised individuals such as patients with cancer or acquired immunodeficiency syndrome (Samonis and Bafaloukos, 1992; Momin and Chandrasekar, 1995; Dixon *et al*., 1996; De Marie, 2000; De Pauw, 2001; Wheat *et al*., 2002). *Histoplasma capsulatum*, the etiologic agent of histoplasmosis, is a primary fungal pathogen that infects healthy as well as immunocompromised individuals. The latter tend to develop progressive, disseminated disease that can be fatal. *H. capsulatum* is endemic in the Ohio River Valley through the midwestern United States into Texas.

H. capsulatum exists in two morphological forms: a mycelial (or filamentous) form in soil and a yeast form in the host. The mycelial form produces vegetative spores, or conidia. Conidia or mycelial fragments are inhaled by the host and then taken up by macrophages and other phagocytic cells (Eissenberg and Goldman, 1994). Once inside the host, conversion of the mycelial form to the budding yeast form is triggered within hours. Yeast cells evade killing and multiply within macrophages (Bullock, 1993). Subsequently, yeast cells spread to multiple organs of the reticuloendothelial system such as the spleen, liver, lymph nodes, and bone marrow. In patients with disseminated disease, a variety of additional organs can be colonized (Eissenberg and Goldman, 1991).

The ability of *H. capsulatum* to grow in the mycelial form in soil and shift to the yeast form in the host is important for infection and disease. On disturbance of the soil, mycelial fragments and associated conidia are aerosolized and inhaled by the host, unlike yeast cells. Once introduced into the host, transformation to the yeast form must occur for the fungus to survive and proliferate (Maresca *et al*., 1977; Medoff *et al*., 1986). Despite the fundamental roles that these two forms play in infection and disease, little is known about their molecular differences or what regulates the transition between them.

The transformation between mycelial and yeast phases, or vice versa, can be recapitulated in culture by shifting the

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growth temperature from 25 to 37°C, or the reverse (Maresca and Kobayashi, 1989; Maresca *et al*., 1994). This characteristic has made it possible to identify phase-specific genes. Five genes have been identified that are expressed predominantly in the yeast phase (Keath *et al*., 1989; Di Lallo *et al*., 1994; Keath and Abidi, 1994; Gargano *et al*., 1995; Patel *et al*., 1998). One of these genes, *CBP1* (calcium binding protein), is a virulence factor in the host (Sebghati *et al*., 2000). Several genes specific to the mycelial phase have also been identified (Harris *et al*., 1989a,b; Tian and Shearer, 2001; Johnson *et al*., 2002; Tian and Shearer, 2002).

To develop a more complete description of the two morphological phases, we performed a large-scale analysis of gene expression in *H. capsulatum*. Because the sequence of the *H. capsulatum* genome has not yet been completed, we constructed a 10,000-element array containing random genomic fragments. Using this array, we identified ~500 clones whose expression was differentially induced in either the yeast or mycelial forms, including several potential regulatory genes. This work sets the stage for uncovering the function of these genes in the growth phases of *H. capsulatum* as well as for applying genomic approaches to other questions in this fungal pathogen.

MATERIALS AND METHODS

Strains and Culture Conditions

H. capsulatum strain G217B (ATCC 26032; obtained from the laboratory of William Goldman, Washington University, St. Louis, MO) was grown in histoplasma macrophage medium broth or on HMM plates (Worsham and Goldman, 1988). To grow *H. capsulatum* in the yeast form, cultures were shaken in HMM broth at 37°C on an orbital shaker under 5% CO_2. Stock cultures were maintained in HMM broth, with passage of cells every 2–3 d at 1:25 dilution. For yeast-phase cultures grown to stationary phase, a 1-liter culture of yeast-phase cells was inoculated on day 0 at a final concentration of 2.6×10^4 cells/ml. By day 3, the cells reached a density of 6×10^7 cells/ml, and remained stationary for the remainder of the experiment. 100 ml of culture was harvested each day from days 2 to 10. At each time point, the morphology of the cells was monitored microscopically. The mycelial form was grown in HMM broth at room temperature (22–25°C) on a stationary platform for 4–6 wk.

Library

Genomic DNA was isolated from 100 ml of the virulent G217B strain by using genomic tips and genomic DNA buffer set (both from QIAGEN, Valencia, CA). For construction of the mini-array, DNA was partially digested with Sau3A*I* and size fractionated (0.5–2 kb) on a 1% low melt agarose gel. This procedure was repeated, size fractionating from 1 to 2 kb, to create a library for the large array. The resulting fragments were ligated into pBluescript KS+ precut with *Bam*HI. The library was transformed into SUREII cells (Stratagene, La Jolla, CA).

Colony Polymerase Chain Reaction (PCR) Amplification and Microarray Construction

A 96-well format was used to grow 1536 (mini-array) or 9600 (large array) individual bacterial cultures, each harboring an independent library transformant (see library construction above). Individual colonies from the library transformation were inoculated into 100 μl of Luria Broth plus 100 μg/ml carbenicillin in individual wells of 96-well plates and incubated for 16–18 h at 37°C on an orbital shaker at 150 rpm. Two microliters from each well were used for PCR amplification of the inserts by using M13-forward (5′-GTTTTCCCAGTCACGAC-3′) and M13-reverse (5′-GCGGATAACAATTTCACACAGG-3′) primers; these primers were complementary to the vector. Glycerol was added to the remaining bacterial cultures to 25%, and the 96-well dishes frozen at −80°C. The PCR products were analyzed on 1% agarose gels and then precipitated, washed, and printed on glass slides as described previously (DeRisi *et al*., 1997). Later iterations of the array included ~400 clones from a yeast-phase cDNA library.

Sequencing

Copies of the 96-well bacterial cultures were sent to Incyte Genomics (Palo Alto, CA) and the Genome Sequencing Center (Washington University, St. Louis, MO). Incyte Genomics (Palo Alto, CA) sequenced one side of each clone by using the M13-forward primer. As part of the ongoing genome project, the Genome Sequencing Center sequenced each clone by using the M13-forward and M13-reverse primers. The Incyte sequence is available at http://gregor.berkeley.edu, and the Genome Sequencing Center sequence is available at http://www.genome.wustl.edu/projects/hcapsulatum/.

RNA Preparation

Cultures of yeast and mycelial phase *H. capsulatum* were harvested by filtration. Cells were disrupted in RNA extraction buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl [*N*-lauroyl-sarcosine], 0.1 M β-mercaptoethanol) by vortexing in the presence of glass beads. RNA was extracted once with acid phenol, chloroform, and 0.1 M NaOAc, pH 4.0, and then extracted twice with equilibrated (pH 8) phenol/chloroform. RNA was then precipitated with isopropanol, washed with 70% ethanol and resuspended in double distilled H_2O.

cDNA Synthesis, Labeling, and Analysis

Fluorescently labeled cDNA was made by incorporating amino-allyl dUTP during reverse transcription of poly-adenylated (poly-A)–selected RNA. Cy3 or Cy5 dyes (Amersham Biosciences, Piscataway, NJ) were coupled to the amino-allyl group as described previously (DeRisi *et al*., 1997). For the yeast stationary-phase experiments, an equal mass of each time point was pooled to generate a reference sample, which was labeled with Cy3. Each time point was individually labeled with Cy5 and competitively hybridized against the reference sample. Yeast and mycelial cDNAs were labeled with Cy3 and Cy5, respectively. Dyes were reversed for the reverse fluor control.

Northern Analysis

Total RNA (5–10 μg) was separated on a 1.5% denaturing agarose-formaldehyde gel and transferred to a GeneScreen Plus membrane (PerkinElmer Life Sciences, Boston, MA). To generate probes, either the entire insert of a library clone was isolated through restriction digest, or gene-specific primers were used to amplify regions specific to the gene of interest. Oligonucleotide sequences can be found in supplemental materials. Probes were labeled using rediprime (Amersham Biosciences) and [α-^{32}P]dCTP. The membrane was probed in hybridization buffer (1 M NaCl, 50% formamide, 1% SDS, 10% dextran sulfate, 33 μg/ml salmon sperm DNA) at 42°C overnight, and then washed twice in 2× SSC, 1% SDS at 65°C for 1 h each before exposure to film and PhosphorImager screen (Molecular Dynamics, Amersham Biosciences, Piscataway, NJ).

Data Analysis

Arrays were scanned on a GenePix 4000B scanner (Axon Instruments, Foster City, CA) and analyzed using GENEPIX PRO version 3.0, NOMAD (http://derisilab5.ucsf.edu/NOMAD), CLUSTER, and TREEVIEW (Eisen *et al*., 1998). For yeast stationary-phase experiments, because the reference was a pooled sample, ratio mea-

surements from the time-course data were normalized relative to the first time point (day 2). The expression ratios for each clone on a given array were divided by the corresponding ratios measured from the day 2 array. CLUSTER analysis was performed on two independent stationary-phase experiments and three yeast-versus-mycelial experiments. Only one stationary-phase experiment is shown in Figure 3. Data from all experiments are available in supplemental materials.

BLAST Analysis and E-Values for Homologs

For initial annotation of partial array clone sequences, DNA sequences from Incyte Genomics were compared against available protein databases by using BLASTx (Gish and States, 1993). BLASTx hits with an E-value less than or equal to 1×10^{-6} were evaluated. For further annotation of yeast and mycelial induced clones, array clone end-sequences from Incyte Genomics and the Genome Sequencing Center were compared against the Genome Sequencing Center *H. capsulatum* genome sequencing project contigs by using BLASTn. Contigs corresponding to microarray clones were compared against the National Center for Biotechnology Information nr protein database by using BLASTx. Array clones and potential BLASTx hits were mapped to contig sequences and evaluated for overlap. Array clones that clearly contained a single open reading frame (ORF) BLASTx hit with an E-value $\leq 1 \times 10^{-12}$ were annotated. Those that contained more than one ORF were not annotated.

5′ and 3′ Rapid Amplification of cDNA Ends (RACE) and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Poly-A RNA was purified from total RNA isolated from yeast or mycelia by using Oligotex mRNA kit (QIAGEN). FirstChoice RNA-ligase mediated (RLM)-RACE kit (Ambion, Austin, TX) was used to identify 5′ and 3′ ends of cDNAs as specified in the kit. Oligonucleotide sequences used for these analyses can be found in supplemental materials. The coding sequence for open reading frames was amplified by PCR with gene-specific primers from cDNA synthesized from poly-A RNA. PCR products were cloned using TOPO-TA (Stratagene) and sequenced using M13-forward, M13-reverse, and gene-specific primers as needed.

RESULTS

Array Construction

Most surveys of gene expression by microarray analysis have required prior knowledge of the genome sequence of the organism. Because the *H. capsulatum* genome was not sequenced at the time this study began, we constructed a 10,000 element *H. capsulatum* shotgun array by using PCR to amplify clones from a library of random genomic fragments (Figure 1A). Based on the estimated genome size of *H. capsulatum* (~25 Mb) (Carr and Shearer, 1998) and data from other fungal genomes, we anticipated that genomic fragments of 1 kb would likely contain coding sequence. The structure of the ~30 *H. capsulatum* genes with available genomic and cDNA sequences predicted that the average intron size is fairly small (100 nucleotides) with 0–6 introns per gene. Thus, intron sequences were unlikely to interfere with the ability of a cDNA probe to bind its cognate genomic fragment. These expectations were tested by construction of a 1500-element mini-array containing random genomic inserts of ~1 kb (see MATERIALS AND METHODS). To determine the fraction of elements in this array that contained coding sequence, we carried out a competitive hybridization with two samples: 1) genomic DNA labeled with the green fluor Cy3 and 2) cDNA made from yeast-phase cells labeled with the red fluor Cy5. As expected, the genomic DNA gave a signal for all of the array clones. The cDNA hybridized to ~75% of the array clones (our unpublished data). Because the cDNA represented genes expressed only under one growth condition, these data indicate that a minimum of 75% of the array elements contained coding sequence. We therefore proceeded to construct a large-scale genomic shotgun array (see MATERIALS AND METHODS). Previously identified *H. capsulatum* genes were also spotted at known locations on the array.

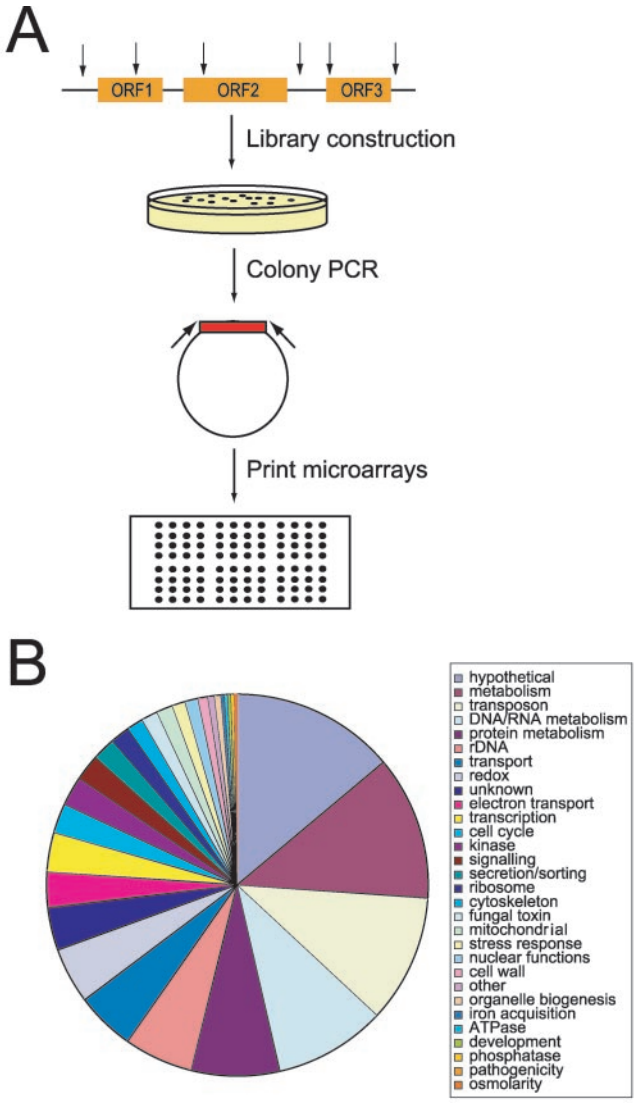

Figure 1. Construction and content of microarray. (A) Construction of microarray. A genomic library was constructed from strain G217B genomic DNA partially digested with Sau3A*I* (represented by top line of figure) and cloned into pBluescript. After transformation, individual colonies were grown up for colony PCR with M13-forward and M13-reverse primers. PCR products were printed onto glass slides. (B) Pie chart of gene functions represented on array, based on partial sequencing and annotation.

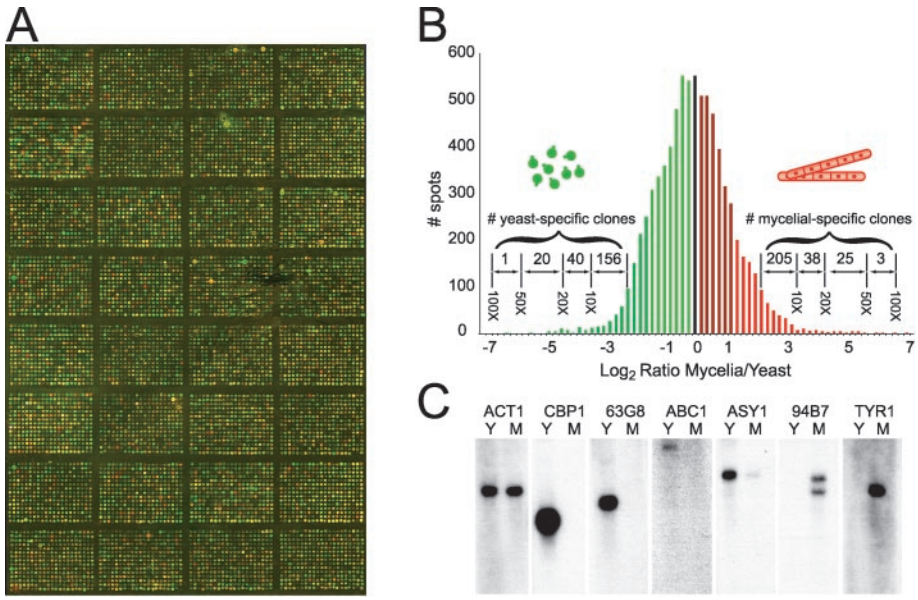

Figure 2. Yeast versus mycelial gene expression. (A) Fluorescence image of yeast cDNA (green) and mycelial cDNA (red) hybridized to genomic microarray. A majority of spots on the genomic shotgun array hybridize to cDNA probes. (B) Histogram of the distribution of yeast-specific (green) and mycelial-specific (red) signal. (C) Northern analysis of yeast and mycelial-specific expression. Total RNA (5 μg) from yeast (Y) and mycelia (M) was probed using clones from candidate yeast- and mycelial-specific spots. Clones showing clear homology to genes of known function have been named accordingly. 63G8 and 94B7 do not have homologs in other organisms. *ACT1* and *CBP1* are included as controls.

Array Content

Based on the estimated genome size, the number of clones on the array, and the average size of the clones, the array covers approximately one-third of the genome. For purposes of clone identification, we obtained partial sequence information (one or two sequence reads per clone) for the majority of clones on the array (see MATERIALS AND METHODS). BLASTx analysis (using an E-value cut-off of 10^{-6}) of single sequence reads revealed that a diverse set of gene families was represented on the array (Figure 1B). Although only 10% of these reads identified a clear homolog by BLAST, mapping and annotation of the remainder of the clones is ongoing as the genome sequencing project develops. Only 2% of the clones contained ribosomal DNA (rDNA); thus, a large percentage of clones likely contain protein-coding sequence.

Identification of Yeast and Mycelial Phase-regulated Clones

To compare the gene expression profiles of yeast cells with the mycelial form of *H. capsulatum*, yeast cells were grown to mid-logarithmic phase at 37°C. Mycelial cells were grown by shifting a dilution of yeast cells from 37°C to room temperature; mycelial form cells were allowed to accumulate for ~3–4 wk without shaking. Microscopic observation of these cells confirmed the expected morphology and revealed that the mycelial sample also contained conidia, or vegetative spores, which arise from the mycelial form. Poly-adenylated RNA was isolated from both yeast and mycelial cells. Lower yields of poly-adenylated RNA (but not total RNA) were obtained from mycelia than from yeast for unknown reasons. cDNAs generated from yeast and mycelial RNA were labeled differentially (Cy3 for the yeast sample and Cy5 for the mycelial sample) and subjected to competitive hybridization on the microarray. As expected from the pilot mini-array, the majority of the array elements contained protein coding sequence, as indicated by the ability of the cDNA probe to bind to most array elements (Figure 2A). A histogram showing the distribution of the signal for each fluorophore over the entire array indicated that at least 500 genes were expressed at significantly higher levels (≥5-fold) in one phase compared with the other (Figure 2B).

CBP1 and *yps-3*, two previously identified yeast-specific genes (Keath *et al*., 1989; Patel *et al*., 1998), were used as control spots on the array. As expected, both were highly expressed in yeast cells. In addition, *CBP1* and *yps-3* were also represented on the array in the set of random genomic array clones; these spots also showed the same differential expression. To provide an independent test of whether other clones identified as differentially expressed in the array analysis were phase-regulated, Northern analysis was performed on total RNA from yeast and mycelial cells for six of these clones (Figure 2C). These clones were recovered from the bacterial archives of the array library, and the insert corresponding to the genomic DNA fragment on the array was purified from these clones and used as a probe. Four yeast-specific clones, including the previously identified *CBP1*, were confirmed as being primarily expressed in the yeast form. The enhanced expression of two mycelial-specific clones was also confirmed. It should be noted that we use "yeast specific" and "mycelial specific" to refer to quantitatively different expression levels in the two growth phases.

Annotation of Phase-regulated Genes

Because the Northern analysis confirmed the validity of the array data, the most highly phase-regulated array clones were annotated. The single-sequence reads that marked the boundaries of each array clone were mapped onto contigs from the ongoing genome-sequencing project (http://genome.wustl.edu/projects/hcapsulatum) and then a BLASTx analysis of each contig was performed

Table 1. Phase-regulated genes

A. Mycelial-induced Genes

Gene name	Annotation	E-value[a]	Accession no. of ortholog	M/Y ratio[b]
TYR1	Tyrosinase	1E-17	BAC22112	118.9
MPS1[c]	Hypothethical protein	3E-19	T48700	33.8
LPL1	Lysophospholipase	2E-37	CAC10084	29.0
FDH1	Formate dehydrogenase	9E-84	1905380A	23.4
OXO1	3 oxoacyl-(acyl-carrier protein) reductase	1E-37	ZP_00026098	22.1
MPD1	Mannitol-1-phosphate dehydrogenase	7E-50	AAL89587	20.8
NIR1	Nitrite reductase	4E-37	AAF41975	14.4
HST4	Homolog of SIR2 (*HST4*)	2E-66	CAB66167	13.1
ABC4	ABC transporter 4	0E+00	CAC42218	12.4
MPS2	5′-nucleotidase containing protein	4E-73	BAB30497	11.9
NOP2	Nucleolar protein (*NOP2*)	0E+00	2103264A	8.8
FBC1	flbC	3E-28	AAC33347	8.4
YTM1	Putative microtubule-associated protein (*YTM1*)	2E-22	CAB54817	8.0
FLU1	fluG	6E-97	AAC37414	6.5
PRP8	Pre-mRNA splicing (*PRP8*)	0E+00	EAA04255	6.3
DPP1	Ydr284	6E-13	XP_134059	6.2
SDH1	Sorbitol dehydrogenase	4E-53	EAA08770	6.2
MCP1	Mitochondrial carrier protein	8E-58	CAD01131	6.1
NOV1	Related to novobiocin biosynthesis protein (novR)	1E-76	CAD21319	5.9
MCP2	Putative mitochondrial phosphate carrier protein	1E-80	CAB55764	5.8
MFS4	MFS transporter 4	1E-42	AAF64435	5.8
PRO1	Related to 26s proteasome subunit p28	4E-49	CAB97304	5.5
GST1	GST	1E-25	AAF21054	5.3
WET1	wetA	2E-30	AAA33330	5.2
RPS2	40S ribosomal protein S2	4E-76	CAA63835	5.1
TUB1	Tubulin	0E+00	S13336	4.7
TRF1	Phenylalanyl-tRNA synthetase alpha subunit-like protein	0E+00	AAF79201	4.4
MS95	MS95[d]	4E-34	CAA89906	3.9
SMT1	Serine hydroxymethyl transferase	0E+00	AAB64197	3.3
PMT1	Mannosyltransferase	2E-41	AAK40024	3.1

[a] E-value comparing *H. capsulatum* gene and its ortholog.

[b] Ratio of expression from YvsM 1 experiment.

[c] Mycelial-phase specific 1.

[d] 127bp of the *MS95* open reading frame overlaps with the microarray clone. A second open reading frame may be represented on the clone.

against the National Center for Biotechnology Information nr database. Clones were annotated if 1) the entire sequence of the clone encoded a single BLASTx hit, or 2) the majority of the clone's sequence corresponded to a single BLASTx hit and flanking sequence was unlikely to correspond to a second gene (Table 1). Each *H. capsulatum* gene was given a three-letter name based on the putative function of its ortholog. The three-letter code, annotation of each gene, accession number of its ortholog, and ratio of expression in the two morphological forms are displayed in Table 1. Differentially expressed clones that contained sequence from transposable elements or rDNA are excluded from this table.

Identification of Mycelial-specific Genes

The data revealed several categories of genes showing significantly higher expression in mycelia compared with yeast. Several orthologs of genes involved in conidiation (a process confined to mycelia) in other fungi were observed. For example, an ortholog of *fluG*, an *Aspergillus nidulans* gene that regulates conidial differentiation and secondary metabolite production was mycelial specific. The *H. capsulatum* ortholog *FLU1* may be of particular interest because *A. nidulans fluG* is required for the accumulation of a presumed extracellular, diffusible factor that stimulates the differentiation of mycelia into conidia (Lee and Adams, 1994). In *A. nidulans*, *fluG* functions as an upstream member of a conidiation pathway that includes the transcription factor *flbC* and the regulator *wetA* (Marshall and Timberlake, 1991; Wieser *et al.*, 1994). *H. capsulatum* orthologs of both of these genes (*FBC1* and *WET1*) were among the set of genes expressed in mycelia. A formate dehydrogenase homolog (*FDH1*) was also expressed at higher levels in mycelia than in yeast. Expression of the *N. crassa* formate dehydrogenase is induced under conditions that favor the formation of conidia (Chow and RajBhandary, 1993). Because the mycelial sample contained both mycelia and conidia, it is unknown whether these *H. capsulatum* orthologs were expressed in mycelia because they differentiate into conidia, in the conidia themselves, or both.

Table 1. Continued

B. Yeast-induced Genes

Gene name	Annotation	E-value[a]	Accession no. of ortholog	M/Y ratio[b]
MFS1	MFS efflux transporter 1	7E-40	CAA20729	26.9
BUB1	Mitotic checkpoint kinase (*BUB1*)	0E+00	AAC39457	25.9
GPD1	Glycerol-3-phosphate dehydrogenase	0E+00	CAD01115	18.9
TRI11	Trichothecene C-15 hydroxylase	2E-63	AAM48897	18.4
CHO1	Choline sulfatase	0E+00	ZP_00083149	16.7
LOM1	Ornithine-N5-oxygenase	0E+00	BAC15565	14.0
ABC1	ABC transporter 1	7E-52	AAM27211	13.0
LYP1	Lysine permease	1E-64	CAD37145	10.9
CDO1	Cysteine dioxygenase	8E-19	AAB36852	10.9
PLC1	Nonhemolytic phospholipase C	7E-35	AAM55475	10.8
ARH2	Probable ATP-dependent RNA helicase	2E-44	Q9H5Z1	10.6
MTP1	Probable metabolite transporter	3E-25	BAB43911	10.1
MFS3	MFS efflux transporter 3	4E-18	CAA21876	9.3
RTT1	Regulator of Ty1 transposition	5E-13	CAA96106	8.1
CRP1	Copper transporter	0E+00	AAF04593	8.0
SMC1	Sister chromatid cohesin (*SMC1*)	0E+00	CAC18213	7.5
NPP1	Putative nuclear pore protein	1E-44	CAB55845	7.1
TIF3	Translation initiation factor 3 alpha subunit (eIF3)	5E-78	CAA19172	7.1
MFS2	MFS efflux transporter 2	4E-32	CAB83151	7.0
MET3	ATP sulfurylase (*MET3*)	2E-83	AAN04497	7.0
SIP1	Septin interacting protein homolog	2E-22	CAA22599	7.0
GCL1	Glutamate-cysteine ligase	0E+00	CAD21373	6.9
GAM1	Glucoamylase precursor	3E-41	AAB02927	6.8
COP1	Coatamer	5E-86	AAK92952	6.5
HPD1	4-Hydroxyphenylpyruvate dioxygenase	2E-42	AAA82574	6.4
ABC2	ABC transporter 2	1E-50	CAD28433	6.4
KIN1	Protein kinase (kin1)	0E+00	CAA20726	5.7
CSE1	Cephalosporin esterase	2E-15	AAB93483	5.6
CAD1	Chromosome adherin (Nipped B)	1E-28	BAC15836	5.6
MEP1	Methionine permease	4E-76	CAA97055	5.6
TIF2	Translation initiation factor 2 (eIF2) gamma	2E-76	AAB64558	5.5
DLD1	Dihydrolipoamide dehydrogenase	0E+00	CAB65609	5.5
OPR1	Putative oxoprolinase	0E+00	CAA92316	5.3
ABC3	ABC transporter 3	0E+00	CAC42217	5.3
DYN1	Dynein	0E+00	AAA18338	5.2
SNR2	U2 snRNP	0E+00	CAA93298	5.1
ASY1	Asparagine synthetase	9E-44	T42010	2.4

Genes that affect mycelial or polar growth in other organisms were also identified. *TYR1* is an ortholog of a tyrosinase gene (*MelC2*) from the bacterium *Streptomyces griseus*. Expression of *MelC2* is both necessary and sufficient to produce melanin and stimulate the production of aerial hyphae (Endo *et al*., 2001). The connection between the production of melanin and morphology is unclear, but under normal culture conditions, the *H. capsulatum* mycelial form produces melanin, whereas yeast cells do not (Nosanchuk *et al*., 2002). *TYR1* is an intriguing candidate for a regulator of melanin production and establishment or maintenance of the mycelial phase in *H. capsulatum*. We also found an ortholog of phenylalanyl-tRNA synthetase (*TRF1*), which is required for polar hyphal growth in *A. nidulans* (Osherov *et al*., 2000).

Genes that might affect the accumulation of other phase-regulated transcripts, such as orthologs of the splicing factor *PRP8* (Strauss and Guthrie, 1991) and the silencing factor *HST4*, were also induced in mycelia. The latter is a presumptive NAD^+-dependent protein deacetylase that has roles in silencing in *Schizosaccharomyces pombe* (Freeman-Cook *et al*., 1999) as well as other cellular processes in *S. cerevisiae* (Brachmann *et al*., 1995). *PRP8* and *HST4* might influence the overall mycelial gene expression program by affecting the splicing and expression of mycelial genes.

Finally, we identified a set of genes that might reflect the unique physiology of the mycelial form. One of these, *NIR1*, encodes a nitrite reductase most similar to the *Neisseria gonorrhea aniA* gene, which is required for growth under oxygen-limiting conditions (Hoehn and Clark, 1992; Mellies *et al*., 1997). In other organisms, nitrite reductase functions in denitrification (Zumft, 1997), converting nitrogen oxides into molecular nitrogen. Denitrification is performed by many soil organisms. Because soil is the natural environment of the mycelial form of *H. capsulatum*, the expression of enzymes required for denitrification may be coupled with growth in the mycelial form. Similarly, we identified genes that encode several transporters and enzymes (such as *DPP1*, which encodes a zinc-regulated diacylglycerol pyrophosphate phosphatase, and *OXO1*, which encodes a 3-oxoacyl reductase) whose differential expression might

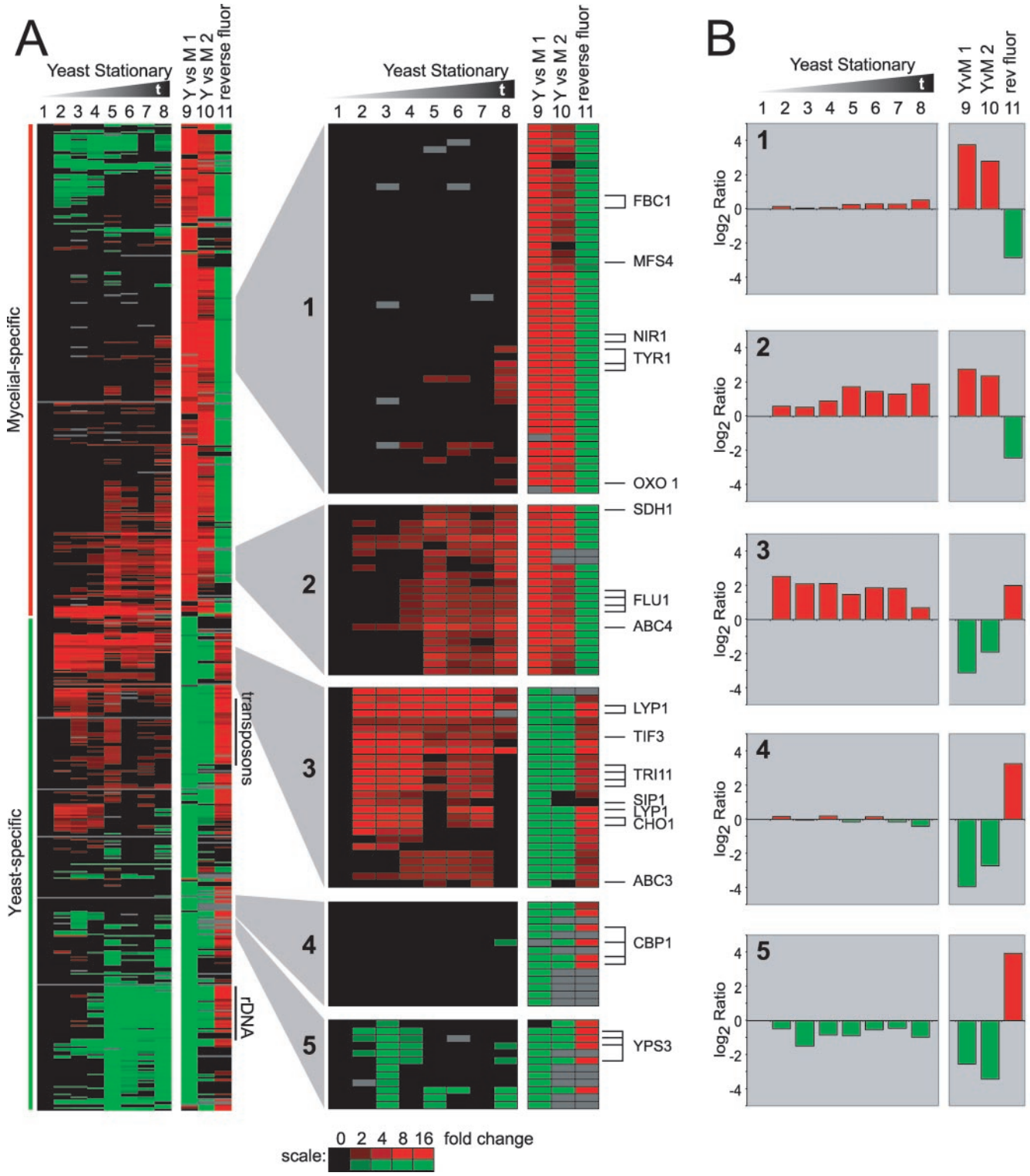

Figure 3. Gene expression profile in yeast-stationary phase, yeast, and mycelia. (A) Hierarchical clustering of spots whose expression changes by fivefold or more in at least one yeast versus mycelial microarray experiment. Phase-specific clones (mycelial or yeast) are marked by a vertical line at the left. Each column represents a microarray experiment and each row represents expression of a spot on the microarray. For the yeast stationary-phase experiment, cDNAs from 2-, 3-, 4-, 5-, 6-, 7-, 8-, and 10-d cultures (columns 1–8) were labeled with the Cy5 (red) fluorophore, and a reference sample containing equal amounts of each time point was labeled with the Cy3 (green) fluorophore. Each

reflect the different growth requirements of mycelia and yeast. In addition, we observed two genes previously identified as mycelial-specific in *H. capsulatum*: the *MS95* gene (GenBank, Glen Shearer), as well as tubulin, which has been reported to be expressed fivefold higher in the mycelial phase (Harris *et al*., 1989b).

Identification of Yeast-specific Genes

Genes with higher expression in the yeast phase than the mycelial phase were also identified (Table 1), including several involved in sulfur metabolism. Sulfur metabolism influences the morphological state of *H. capsulatum* and other dimorphic fungi (Medoff *et al*., 1987; Maresca and Kobayashi, 1989). In *H. capsulatum*, at least some strains exhibit a requirement for the presence of cystine or cysteine in the culture medium to establish the yeast phase (Salvin, 1949; Scherr, 1957; Maresca *et al*., 1977). Additionally, a cysteine dioxygenase activity was previously identified as specific to the yeast phase of *H. capsulatum* (Kumar *et al*., 1983), but the gene encoding this protein had not been identified. We identified a yeast-specific cysteine dioxygenase gene that was expressed 11-fold higher in yeast than mycelia. In addition, we identified several yeast-expressed genes, which share sequence similarity to genes involved in sulfur metabolism in other organisms: choline sulfatase; ATP sulfurylase (the first enzyme in the sulfate-assimilation arm of the methionine/cysteine biosynthetic pathway); glutamate-cysteine ligase (which affects glutathione and glutamate metabolism); and methionine permease (which can mediate both methionine and cysteine uptake in *S. cerevisiae*; Kosugi *et al*., 2001).

The yeast-expressed genes included those that might affect other aspects of metabolism and nutrient availability, such as a lysine permease, an oxoprolinase (which affects L-glutamate production), a 4-hydroxyphenylpyruvate dioxygenase (which affects tyrosine metabolism), a dihydrolipoamide dehydrogenase (which affects carbon and amino acid metabolism), and several ATP-binding cassette and major facilitator superfamily efflux transporters. Expression of these genes predominantly in the yeast form over the mycelial form may reflect different metabolic requirements for the two forms independent of the growth medium. Because the yeast phase represents the form of *H. capsulatum* found in the host, expression of these genes could reflect the ability of the yeast form to adapt to growth-limiting environments during infection.

Other interesting yeast-regulated genes included those that may affect aspects of the cell cycle, such as *BUB1*, which encodes a spindle assembly checkpoint kinase (Roberts *et al*., 1994), and *SMC1*, which is involved in sister chromatid cohesion (Strunnikov *et al*., 1993). The enhanced expression of these genes in the yeast phase may reflect differences between cell cycle processes in a unicellular yeast versus a multicellular mycelium. Additionally, there was markedly increased expression of an ortholog (*TRI11*) of trichothecene C-15 hydroxylase in yeast compared with mycelia. The ortholog of this gene from the plant pathogen *Fusarium sporotrichioides* encodes a cytochrome P450 monooxygenase required for production of the toxin trichothecene (Alexander *et al*., 1998). Trichothecene is thought to inhibit protein synthesis in many eukaryotes. The role of *TRI11* in the virulence of *H. capsulatum* yeast is unknown.

Comparison of Yeast and Mycelial Phase-regulated Genes with Genes Induced during Stationary Phase

The 500 yeast- and mycelial-phase–regulated clones were further characterized by examining their expression under other conditions. Analysis of the expression profile of these clones during stationary phase of yeast cells was useful for the following reasons: Because the mycelial cultures must be grown for extended periods, a subset of the putative mycelial-expressed genes might be genes that are induced during stationary phase independent of morphology. Additionally, because yeast-phase cells transform into mycelia if kept in stationary phase for extended periods (Maresca and Kobayashi, 1989), genes required for establishment of the mycelial phase might be induced in stationary-phase yeast cells. In contrast, genes that are required only for the maintenance of the mycelial phase might show no induction in stationary-phase yeast cells. A large culture of yeast-phase cells was inoculated on day 0 and the culture sampled daily from days 2 to 10. The culture grew exponentially until day 3 and then remained stationary. Morphologically, the cells remained in the yeast phase throughout the experiment. Poly-adenylated RNA was used to produce a labeled cDNA probe from each sample, and the gene expression profile of each time point was determined relative to the first logarithmic phase time point (day 2).

Cluster analysis, which groups genes that show similar expression profiles (Eisen *et al*., 1998), was used to determine which of the 500 phase-regulated genes were also transcriptionally regulated during stationary phase. This analysis revealed a variety of clusters, several of which are highlighted in Figure 3. Cluster 1 (Figure 3A) represents clones that were induced only in mycelial cells and not in stationary-phase yeast cells. In contrast, cluster 2 represents clones that were induced in both mycelial cells and stationary-phase yeast cells. By graphing the average fold change of all clones in each of the highlighted clusters for each time point, it is apparent that only the second cluster showed significant expression in stationary phase (Figure 3B).

Cluster 1 includes multiple clones encoding *FBC1*, *NIR1*, and *TYR1*, as well as the *MFS4* transporter. The expression of *WET1*, which falls outside cluster 1, was also restricted to mycelia. Because these genes were not induced in stationary phase, their expression was unlikely to reflect simple nutri-

Figure 3 (cont). time point was competitively hybridized to a microarray versus the reference. The stationary-phase experiment was normalized against the first time point (2-d culture, first column) as described in MATERIALS AND METHODS. In YvsM 1 (column 9) and YvsM 2 (column 10), yeast cDNA was labeled with Cy3 and mycelial cDNA was labeled with Cy5. The reverse fluorophore experiment (column 11) used the same RNA from YvsM 2; however, yeast cDNA was labeled with Cy5 and mycelial cDNA was labeled with Cy3. In the stationary-phase experiment, red indicates up-regulation and green indicates down-regulation. In YvsM experiments 1 and 2, red indicates differentially induced mycelial expression and green indicates differentially induced yeast expression. The color indications are switched for the reverse fluor experiment. Clusters of interest have been enlarged on the right and are described in the text. Changes in expression under twofold are in black. Missing data are represented as a gray box. For each enlarged cluster, all clones with annotation are labeled. B. Bar graph representation of average fold change in expression of all clones in clusters 1–5.

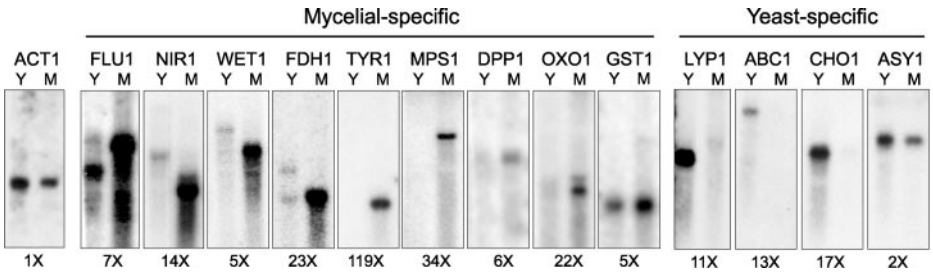

Figure 4. Northern analysis of phase-specific genes. Total RNA from yeast (Y) and mycelia (M) was probed using gene-specific probes. The fold expression change seen in the YvsM 1 microarray experiment is indicated below each Northern blot. *ACT1* is included as a control.

ent deprivation due to extended growth time. Instead, these genes may be expressed only once cells have switched to mycelial growth.

In contrast, cluster 2 includes clones that were induced in stationary phase of yeast cells as well as in the mycelial form. This cluster includes *FLU1*. Because the stationary-phase cultures contained only yeast cells and no mycelia or conidia, the expression of *FLU1* may precede mycelial conversion and the expression of *WET1* and *FBC1*, which are likely to be involved in production of conidia. The cluster also contains *SDH1* and *ABC4*, which encode sorbitol dehydrogenase and an ATP-binding cassette transporter, respectively.

Some of the yeast-specific clones also showed informative expression patterns during stationary phase. Cluster 3 represents yeast-specific clones that become even more highly expressed in stationary-phase yeast cells compared with mid-logarithmic yeast cells. This cluster includes *CHO1*, *LYP1*, *TIF3*, *TRI11*, *SIP1*, and *ABC3*. Because these genes were induced in stationary-phase yeast cells but were not highly expressed in mycelia, they may reflect nutrient requirements that are specific to yeast-phase cells. Genes such as *TRI11* could be induced as the density of the yeast culture increases if quorum sensing regulates production of a toxin.

Because coregulated genes cluster together over a variety of conditions, we were able to draw some conclusions about the regulation of phase-specific genes by examining the content of different clusters. Most notably, the previously identified yeast-specific genes *yps-3* and *CBP1* fall into two different clusters (Figure 3, A and B, cluster 4 and 5). Although each of these genes was significantly more expressed in yeast cells than mycelia, *yps-3* was repressed as yeast cells went into stationary phase, whereas the expression of *CBP1* remained constant as yeast cells aged. This observation was consistent with the prior suggestion that *yps-3* and *CBP1* are subject to different regulation (Rooney *et al*., 2001).

Finally, two other clusters were identified. First, clones that contain rDNA sequence were expressed more strongly in yeast than in mycelia and were repressed in stationary-phase yeast cells. These results likely reflect a difference in metabolic activity among mycelial cells, mid-logarithmic yeast cells, and stationary-phase yeast cells. Second, a group of array clones contains regions of DNA homologous to retrotransposon sequences, which are frequent in the *H. capsulatum* genome (Mardis, unpublished data). These sequences exhibit increased expression in the yeast form over the mycelial form. It is unclear whether these transposons are active and whether expression of these genes correlates with transposition.

Confirming Differential Expression with Northern Analysis

Northern analysis was used to confirm the phase regulation of a subset of the genes described above. Although most of the annotated clones clearly contained a single ORF, a subset of array clones (*FLU1*, *NIR1*, *WET1*, *FDH1*, *TYR1*, *MPS1*, *DPP1*, *OXO1*, *GST1*, *LYP1*, *ABC1*, *CHO1*, *MS95*, and *ASY1*) contained significant amounts of flanking sequence in addition to the complete or partial homologous ORF that specified the annotation. For these clones (with the exception of *MS95*), the annotation was confirmed by designing ORF-specific probes for each of the previously mentioned genes. Northern analysis of yeast and mycelial total RNA confirmed that these genes were differentially expressed (Figure 4). *FLU1*, *NIR1*, *WET1*, *FDH1*, *TYR1*, *MPS1*, *DPP1*, *OXO1*, and *GST1* were more highly expressed in mycelia than in yeast, whereas *LYP1*, *ABC1*, *CHO1*, and *ASY1* were more highly expressed in yeast than in mycelia.

Molecular Basis of Difference in Transcript Size

Surprisingly, Northern analysis revealed that, in addition to their differential expression, six of the nine mycelial-regulated genes (*FLU1*, *NIR1*, *WET1*, *FDH1*, *DPP1*, and *OXO1*) exhibited differences in transcript size in yeast and mycelia. To determine whether this difference was due to differential transcription initiation sites, poly-adenylation sites, or splicing, we used 5′ RLM-RACE to map the transcription initiation sites of capped transcripts and 3′ RACE to map the sites of poly-adenylation for *FLU1*, *NIR1*, *WET1*, and *FDH1*. In addition, RT-PCR was used to clone the full-length cDNAs from the yeast and mycelial forms. The sequence of the full-length cDNAs as well as the 5′ and 3′ RACE products was compared with the genomic sequence to determine the location of introns. For all of these genes, we observed major differences in the length of the 5′ untranslated region (UTR), likely due to differences in the start site of transcription (Figure 5B); these differences are reflected by the size of the 5′ RACE products from yeast and mycelia (Figure 5A). The start site of the longest yeast transcripts ranged from between 0.8 and 1.5 kb upstream of the mycelial transcripts. In contrast, *TYR1*, a mycelial-specific gene that did not show an altered form in yeast cells by Northern, gave no evidence of an altered transcript by 5′ RACE analysis (Gebhart and Sil, unpublished data). In addition to altered transcription initiation sites, the yeast-form transcripts of *WET1*, *FDH1*, and *NIR1* have introns in their long 5′UTRs. *FLU1, FDH1*, and *NIR1* had short 5′ UTR transcripts in the yeast form in addition to the long 5′ UTR transcripts. In the case of *NIR1*,

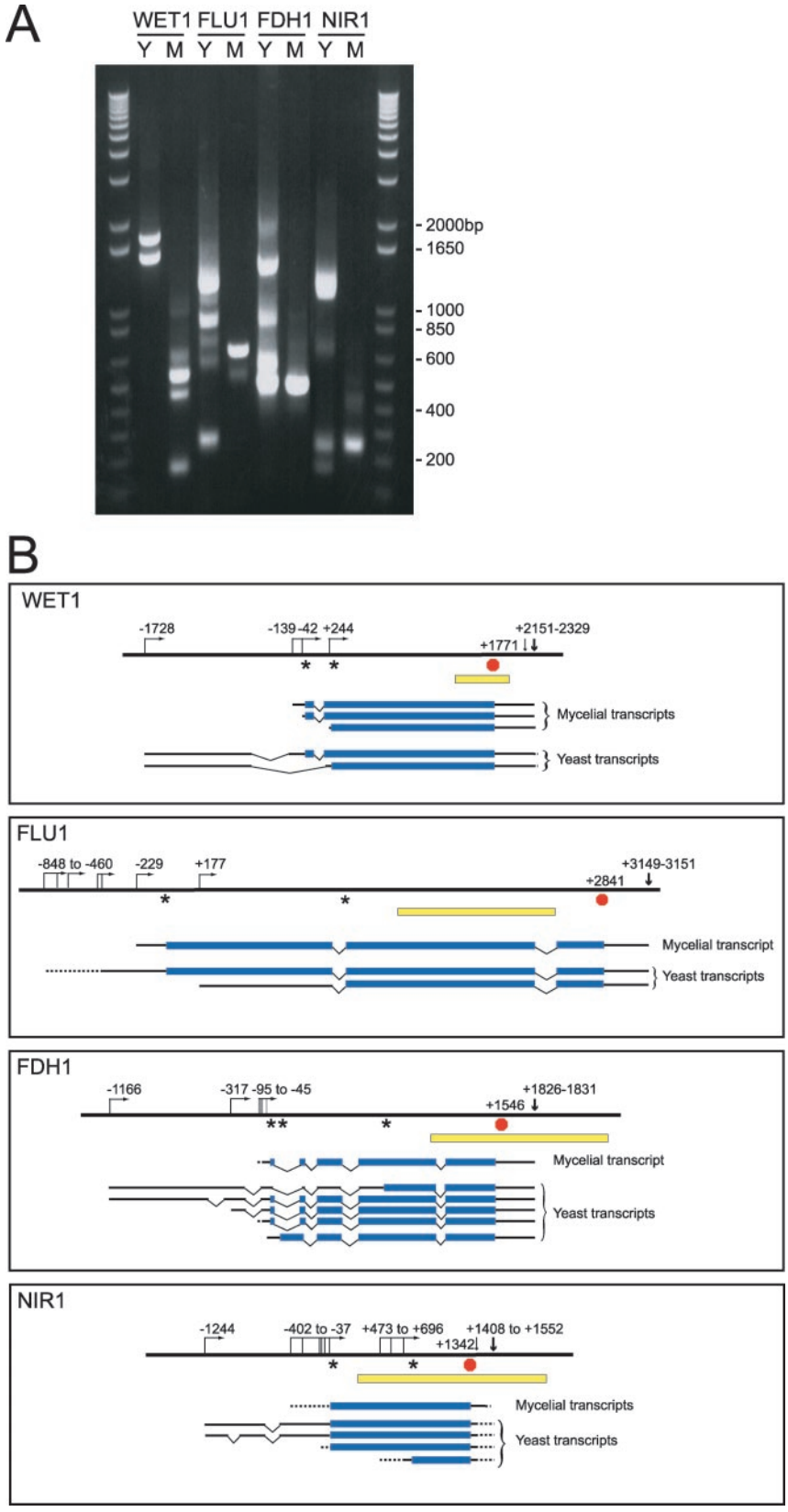

Figure 5. Differential site of transcript initiation for mycelial-induced genes. (A) The 5′ ends of intact, capped yeast poly-A RNA (Y) or mycelial poly-A RNA (M) for *WET1*, *FLU1*, *FDH1*, and *NIR1* were amplified using 5′ RLM-RACE, as described in MATERIALS AND METHODS. These PCR products were run on a 1% agarose gel. (B) Schematic of genomic DNA and mRNA structures, determined by 5′ RLM-RACE, 3′ RACE, and RT-PCR. Genomic DNA is at the top of each gene diagram represented by thick, black lines. Transcription initiation sites are marked by right arrows, poly-adenylation sites by vertical arrows. Thick vertical arrows indicate multiple closely spaced poly-adenylation sites. Potential initiation codons are indicated by asterisks. The first nucleotide of the first in-frame translation initiation codon is designated as +1. Downstream translation initiation codons are included to indicate the first in-frame ATG in the transcripts that initiate downstream of the + 1 ATG, or when differential splicing makes the + 1 ATG out of frame (*FDH1*, bottom yeast transcript). The stop codon is marked by a red octagon. Exons are represented by a blue bar, introns by a "V" that falls below the level of the exon, and 5′- and 3′-UTR sequences by thin, black horizontal lines. Thin, dashed black lines indicated multiple initiation or poly-adenylation sites. The yellow bar delineates the sequence of the spot represented on the microarray.

this short yeast-form transcript may encode a cytosolic nitrite reductase rather than the larger predicted membrane-bound form encoded in mycelia.

Although minor differences at the 3′ ends of the transcript were revealed by sequencing the 3′ RACE clones, these differences contributed to, but could not account for, the large difference in transcript size between yeast and mycelia. Similarly, differential splicing was observed (Figure 5B) but did not account for the large difference in transcript size. Thus, the major mechanism underlying differential transcript size between yeast and mycelia was the use of different transcript initiation sites. These observations suggest that *H. capsulatum* uses both differential expression and differential usage of transcript initiation site to regulate the transcriptional profile of the two morphological forms.

DISCUSSION

We used a genomic shotgun array to identify genes with phase-specific patterns of gene expression in the pathogenic fungus *Histoplasma capsulatum*. When this work began, the sequence of the *H. capsulatum* genome was unknown. Our microarray data, in conjunction with sequence information from array clones and the sequence generated by the ongoing genome project, allowed the identification of genes whose expression was induced in different morphological phases of the organism. This work demonstrates the utility of a genomic shotgun array for identification of genes that exhibit differential expression patterns and for the identification of regulatory circuits in the absence of complete genome sequence information.

We sorted the phase-regulated genes identified herein into broad functional categories (Figure 6). Yeast and mycelial cells differ in morphology, sulfur metabolism, growth rate, growth environment (host vs. soil), melanin production, mating competence, and conidiation. Based on the function of their orthologs (Table 1), we have identified phase-regulated genes that are implicated in several of these processes (Figure 6). Because the ability of yeast cells to survive in the phagosome of host cells may be dependent on nutrient acquisition, we hypothesize that genes that may affect growth rate might also affect host survival (Figure 6). A deeper analysis of the genes identified in this study is necessary before their function can be determined.

It may be particularly informative to examine the function of the yeast-specific genes involved in sulfur metabolism. Although little is understood about the regulation of the morphological phases, for *H. capsulatum* and other systemic dimorphic fungi, the addition of exogenous sulfhydryl reducing agents (dithiothreitol) to the media traps cells in the yeast form independent of temperature, whereas the addition of sulfhydryl oxidizing agents (*p*-chloromercurphenisulphonic acid) traps cells in the mycelial form independent of temperature (Maresca *et al*., 1977; Medoff *et al*., 1986, 1987) (Figure 6). Presumably, the effectors and downstream targets of these regulatory pathways will emerge from examining gene expression in response to dithiothreitol and *p*-chloromercurphenisulfonic acid. Disrupting the genes involved in sulfur metabolism identified by this work will test the contributions of these genes to the two growth forms.

We expect that these findings on *H. capsulatum* will be relevant to other fungi. There have been only a few published examples of gene expression profiling by microarray in fungal

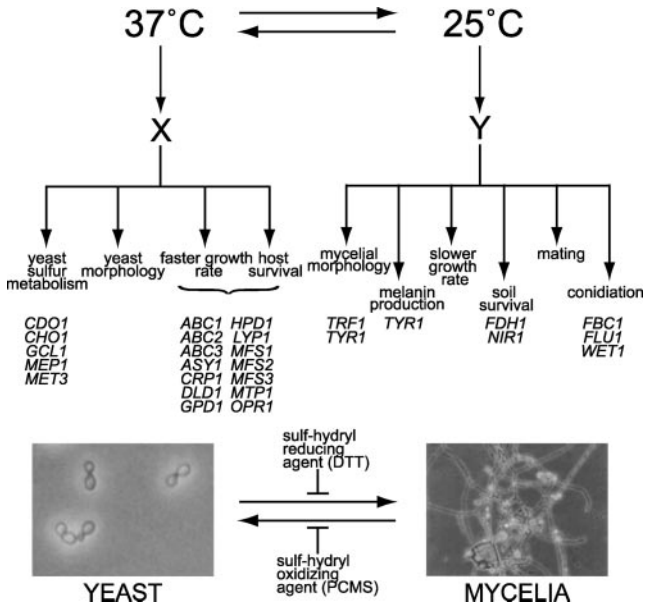

Figure 6. Model for the proposed function of differentially expressed genes. X and Y represent multiple components of an unknown signal transduction pathway that responds to temperature. Some of the known phenotypes of the yeast and mycelial forms are represented downstream of X and Y, respectively. The targets of sulfhydryl-oxidizing and -reducing agents are unknown. Annotation for each gene can be found in Table 1.

pathogens (mainly *Candida albicans*) (De Backer *et al*., 2001; Kahmann and Basse, 2001; Murad *et al*., 2001; Lan *et al*., 2002; Lorenz, 2002; Rogers and Barker, 2002), and no examples of large-scale microarray studies in the systemic dimorphic fungi (*H. capsulatum*, *Coccidioides immitis*, *Blastomyces dermitiditis*, *Paracoccidioides brasiliensis*, and *Penicillium marneffii*). Because alterations in physiology and morphology play key roles in the pathogenesis of many fungi, the genes and regulatory circuits we have identified will be relevant to understanding similar switches in other species. For example, we have identified orthologs of three *Aspergillus* regulatory factors required for conidiation: *fluG*, which initiates conidiophore development; *wetA*, whose expression is both necessary and sufficient for conidia-specific gene expression; and *flbC*, which encodes a zinc-finger transcription factor that is thought to affect the timing of conidiation (Marshall and Timberlake, 1991; Lee and Adams, 1994; Wieser *et al*., 1994). The expression patterns of the *FLU1/fluG*, *WET1/wetA*, and *FBC1/flbC* orthologs from *Histoplasma* and *Aspergillus* suggest the evolutionary conservation of conidial development from mycelial cells. This hypothesis is strengthened by the presence of orthologs of other genes required for conidiation in *Aspergillus*, such as *brlA* and *flbA* (Wieser *et al*., 1994) in the *H. capsulatum* genome. Further annotation of the genes identified in these experiments will deepen these insights.

In addition to differential gene expression, we observed further regulation of a subset of phase-regulated genes. Several genes predominantly expressed in mycelia unexpectedly displayed modest accumulation of transcripts of significantly altered size in the yeast phase. This phenomenon arose from different sites of transcript initiation in yeast and

mycelia. The underlying rationale for the production of longer yeast transcripts, some of which initiated >1 kb upstream of the mycelial transcript, is unclear. Perhaps the long 5′ UTR might be refractory to translation at 37°C but permissive for translation at 25°C. Such a mechanism might allow the initial production of mycelial-specific proteins by yeast cells that are shifted to 25°C until the normal mycelial transcript is produced. Temperature-dependent regulation of translation of a 5′ UTR has been observed recently in the bacterial pathogen *Listeria monocytogenes* (Johansson *et al*., 2002). Translation of transcripts with particular 5′ UTRs can also be influenced by the abundance of translation initiation factors (Browning *et al*., 1988; Calkhoven *et al*., 2000). Interestingly, we observed two translation initiation factors, eIF2 and eIF3, that were significantly more expressed in the yeast form over the mycelial form.

At present, the mechanism of generating the longer 5′ UTR transcripts is unknown. Our observations suggest that an *H. capsulatum* ortholog of the *SPT6* gene might be significantly expressed in mycelia compared with yeast. In *S. cerevisiae*, *SPT6* can influence the site of transcription initiation (Clark-Adams and Winston, 1987). Future experiments will be necessary to determine the prevalence, implications, and mechanism of this regulation of transcript initiation in *H. capsulatum*.

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