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

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Submitted January 20, 2002; Revised February 13, 2003; Accepted February 19, 2003

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
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% CO2. 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 HMM broth, with passage of cells every 2–3 d at 1:25 dilution. For yeast form, cultures were shaken in HMM broth at 37°C on a stationary platform for 4 days 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 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 K5+ precut with BamHI. 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'-GTTC-

TCCAGTCAAGAC-3') and M13-reverse (5'-GGCGATAACATT-TCAACAGG-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 H2O.

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-32P]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 2X 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 400B scanner (Axon Instruments, Foster City, CA) and analyzed using GENEPX 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-
measurements 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 $1 \times 10^{-6}$ 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.

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Figure 1. Construction and content of microarray. (A) Construction of microarray. A genomic library was constructed from strain G217B genomic DNA partially digested with Sau3AI (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.
mid-logarithmic phase at 37°C. Mycelial cells were grown
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H. capsulatum
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To compare the gene expression pro
Clones
Identi
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cover approximately one-third of the genome. For purposes
on the array, and the average size of the clones, the array
Based on the estimated genome size, the number of clones
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. Polyadenylated 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.
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. Phase-regulated genes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Annotation</th>
<th>E-value</th>
<th>Accession no. of ortholog</th>
<th>M/Y ratio</th>
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- E-value comparing *H. capsulatum* gene and its ortholog.
- Ratio of expression from YvsM 1 experiment.
- Mycelial-phase specific 1.
- 127bp of the MS95 open reading frame overlaps with the microarray clone. A second open reading frame may be represented on the clone.
HST4, were also induced in mycelia. The latter is a presump-
tive transcript, such as orthologs of the splicing factor
GPD1, Glycero-3-phosphate dehydrogenase
TRI11, Trichothecene C-15 hydroxylase
CHO1, Choline sulfatase
LOM1, Ornithine-N5-oxygenase
ABC1, ABC transporter 1
LYP1, Lysine permease
CD01, Cysteine dioxygenase
PLC1, Nonhemolytic phospholipase C

Finally, we identified a set of genes that might reflect the uniquophysiology of the mycelial form. One of these, NRI1, encodes a nitrite reductase most similar to the Neisseria gonorrhoeae 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 pyro-phosphate phosphatase, and OX1, which encodes a 3-oxoacyl reductase) whose differential expression might

Table 1. Continued

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<thead>
<tr>
<th>Gene name</th>
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<th>E-value</th>
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<th>M/Y ratio</th>
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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 _MF54_ 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-
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 TIF3, LYP1, ABC1, CHO1, 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.

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 YsM 1 microarray experiment is indicated below each Northern blot. ACT1 is included as a control.

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, NRI1, WET1, FDH1, TYR1, MIPS1, DPP1, OXO1, GST1, LYP1, ABC1, CHO1, MS59, 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 MS59), 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, NRI1, WET1, FDH1, TYR1, MIPS1, 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, NRI1, 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, NRI1, 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 NRI1 have introns in their long 5’UTRs. FLU1, FDH1, and NRI1 had short 5’ UTR transcripts in the yeast form in addition to the long 5’ UTR transcripts. In the case of NRI1,
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'/H11032 fungus patterns of gene expression in the pathogenic phase-specific si.

We used a genomic shotgun array to identify genes with bound form encoded in mycelia. This short yeast-form transcript may encode a cytosolic nis.

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**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, which 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-chloromercuriphenylsulfonic 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-chloromercuriphenylsulfonic 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 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, Coccioides immitis, Blastomyces dermatitidis, Paracoccidioides brasiliensis, and Penicillium marneffei*). 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 conidial development; 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.

ACKNOWLEDGMENTS

We thank Ira Herskowitz for the inspiration behind this work. We are indebted to William Goldman and Linda Eisenberg for generously providing strains and expert advice. We thank Joseph DeRisi and Holly Bennett for invaluable assistance with all aspects of microarray construction, experimental design, execution, and data analysis. We thank Dale Webster for making it possible to annotate the array clones discussed in this article. We thank Incyte Genomics for the Discovery Grant (awarded to L.H.) that funded both the initial end-sequencing of the array clones, and BLAST analysis of these sequences. We are extremely grateful to Elaine Mardis, Michael Kobor, and members of the Sil laboratory for their advice and expert help. We are indebted to William Goldman and Linda Eissenberg for generously providing strains and expert advice. We thank Joseph DeRisi and Holly Bennett for invaluable assistance with all aspects of microarray construction, experimental design, execution, and data analysis.

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