TUP1, *CPH1* and *EFG1* Make Independent Contributions to Filamentation in *Candida albicans*

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ABSTRACT

The common fungal pathogen, *Candida albicans*, can grow either as single cells or as filaments (hyphae), depending on environmental conditions. Several transcriptional regulators have been identified as having key roles in controlling filamentous growth, including the products of the *TUP1*, *CPH1*, and *EFG1* genes. We show, through a set of single, double, and triple mutants, that these genes act in an additive fashion to control filamentous growth, suggesting that each gene represents a separate pathway of control. We also show that environmentally induced filamentous growth can occur even in the absence of all three of these genes, providing evidence for a fourth regulatory pathway. Expression of a collection of structural genes associated with filamentous growth, including *HYR1*, *ECE1*, *HWP1*, *ALS1*, and *CHS2*, was monitored in strains lacking each combination of *TUP1*, *EFG1*, and *CPH1*. Different patterns of expression were observed among these target genes, supporting the hypothesis that these three regulatory proteins engage in a network of individual connections to downstream genes and arguing against a model whereby the target genes are regulated through a central filamentous growth pathway. The results suggest the existence of several distinct types of filamentous forms of *C. albicans*, each dependent on a particular set of environmental conditions and each expressing a unique set of surface proteins.

CANDIDA albicans causes common mucosal infections of varying severity in both normal and immunocompromised patients, as well as life-threatening systemic infections in immunocompromised patients (Odds 1988; Fidel and Sobel 1996). The capability of growing in a spectrum of morphologies, ranging from ellipsoidal (yeast form) blastospores to true hyphae (filaments), has been proposed to be a specialized virulence factor (reviewed by Odds 1994; Kobayashi and Cutler 1998). Many conditions induce filamentous growth in *C. albicans*, though only a few have been well characterized. Mammalian serum is a potent inducer of filamentous growth (particularly of the nascent form known as germ tubes), as are a temperature of 37° and neutral pH. Filaments are characteristically seen in tissue samples from disseminated infections and have been observed invading mucosal membranes (reviewed by Gow 1997). Certain forms of nutrient deprivation on solid media (such as Spider agar, cornmeal agar, and milk-tween agar) prompt a slower filamentous growth response that can be conveniently studied in the laboratory (Liu *et al.* 1994; Gale *et al.* 1998).

The recent advent of molecular genetics in *C. albicans* has allowed several genes to be identified that specifically influence filamentous growth. These genes include *EFG1* (Stoldt *et al.* 1997), *TUP1* (Braun and Johnson

1997), *CPH1* (Liu *et al.* 1994), *INT1* (Gale *et al.* 1998), *PRA1* (Sentandreu *et al.* 1998), and *RBF1* (Ishii *et al.* 1997). In addition, mitogen-activated protein (MAP) kinase pathway members *CEK1* (Csank *et al.* 1998), *CST20* (Köhler and Fink 1996), *HST7* (Leberer *et al.* 1996), *CPP1* (Csank *et al.* 1997), and *RAS1* (Feng *et al.* 1999) that impinge on *CPH1* have been identified (for reviews, see Mitchell 1998; Brown and Gow 1999). In previous work, we identified *TUP1* from *C. albicans* and showed that deletion of both copies in the diploid organism generated cells that are always filamentous (Braun and Johnson 1997). Based on close sequence similarity and its complementation of the phenotypes of the well-characterized repressor *TUP1* of *Saccharomyces cerevisiae*, the Candida *TUP1* is very likely to be a transcriptional repressor. In *S. cerevisiae*, regulated DNA-binding proteins direct the *TUP1*-containing complex to target genes (Keleher *et al.* 1992; Komachi *et al.* 1994; Varanasi *et al.* 1996; Treitel *et al.* 1998). Based on these considerations, we hypothesized that *C. albicans TUP1* represses genes responsible for carrying out filamentous growth.

Conversely, *EFG1* and *CPH1* appear to be transcriptional activators of filamentous growth, as deletion of either one causes decreased filamentous growth. *CPH1*, a homeodomain protein, is known to be downstream of a MAP kinase cascade in *C. albicans* (Csank *et al.* 1998). This cascade resembles the pseudohyphal growth and pheromone response MAP kinase cascade of *S. cerevisiae* that acts through the *CPH1* orthologue, *STE12* (Madhani and Fink 1998a,b). The *cph1Δ* mutant of *C.*

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TABLE 1

C. albicans strains used in this study

	Abbreviation	Parent	Genotype	Source
SC5314	wt		ura3Δ::imm434/URA3	Fonzi and Irwin (1993)
CAF2-1	wt	SC5314	ura3Δ::imm434/ura3Δ::imm434 (termed "ura3Δ/ura3Δ" below)	Fonzi and Irwin (1993)
CAI4	ura3Δ	CAF2-1		Fonzi and Irwin (1993)
JKC19	cph1Δ	CAI4	cph1Δ::hisG/cph1Δ::hisG-URA3-hisG, ura3Δ/ura3Δ	Liu et al. (1994)
BCa2-9	tup1Δ(ura3)	CAI4	tup1Δ::hisG/tup1Δ::hisG, ura3Δ/ura3Δ	Braun and Johnson (1997)
BCa2-10	tup1Δ	BCa2-9	tup1Δ::hisG/tup1Δ::hisG::p405-URA3, ura3Δ/ura3Δ	Braun and Johnson (1997)
BCa9-4	efg1Δ	CAI4	efg1Δ::hisG/egfΔ::hisG-URA3-hisG, ura3Δ/ura3Δ	This work
BCa4-3	tup1Δ, cph1Δ	JKC19	cph1Δ::hisG/cph1Δ::hisG, tup1Δ::hisG/tup1Δ::hisG-URA3-hisG, ura3Δ/ura3Δ	Braun and Johnson (1997)
BCa10-3	tup1Δ, efg1Δ	BCa2-9	efg1Δ::hisG/efg1Δ::hisG-URA3-hisG, tup1Δ::hisG/tup1Δ::hisG, ura3Δ/ura3Δ	This work
BCa13-2	tup1Δ, efg1Δ, cph1Δ	BCa4-3	efg1Δ::hisG/efg1Δ::hisG-URA3-hisG, cph1Δ::hisG/cph1Δ::hisG, tup1Δ::hisG/tup1Δ::hisG, ura3Δ/ura3Δ	This work

albicans shows delayed initiation of filamentous growth on Spider plates, relative to wild-type cells, but shows wild-type levels of filamentous growth in response to liquid serum treatment (Liu *et al.* 1994). In contrast, *efg1*Δ mutant cells are strongly attenuated in responding to serum, showing no germ tubes or hyphae within 2 hr of being introduced to 20% serum in YPD at 37° (Lo *et al.* 1997). They show variable filamentation defects in response to other stimuli as well as other abnormalities of growth and morphology (Lo *et al.* 1997; Stoldt *et al.* 1997). The Efg1p protein contains a basic helix-loop-helix DNA-binding motif similar to *Myc*-related proteins and may have both activation and repression activities. By analogy to its relatives in *S. cerevisiae* (*PHD1* and *SOK2*), *EFG1* has been proposed to lie downstream of a cAMP signaling system (Stoldt *et al.* 1997). The complementary phenotypes of *cph1*Δ and *efg1*Δ mutants prompted Lo *et al.* to make a double mutant. They found that filamentous growth was abolished and suggested that *EFG1* and *CPH1* together account for the pathways that activate filamentous growth in *C. albicans* (Lo *et al.* 1997).

Since deletion of *TUP1* caused constitutive filamentous growth while deletion of *CPH1* and *EFG1* abolished filamentous growth, we combined deletions in all three genes to determine their epistatic relationships. In this article we describe this set of mutant strains and assay their phenotypes in terms of colony morphology, cell morphology, and expression of a battery of filament-specific genes. The experiments delineate the relative roles of each regulatory gene in filamentous growth and suggest the existence of an additional pathway of filamentous growth induction. In addition, the results demonstrate that downstream genes respond differently to the different regulatory pathways.

MATERIALS AND METHODS

Strains and culture conditions: The strains used are listed in Table 1. *cph1*Δ strain JKC19 was generously provided by G. Fink and colleagues (Liu *et al.* 1994). Disruption strains were produced by integrating a *URA3*-marked disruption cassette into the genome (see next section for DNA constructions) by lithium acetate transformation and homologous recombination (Gietz *et al.* 1995; Braun and Johnson 1997). Transformants were screened by PCR to verify the correct integration of each end of the disruption cassettes and the removal of the wild-type gene (Liu *et al.* 1994).

Routine growth was on YPD plates and liquid at 30°, and selections for *URA3* prototrophy were performed on minimal SD plates (Guthrie and Fink 1991). Counter-selections against *URA3* were performed on uridine and 5-fluoroorotic acid-containing SD plates (Fonzi and Irwin 1993). Spider (Liu *et al.* 1994) and minimal SD plates were used for the colony growth assays, and the plates were incubated in moist, well-humidified sleeves at 30°. We have found that humidity strongly affects the degree of hyphal growth obtained on Spider plates. For gene expression analysis, YPD (30°) was used to pregrow cells to saturation overnight. Portions of either 2.5 or 5 ml were then innoculated to 100 ml of YPD (30°), Spider

liquid media (37°), and YPD with 10% fetal calf serum (37°) and growth allowed for 2 hr (Spider medium) or for 1 hr (YPD and YPD plus serum). Cells were harvested at an OD_{600} of ~2.0.

Plasmids and strain construction: We followed the technique of Fonzi and Irwin (1993) to delete genes in *C. albicans*, with small modifications. Following an idea of Perez-Martin *et al.* (1999), a derivative of pMB7 (Fonzi and Irwin 1993) was made to allow the *hisG-URA3-hisG* cassette to be cloned in two opposite orientations within the target gene flanks. The two resulting disruption plasmids allow the sequential disruption of two alleles to be followed by PCR that identifies the four unique new ends (Perez-Martin *et al.* 1999). pBB510 was derived from pMB7 by filling in the *Bam*HI sites near the *URA3* marker and 250 bp downstream of the *Sal*I-containing polylinker sequence, leaving a unique *Bam*HI site next to the *Sal*I site and also inserting an unphosphorylated *Nsi*I linker (5′gatccatgcatca/5′gatctgatgcatg) at the *Bgl*II site. This produced a vector where either *Bam*HI + *Nsi*I or *Bgl*II + *Pst*I can be alternately used to force the central *hisG-URA3-hisG* cassette into opposite orientations relative to the gene-specific flanking fragments in a four-way ligation. For the disruption of *EFG1*, DNA sequences flanking the gene were obtained from wild-type genomic DNA by PCR, using the following primers: BBo238, 5′tttgcctccAAGCTTagttgctca (*Hin*dIII); BBo239, 5′tagttataaggCTGCAGGgactgca (*Pst*I); BBo240, 5′gcaggaactAGATCTgtgcacca (*Bgl*II); and BBo241, 5′cctgcttGGTACCtcatgtctta (*Kpn*I). Primers BBo238 and BBo239 generate a 347-bp fragment from the region upstream of *EFG1* (later reduced to 222 bp by an internal *Pst*I site), and BBo240 and BBo241 generate a downstream flanking fragment of 650 bp. These fragments were cut with the enzymes matching their respective ends and cloned for the first orientation into pMB7 (Fonzi and Irwin 1993) cut with *Hin*dIII *Pst*I, *Bgl*II, and *Kpn*I (creating pBB503) and for the second orientation into pBB510 cut with *Hin*dIII, *Bam*HI, *Nsi*I, and *Kpn*I (creating pBB515). For transformation of *C. albicans*, either pBB503 or pBB515 was cut with *Hin*dIII and Asp 718, phenol extracted and ethanol precipitated, and transformed as stated above.

Transformants were checked by PCR, using the following primers that anneal to regions of the *EFG1* locus outside the cloned flanking regions: BBo242, 5′catatttgtaccttccgcattaga; BBo243, 5′attcattaccaggcgtgtttatta; and two primers that anneal to the *hisG* repeats flanking the *URA3* cassette, URA 3KO-2 and URA 3KO-4 (Perez-Martin *et al.* 1999). After setting up the reaction mixes with various primer pairs, 1–2 μl of template cells was briefly ground against the wall of the tube with a sterile yellow pipettman tip, then dispersed into solution. PCR conditions were 1.5 min at 95°, then 32 cycles of 15 sec at 95°, 30 sec at 52°, 2 min at 72°. The resulting bands were diagnostic of each end of the desired genomic insertion. The diagnostic PCR was repeated on positive colonies after purification by restreaking to confirm the newly constructed ends, and for strains deleted for both *EFG1* copies, PCR was done with intragenic primers to confirm that the native gene had been removed.

RNA expression analysis: Appropriate primer pairs to generate unique DNA probes were made using information from the literature, gene databases, or our own sequencing for the *RBT* and *WAP1* genes. The identification of the *RBT* series of genes, which were isolated in a molecular screen for *TUP1*-repressed genes, will be reported elsewhere. PCR was performed on SC5314 genomic DNA, the resulting product (from 500 bp to 1 kb long) purified with a QIAquick column (QIAGEN, Chatsworth, CA), and a ^{32}P probe made using a random priming kit from Amersham Pharmacia (Arlington Heights, IL). *C. albicans* strains were grown as specified above and their whole RNA was isolated using hot phenol (Ausubel *et al.*

1992). RNAs were run in formaldehyde gels and capillary blotted onto nylon (GeneScreen) membranes. Identical aliquots of RNA were run on each gel, and stained rRNA was used to check each gel and blot for uniform loading and transfer. Blots were used only once, prehybridizing and hybridizing (Church and Gilbert 1984) at 67°, and washing at room temperature. All exposures shown were taken without an intensifying screen. Autoradiograms were digitized using a transmitted light scanner and their backgrounds were reduced slightly in Photoshop for presentation.

Photography: Colony micrographs were taken on a Leica (Deerfield, IL) M420 microscope with unidirectional illumination at zoom settings 5.6 (Spider plates) and 11.5 (SD plates). Cell micrographs were taken on a Leica DM RD microscope with ×100 objective and differential interference contrast optics.

RESULTS

Various combinations of *cph1Δ*, *efg1Δ*, and *tup1Δ* were constructed and their colony and cellular morphologies on both filament-inducing and noninducing media were observed (Figure 1). In our hands, the *cph1Δ* strain had a less dramatic impairment of filamentous growth on Spider agar than has been described previously (Figure 1, I and J; Liu *et al.* 1994), which could be due to slight variations in our formulation of Spider medium or in the incubation conditions.

Cells carrying deletions of *EFG1* behaved as described (Lo *et al.* 1997; Stoldt *et al.* 1997), showing slight growth defects, small and abnormally oblong cell bodies when grown in YPD (not shown), and a lack of filamentous growth in response to serum in liquid media. The *efg1Δ* strain also showed reduced filamentous growth on most but not all solid media (Figure 1K and data not shown). This mixture of defects and enhancements with regard to filamentous growth has been noted previously for *efg1Δ* cells (Stoldt *et al.* 1997). Combining *efg1Δ* with *cph1Δ* yielded a reduction in filamentous growth compared to the wild-type strain, as described (Lo *et al.* 1997; Figure 1L). We did find, however, in aggreement with others (Riggle *et al.* 1999) that these cells were still capable of weak filamentous growth, especially in response to incubation under coverslips on cornmeal agar plates (not shown). This suggested that both the capability for filamentous growth and one or more of the signaling pathways that activate it remain intact in *efg1Δ cph1Δ* cells.

The role of EFG1 in the morphology of the *tup1* mutant: Deletion of *EFG1* in the *tup1Δ* background led to a marked reduction in both constitutive and induced filamentous growth (Figure 1, G and O *vs.* E and M). Without environmental induction, a substantial proportion of the *efg1Δ tup1Δ* cells were nonfilamentous, as shown in the high-magnification micrograph and by the shiny (though wrinkled) surface of the colonies on SD plates (Figure 1G). This was in strong contrast to *tup1Δ* cells, which always grew as filaments (Figure 1E). On SD plates, the *tup1Δ* deletion strain consisted of highly
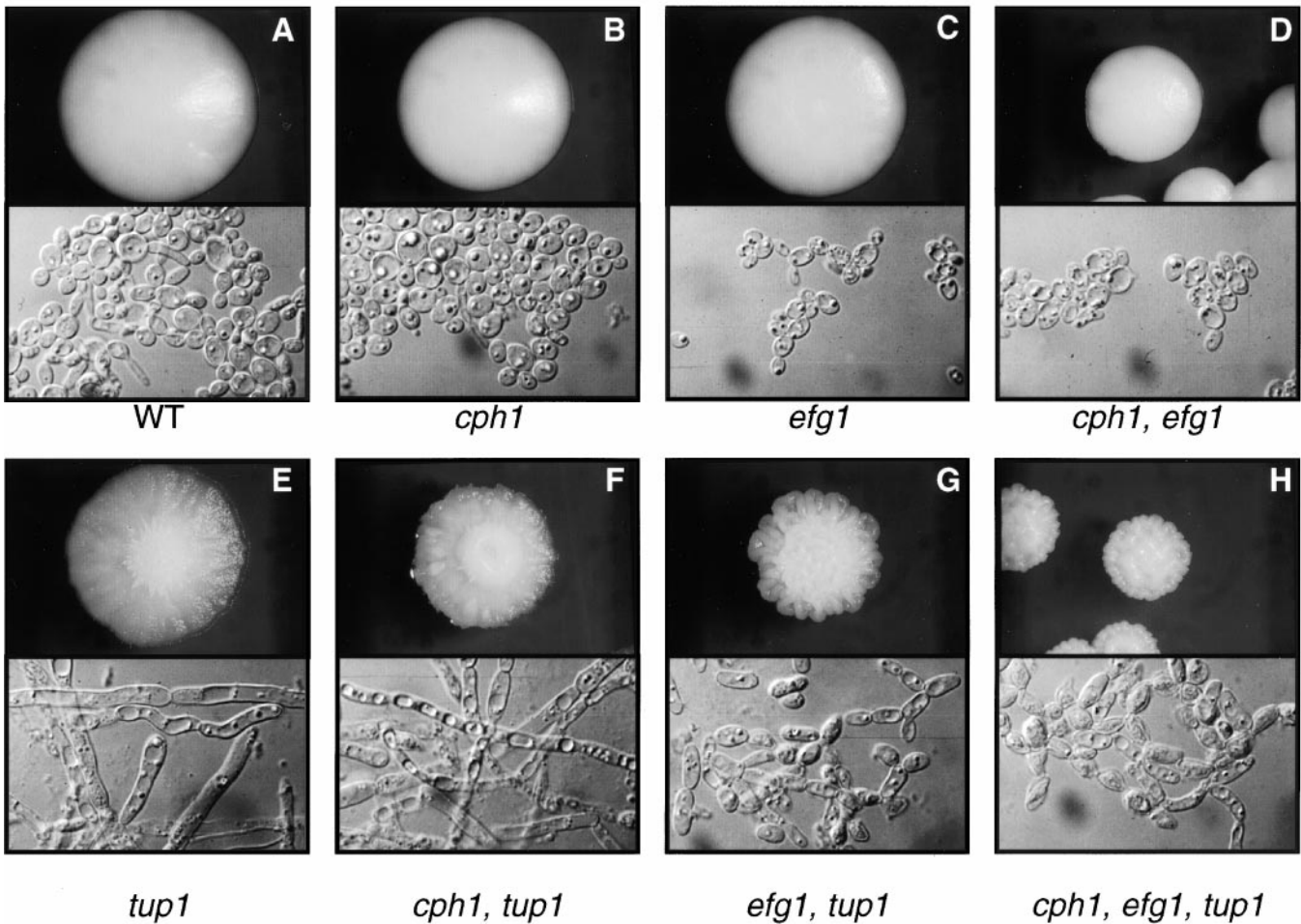
2X Non-inducing plate (SD)

Figure 1.—Colony and cell morphologies of mutants in *tup1*, *cph1*, and *efg1* indicate separate pathways of regulation. (A–H) colonies were grown on SD (minimal medium) agar for 7 days at 30°, conditions that do not induce filamentous growth, whereas for I–P, colonies were grown on Spider (inducing) plates under the same conditions. Whole colonies were scraped or excavated from the plates, ground briefly in an Eppendorf tube with a pestle to disperse clumps of filaments and agar, and mounted on a slide for microscopy. The relevant genotypes are noted below A–P—each gene refers to the deletion of both alleles, and WT refers to the wild-type strain used for comparison, CAF2-1, which carries one copy of the URA3 gene, as do all the mutant strains. Magnification in A–H (whole field, 8 mm wide) was twice that of I–P (whole field, 16 mm wide). The fields shown in the bottom halves of A–P correspond to 73 μm in total width.

elongated, strongly attached cells growing in a tangled mass with a seemingly infinite chain length despite the lack of hyphae projecting into the medium. In comparison, cells in colonies of the *efg1Δ tup1Δ* deletion strain were much shorter than the corresponding *tup1Δ* cells, and the strength of their cell-cell attachment appeared much weaker. Upon dispersion of the colony for microscopy, only about half of the cells were observed to be in chains. We have found a rough correlation between these aspects of filamentous growth and the degree of wrinkling of colonies, making colony morphology a convenient integrated assay of filamentous growth (Slutsky *et al.* 1985; Dutton and Penn 1989; Pesti *et al.* 1999).

After induction of filamentous growth on Spider

plates for a week at 30°, colonies of *efg1Δ tup1Δ* cells had a small, smooth central area of nonfilamentous cells, surrounded by a wide fringe of agar-invading filaments (Figure 1O). This fringe was distinctly smaller than that of either wild-type or *tup1Δ* colonies (Figure 1, I and M). These results indicate that the pathway represented by *EFG1* is required for much of the filamentous growth seen in *tup1Δ* cells under inducing environmental conditions and most of the filamentous growth seen under noninducing conditions. *efg1Δ tup1Δ* cells also showed no germ tube formation when transferred from YPD medium at 30° to YPD + 10% serum at 37° (not shown). In contrast, wild-type cells show uniform and rapid induction of germ tubes under these conditions. Since neither *efg1Δ* nor *tup1Δ* cells show any
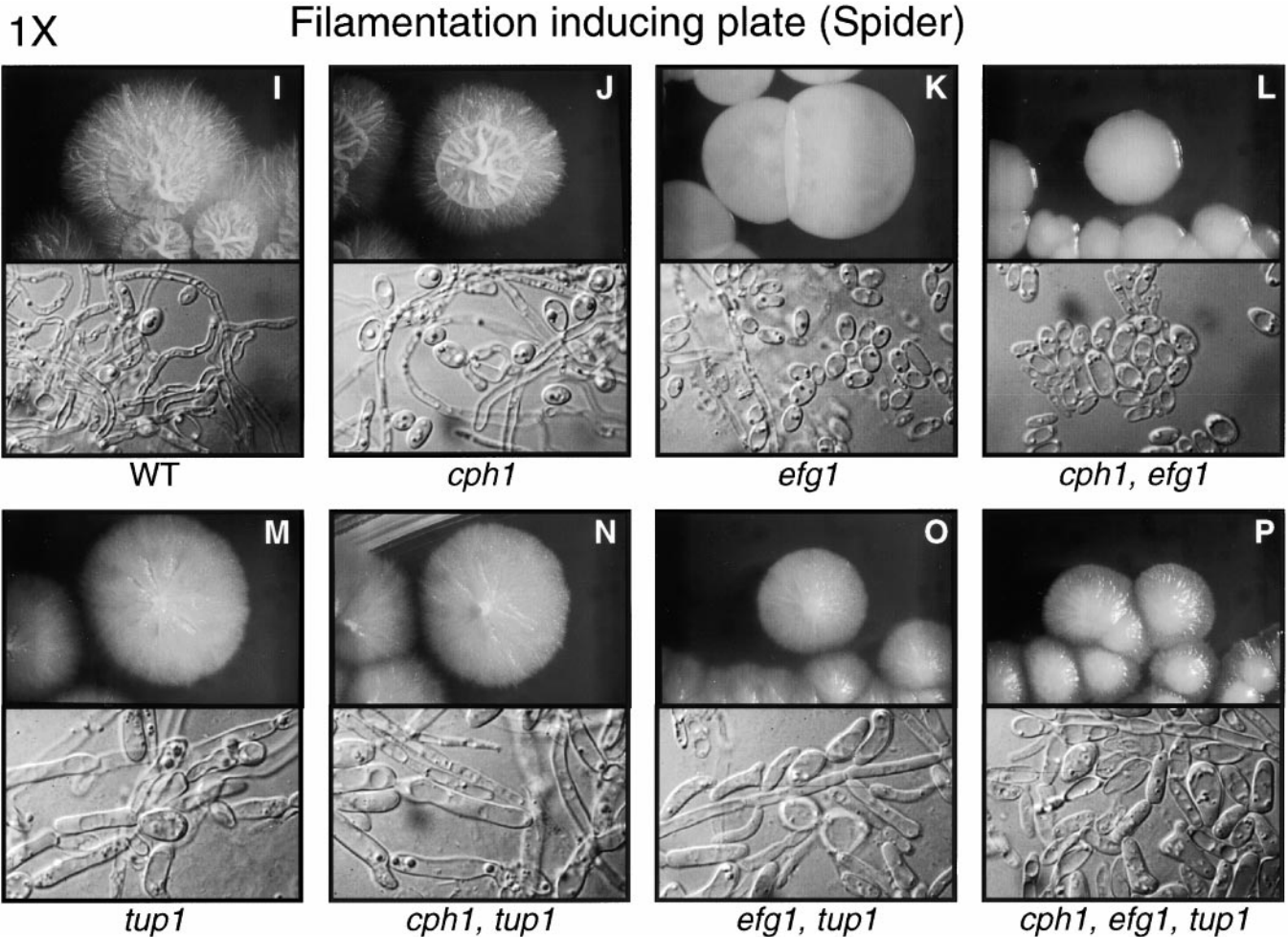
Filamentation inducing plate (Spider)

Figure 1.—*Continued.*

visible response under these conditions (Braun and Johnson 1997; Lo *et al.* 1997; and data not shown), this result was not surprising.

In previous work, we described a *cph1Δ tup1Δ* strain and noted that its morphology appeared identical to that of cells carrying the *tup1Δ* mutation alone (Braun and Johnson 1997). In this study we found that these strains were indeed distinguishable under certain conditions (Figure 1, E and F; SD plate for 7 days), and furthermore, that the additional deletion of *CPH1* detectably decreased the degree of filamentous growth of *efg1Δ tup1Δ* mutant cells (Figure 1, H and P). Colonies of *cph1Δ efg1Δ tup1Δ* cells grown on inducing (Spider) media had a large central patch of nonfilamentous cells surrounded by a small fringe of filamentous cells invading the agar. This fringe was much smaller than that of either wild-type, *tup1Δ*, *cph1Δ tup1Δ*, or *efg1Δ tup1Δ* colonies (Figure 1, compare M–P). This reduction in filamentation is consistent with previous work indicating that *CPH1* represents an environmentally responsive activating pathway that contributes to filamentous growth on Spider medium and that is independent of *EFG1* (Liu *et al.* 1994; Lo *et al.* 1997). More importantly, it

also indicates that the *TUP1* and *CPH1* pathways are separate and make additive contributions to filamentous growth, although the contribution from *CPH1* appears to be relatively small.

Even in the absence of environmental signals (on the noninducing SD medium), deletion of *CPH1* caused an additional reduction in hyphal growth, as indicated by the lower degree of wrinkling of the *cph1Δ efg1Δ tup1Δ* colonies compared with the *efg1Δ tup1Δ* colonies (Figure 1, G and H). This result was not simply due to differences in colony size, since the finding was consistent over other areas of these plates that had colonies in various sizes and densities and is consistent with the idea that the *CPH1*, *EFG1*, and *TUP1* pathways make additive contributions to filamentous growth.

Evidence for a fourth filamentous growth pathway: Triple mutant cells lacking the *CPH1*, *EFG1*, and *TUP1* regulators still exhibited a small amount of filamentous growth (Figure 1P). This filamentous growth was environmentally responsive (Figure 1, compare H and P), indicating that one or more signaling pathways that activate filamentous growth remained intact in the triple mutant. The slight filamentous growth seen under non-

inducing conditions (Figure 1H) could be due to an unregulated (basal) activity that has been uncovered by removal of *TUP1* repression.

Expression analysis of filament-specific genes: Since *TUP1*, *EFG1*, and *CPH1* each regulate filamentous growth, it seemed likely that their deletion should have corresponding effects on the expression patterns of filament-specific genes. Two types of models for these effects can be envisioned (Figure 3). In the first, "central processor" model, the regulatory pathways feed into a central pathway that monitors the strength of various inducing signals, integrates the signals, and expresses all the filament-specific genes accordingly. By this model, downstream genes should all behave in a parallel fashion in response to various inducing signals and should closely track the morphology of the cells. The second, or "network" model, proposes a diverse set of individual connections between the regulatory pathways and their filament-specific downstream target genes. This model predicts that a given downstream gene may respond to some, but not necessarily all, upstream pathways, and that their levels of expression may not necessarily coincide with the morphological state of the cell. This model also supposes that filamentous growth can arise from the expression of different sets of target genes.

To investigate these models, we tested the expression of a variety of hyphal-specific genes in the mutant strains described above. Several cell wall proteins have been found to be specifically expressed upon induction of hyphal growth, including *HWP1*, *ALS1*, *ECE1*, *CHS2*, and *HYR1* (Birse *et al.* 1993; Hoyer *et al.* 1995; McCreath *et al.* 1995; Bailey *et al.* 1996; Staab *et al.* 1996; Sharkey *et al.* 1999). In a screen to be described elsewhere, we have identified four additional genes that also show hyphal-specific regulation (*RBT1*, *RBT4*, *RBT5*, and *WAP1*). Other genes were chosen as controls, including *ACT1* (Losberger and Ernst 1989), encoding actin, *RBT2*, encoding a *TUP1*-regulated gene that is expressed both in blastospores and hyphae (also to be described elsewhere), and *CHT2*, encoding a chitinase that displays a regulatory pattern that is the converse of the other genes, showing greater expression in non-hyphal cells (McCreath *et al.* 1995). While the experimental genes were chosen because they are induced during filamentous growth, most other aspects of their regulatory profiles are unknown.

Figure 2 displays Northern blots of RNA from our mutant *C. albicans* strains, as noted across the top, hybridized to probes derived from the various genes mentioned above. Each strain was subjected to three growth conditions: uninduced (YPD at 30°) and two conditions of filamentous growth induction: YPD + 10% serum for 60 min at 37° after prior growth in YPD at 30° and liquid Spider medium at 37° for 120 min after prior growth in YPD at 30°. The two inducing conditions caused the majority of wild-type cells to grow germ tubes (nascent hyphae) up to three times the length of the cell body by

the time of harvesting (not shown). Both the ethidium-stained gel and the actin-probed blot (Figure 2, bottom) show that there were roughly equal amounts of RNA in the preparations that were used to load each well.

The first three lanes of each blot show the response of wild-type cells to each growth condition. Since the time of incubation was 2 hr in Spider medium and only 1 hr in YPD + serum, the differences that are seen for some genes in amount of induction between the two conditions (lanes b and c) may derive from this time difference as well as the difference in media. Liquid growth conditions have not yet been described that can simulate the slow starvation on solid media produced by Spider agar, which reveals the lack of filamentous growth in the *cph1Δ* mutant. The induction conditions used in Figure 2 were therefore not designed to accentuate the *cph1Δ* phenotype, and none of the gene expression profiles show significant dependence on *CPH1*. Overall, most of the genes depended on *EFG1* for activation and on *TUP1* for repression, as was true of the filamentous cell morphology itself. However, there were significant exceptions to this rule, and even among the genes regulated by *TUP1* and *EFG1*, there was surprising variation in the degree of response to deletions of these regulators, described in the next sections.

Dependence on *TUP1*: Deletion of *TUP1* caused derepression of most of the genes assayed. Since deletion of *TUP1* causes hyphal growth, it was not surprising that many hyphal-specific genes were turned on in the mutant strain; however, the degree of derepression varied considerably from gene to gene. *RBT4*, for instance, was induced by filament-inducing growth conditions in wild-type cells, but this regulation was lost in strains carrying a deletion of *TUP1* and the gene was fully expressed under all growth conditions. In contrast, *HYR1* was only mildly derepressed in the *tup1* deletion strain when grown on YPD and retained substantial induction in response to Spider and serum media.

A different type of profile was observed for the *ECE1*, *ALS1*, and *HWP1* genes. Deletion of *TUP1* caused constitutive expression of these genes and abolished their environmental induction yet also reduced, sometimes severely, their final level of expression. This behavior probably indicates more complex regulatory control on filamentous growth than the simple direct action of the gene regulatory proteins discussed here. The intermediate expression level of these genes may be connected to the fact that the filamentous growth exhibited by *tup1Δ* cells in YPD is not as fully elongated and straight (*i.e.*, "hyphal") as is possible in wild-type cells. Correspondingly, the hyphal-specific genes in these cells may not be fully induced. In addition, *TUP1* may regulate more than one pathway that impinges on filamentous growth.

Dependence on *EFG1*: Many of the tested genes (*HYR1*, *ALS1*, *ECE1*, *HWP1*, *RBT1*, and *RBT4*) were heavily dependent on *EFG1* for their full expression. This
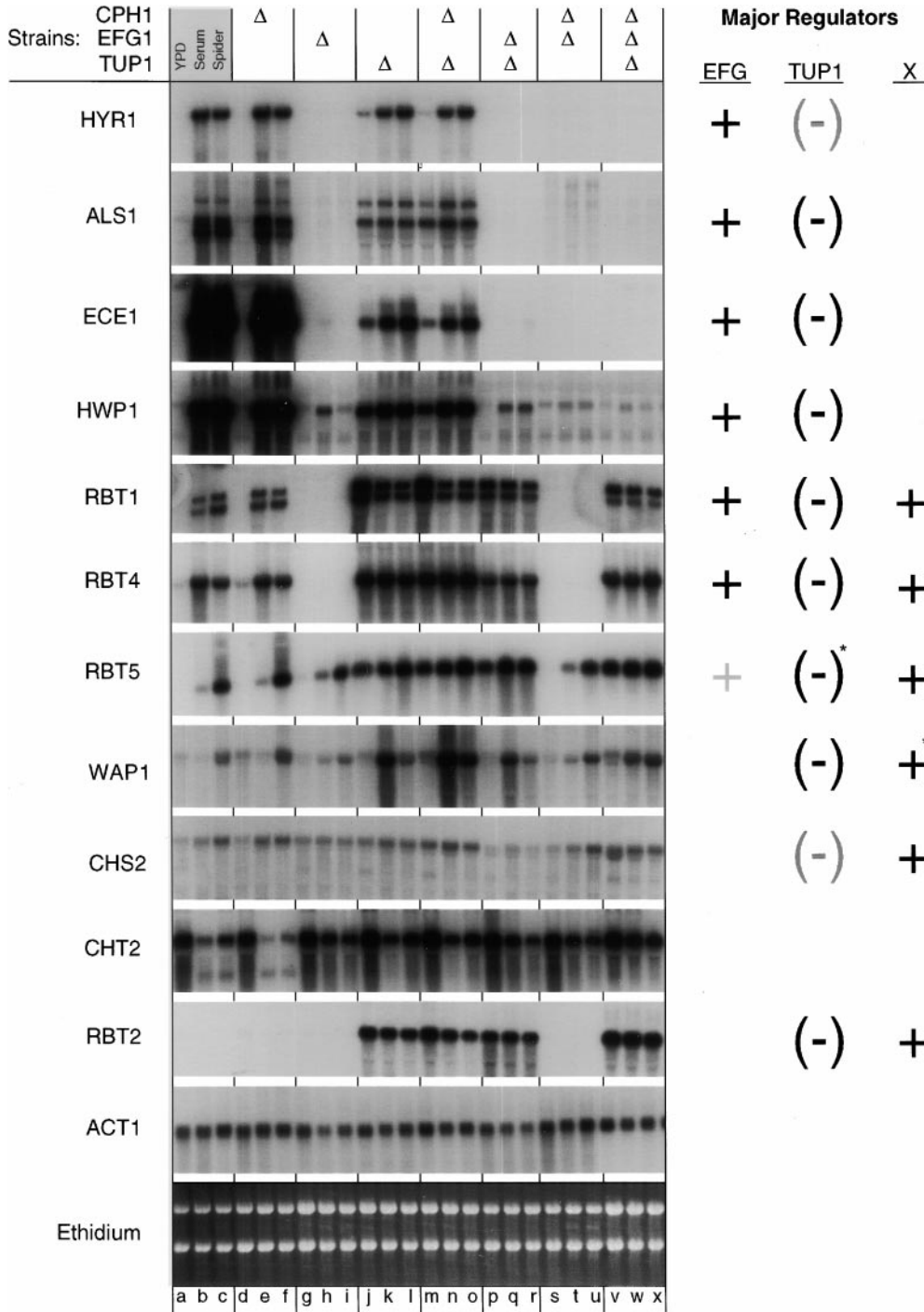
Figure 2.—Inducible gene expression from several filament-specific genes is altered in regulatory mutants. Total RNA from each strain under three growth conditions was probed with the gene indicated. The YPD liquid (30°) growth condition (lanes a, d, g, j, m, p, s, and v) did not induce filamentous growth, whereas both serum treatment for 1 hr at 37° (lanes b, e, h, k, n, q, t, and w) and Spider treatment for 2 hr at 37° (lanes c, f, i, l, o, r, u, and x) did induce filamentous growth (mostly in the form of germ tubes). At the right is an interpretation of the results indicating which regulators appear to influence the observed expression pattern, with shading indicating the relative importance of the regulator. Pluses represent activation and minuses represent repression. The asterisk indicates, in the case of *TUP1* and *X*, that the regulator in this case not only represses or activates gene expression but may itself be controlled by filamentous growth-specific signals.

observation agrees with the phenotypic behavior of the *efg1Δ* cells, which showed no morphological response to either of these serum or Spider treatments (not shown; see Lo *et al.* 1997). However, *HWP1*, *CHT2*, and especially *RBT5* and *WAP1* showed induction profiles that were to some degree independent of *EFG1*. *HWP1* showed slight induction in the absence of *EFG1*, and this residual induction was almost fully eliminated by the additional deletion of *CPH1* (Figure 2, compare lanes h and i with t and u), indicating that under these conditions, *HWP1* is at least slightly dependent on the

CPH1 pathway (Sharkey *et al.* 1999). The additional deletion of *TUP1* (lanes w and x) uncovered no additional *HWP1* activation and indicates that the activation seen in the *tup1Δ* strain was due principally to activation through *EFG1*.

CHT2 is turned off, instead of on, in filamentous cells (Figure 2, compare lanes a, b, and c; McCreath *et al.* 1995). We were surprised to see that much of this regulation was intact in *tup1* cells (Figure 2, compare lanes j, k, and l with lanes a, b, and c), despite their consistent filamentous growth and lack of morphologi-

cal change under induction conditions. Likewise, the deletion of *EFG1* impaired, but did not eliminate, the reduction of *CHT2* message in serum- or Spider-induced cells. The triple mutant cells still exhibited modest regulation of *CHT2*, which again points to an additional pathway of environmental control.

Unique induction behaviors of *RBT5* and *WAP1*: *RBT5* and *WAP1* are examples of genes whose serum- and Spider-induced expression is almost entirely independent of *EFG1*, as deletion of *EFG1* or both *EFG1* and *CPH1* had only minor effects on their induction. These results indicate that even though the *cph1Δ efg1Δ* cells are morphologically unresponsive to serum and Spider induction under these conditions, at least one regulatory pathway remained intact. For *RBT5*, *TUP1* may represent that pathway, since deletion of *TUP1* caused both high expression and loss of most environmental responses. This result also implies the existence of a transcriptional activator of *RBT5* that is distinct from *CPH1* and *EFG1.* This activator may not be regulated, since *TUP1* may represent the major pathway controlling induction of *RBT5* expression.

WAP1 is affected in complex ways by the deletion of *CPH1*, *EFG1*, and *TUP1*, yet shows robust induction in the absence of all three genes (Figure 2, compare lanes a–c with v–x), indicating that *WAP1* is induced by a pathway separate from *EFG1*, *CPH1*, and *TUP1.* This pathway may correlate with the fourth induction pathway deduced above from morphological criteria.

DISCUSSION

C. albicans cells deleted for the *TUP1* transcriptional repressor show constitutive filamentous growth, and in this article we show that this filamentous growth depends on at least three separate activating pathways. Two of these pathways are represented by the *CPH1* and *EFG1* transcriptional activators, and the other appears to be novel. We also demonstrate that a group of hyphal-specific genes do not respond in unison to defects in these activating pathways, but rather the genes show diverse individual responses. Taken together with previous work, these results suggest a network of regulatory pathways through which different environmental signals induce filamentous growth by activating different combinations of downstream genes.

EFG1 appears to represent the major activating pathway that drives filamentous growth in a *tup1Δ* strain of *C. albicans. CPH1* plays a lesser role in activating filamentous growth, as does a fourth pathway whose existence was suggested when the known pathways represented by *EFG1*, *TUP1*, and *CPH1* were all deleted and the cells still exhibited filamentous growth. Since this fourth pathway was arrived at by deduction, it may represent one or more distinct pathways. Several new genes involved in regulating filamentous growth were described while our work was in progress. These genes,

including *INT1* (Gale *et al.* 1998), *RBF1* (Ishii *et al.* 1997), *HWP1* (Sharkey *et al.* 1999), *CLN1* (Loeb *et al.* 1999), *ASH1* (D. O. Inglis, personal communication), and *ALS7* (P. Leng and A. Brown, personal communication), may lie in the already known pathways or may represent novel pathways such as the one deduced above.

Insights into *TUP1* regulation: One question addressed by this work concerns whether *TUP1* repression is regulated as part of normal filamentous growth control or whether *TUP1* happens to repress filament-specific genes for other reasons. Several lines of argument suggest that *TUP1* directly regulates filamentous growth. We show that a number of diverse genes correlated with filamentous growth appeared to be repressed by *TUP1*, and their regulation was consistent with a model where *TUP1* repression of hyphal-specific genes is lifted in response to environmental conditions. However, the pattern of *RBT5* expression argues most strongly for hyphal-specific repression by *TUP1*, since its induction was normal in the *efg1Δ*, *cph1Δ* background, yet most induction was lost when *TUP1* was deleted (Figure 2, lanes s–x). The simplest model for this behavior is that *RBT5* is regulated through the specific lifting of *TUP1* repression (as opposed to activation by *EFG1* or *CPH1*), and therefore *TUP1* repression appears at least in some cases to be regulated by the environmental conditions that induce filamentous growth. By analogy with the situation in *S. cerevisiae*, we imagine that the local lifting of repression is due to the inactivation (or some other form of regulation) of the DNA-binding protein that brings Tup1p-containing repression complex to the *RBT5* upstream regulatory region.

The work in this article also shows that *TUP1* is not used exclusively to regulate filamentous growth in *C. albicans. RBT2* is strongly repressed by *TUP1*, but not induced during filamentous growth. Indeed the fact that *RBT2* remained repressed during filamentous growth serves to show that *TUP1* itself is not inactivated to regulate the transition to filamentous growth. Work on the closely related *S. cerevisiae* TUP1 gene has shown that several entirely separate regulatory pathways use Tup1p to repress their target genes (Keleher *et al.* 1992; Deckert *et al.* 1995; Friesen *et al.* 1997; Huang *et al.* 1998; Proft and Serrano 1999), and the present results indicate that this is the case in *C. albicans* as well.

Epistasis: *EFG1*, *CPH1*, and *TUP1* each had independent effects on filamentous growth, regardless of the state of the other genes. The simplest explanation for this observation and the inference of an additional (fourth) pathway activating filamentous growth is that at least four different regulatory inputs contribute to filamentous growth in *C. albicans* (also see Brown and Gow 1999). Whether the pathways are entirely separate is not known, but the results indicate that the pathways represented by these genes are not arranged in a dependent, linear pathway. Why does *C. albicans* have multiple
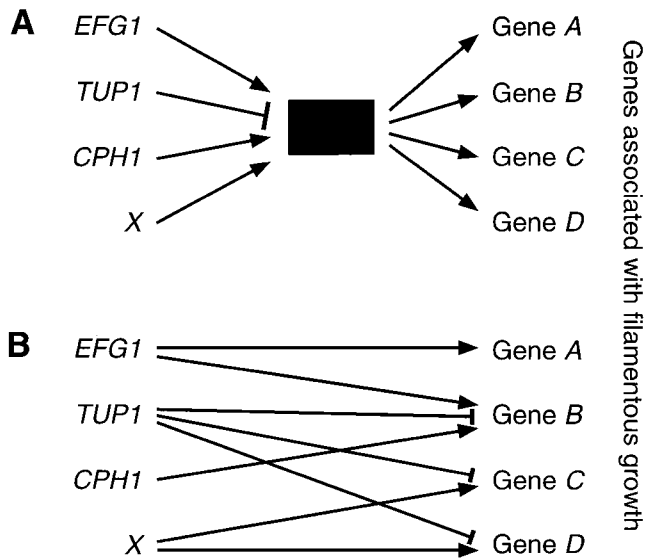
Figure 3.—Models of the regulatory circuit leading to filamentous growth in *C. albicans.* Two models are proposed, as described in the text, for the overall style of connections between upstream regulators and the downstream effectors and related genes that are turned on during filamentous growth. The models are termed the central control model (A) and network control model (B). In B, *HYR1, HWP1, RBT1,* and *RBT5* are examples of genes *A, B, C,* and *D,* respectively.

pathways controlling filamentous growth? A number of fungi respond to their external environments by engaging in filamentous growth; however the conditions that elicit filamentous growth vary among different species (for review, see Madhani and Fink 1998a). For example, filamentous growth in *S. cerevisiae* is activated by limitation for nitrogen or carbon and is thought to allow locomotion toward better nutritional conditions (Vivier *et al.* 1997). *Ustilago maydis* uses filamentous growth as part of its pathogenic life cycle inside plants and requires both mating type and plant signals for its induction (Broach *et al.* 1991). *C. albicans* activates filamentous growth not only in response to starvation conditions (such as Spider, milk, or cornmeal plates), but also to host-specific conditions such as exposure to serum and to 37°, which appear to be unrelated to starvation. It is perhaps not surprising that, given a wide array of inducing conditions, *C. albicans* also possesses a relatively complex set of regulatory pathways to process these signals.

Central *vs.* network control: Finally, our results shed some light on how these regulatory pathways may control filamentous growth. In Figure 3 we diagram two models, termed central control and network control, for the regulation of genes induced during filamentous growth. The central control model posits a master regulator that integrates signals from upstream pathways and provides a single output that controls filamentous growth. Binary regulatory decisions such as *IME1*-activated sporulation in *S. cerevisiae* (Kupiec *et al.* 1997),

induction of phage λ (cI; Ptashne 1992), and sex determination in both flies (*Sxl*) and worms (*xol-1*; Cline and Meyer 1996) are paradigms for this model.

The network model, on the other hand, proposes no distinct integrating step but rather a network of connections between regulatory pathways and downstream genes. The decisions made are not binary but are qualitatively varied (*i.e.,* which downstream genes are expressed) and quantitatively calibrated (*i.e.,* what are their levels of expression?) in response to the nature of the stimulation. Regulation of catabolic gene expression in yeast and other organisms is an example of this form of regulation. Many different signaling inputs indicate the available nutrients, and interrelated sets of circuits determine the amount and type of resources the cell devotes to food uptake (Gancedo 1998). Another example of network control is the stress response. In all organisms, responses to stresses such as heat, oxidation, and high salt make use of overlapping signaling pathways that control a variety of genes. Some of these such genes, such as those involved in heavy metal resistance, are very specialized, and others, such as chaperones, have more general roles. The level of expression of the regulated genes is finely tuned to the needs of the cell, and many of the target genes have overlapping, multiple inputs, creating a complex regulatory network (Ruis and Schuller 1995; Connolly *et al.* 1997).

The results described in this article show that genes turned on during filamentous growth do not respond to a central regulator. Rather, they respond individually to various pathways (at least four) that regulate filamentous growth, suggesting strongly that a network of signaling pathways and transcriptional regulators extends down to target genes without an intervening central level of regulation. Two recent reviews of *C. albicans* morphology have taken a similar view (Mitchell 1998; Brown and Gow 1999). According to this view, the filamentous growth decision in *C. albicans* is not a binary switch, yielding a "dimorphic" yeast, but a complex process that can result in distinct types of filaments, with each type corresponding to a particular set of inducing conditions. (For example, *RBT4* is mildly induced by starvation but strongly induced by serum, whereas *RBT5* shows the opposite pattern.) Thus, even states that look similar morphologically may be dissimilar on a molecular level, especially at the cell wall.

The fact that *EFG1, CPH1,* and *TUP1* all encode transcription regulators also supports a model of direct regulatory control. We think it likely that dissection of the upstream regions of these target genes will reveal distinct DNA binding sites related to each of these pathways, thereby explaining much of the observed regulatory behavior.

LITERATURE CITED

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman *et al.* (Editors), 1992 *Current Protocols in Molecular Biology.* Greene Publishing Associates and Wiley-Interscience, New York.

Bailey, D. A., P. J. Feldmann, M. Bovey, N. A. Gow and A. J. Brown, 1996 The Candida albicans HYR1 gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. J. Bacteriol. **178:** 5353–5360.

Birse, C. E., M. Y. Irwin, W. A. Fonzi and P. S. Sypherd, 1993 Cloning and characterization of ECE1, a gene expressed in association with cell elongation of the dimorphic pathogen Candida albicans. Infect. Immun. **61:** 3648–3655.

Braun, B. R., and A. D. Johnson, 1997 Control of filament formation in Candida albicans by the transcriptional repressor TUP1 [see comments]. Science **277:** 105–109.

Broach, J. R., J. R. Pringle and E. W. Jones, 1991 *The Molecular and Cellular Biology of the Yeast Saccharomyces cerevisiae.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Brown, A. J., and N. A. Gow, 1999 Regulatory networks controlling Candida albicans morphogenesis. Trends Microbiol. **7:** 333–338.

Church, G. M., and W. Gilbert, 1984 Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:** 1991–1995.

Cline, T. W., and B. J. Meyer, 1996 Vive la difference: males vs females in flies vs worms. Annu. Rev. Genet. **30:** 637–702.

Connolly, L., A. De Las Penas, B. M. Alba and C. A. Gross, 1997 The response to extracytoplasmic stress in Escherichia coli is controlled by partially overlapping pathways. Genes Dev. **11:** 2012–2021.

Csank, C., C. Makris, S. Meloche, K. Schröppel, M. Röllinghoff *et al.*, 1997 Derepressed hyphal growth and reduced virulence in a VH1 family-related protein phosphatase mutant of the human pathogen Candida albicans. Mol. Biol. Cell **8:** 2539–2551.

Csank, C., K. Schroppel, E. Leberer, D. Harcus, O. Mohamed *et al.*, 1998 Roles of the Candida albicans mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. Infect. Immun. **66:** 2713–2721.

Deckert, J., R. Perini, B. Balasubramanian and R. Zitomer, 1995 Multiple elements and auto-repression regulate Rox1, a repressor of hypoxic genes in Saccharomyces cerevisiae. Genetics **139:** 1149–1158.

Dutton, S., and C. W. Penn, 1989 Biological attributes of colony-type variants of Candida albicans. J. Gen. Microbiol. **135:** 3363–3372.

Feng, Q., E. Summers, B. Guo and G. Fink, 1999 Ras signaling is required for serum-induced hyphal differentiation in Candida albicans. J. Bacteriol. **181:** 6339–6346.

Fidel, P. L. J., and J. D. Sobel, 1996 Immunopathogenesis of recurrent vulvovaginal candidiasis. Clin. Microbiol. Rev. **9:** 335–348.

Fonzi, W. A., and M. Y. Irwin, 1993 Isogenic strain construction and gene mapping in Candida albicans. Genetics **134:** 717–728.

Friesen, H., S. R. Hepworth and J. Segall, 1997 An Ssn6-Tup1-dependent negative regulatory element controls sporulation-specific expression of DIT1 and DIT2 in Saccharomyces cerevisiae. Mol. Cell. Biol. **17:** 123–134.

Gale, C. A., C. M. Bendel, M. McClellan, M. Hauser, J. M. Becker *et al.*, 1998 Linkage of adhesion, filamentous growth, and virulence in Candida albicans to a single gene, INT1. Science **279:** 1355–1358.

Gancedo, J. M., 1998 Yeast carbon catabolite repression. Microbiol. Mol. Biol. Rev. **62:** 334–361.

Gietz, R. D., R. H. Schiestl, A. R. Willems and R. A. Woods, 1995 Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast **11:** 355–360.

Gow, N. A., 1997 Germ tube growth of Candida albicans. Curr. Top. Med. Mycol. **8:** 43–55.

Guthrie, C., and G. R. Fink, 1991 *Guide to Yeast Genetics and Molecular Biology.* Academic Press, San Diego.

Hoyer, L. L., S. Scherer, A. R. Shatzman and G. P. Livi, 1995 Candida albicans ALS1: domains related to a Saccharomyces cerevis-iae sexual agglutinin separated by a repeating motif. Mol. Microbiol. **15:** 39–54.

Huang, M., Z. Zhou and S. J. Elledge, 1998 The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. Cell **94:** 595–605.

Ishii, N., M. Yamamoto, F. Yoshihara, M. Arisawa and Y. Aoki, 1997 Biochemical and genetic characterization of Rbf1p, a putative transcription factor of Candida albicans. Microbiology **143:** 429–435.

Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson and A. D. Johnson, 1992 Ssn6-Tup1 is a general repressor of transcription in yeast. Cell **68:** 709–719.

Kobayashi, S. D., and J. E. Cutler, 1998 Candida albicans hyphal formation and virulence: is there a clearly defined role? Trends Microbiol. **6:** 92–94.

Köhler, J. R., and G. R. Fink, 1996 *Candida albicans* strains heterozygous and homozygous for mutations in mitogen-activated protein kinase signaling components have defects in hyphal development. Proc. Natl. Acad. Sci. USA **93:** 13223–13228.

Komachi, K., M. J. Redd and A. D. Johnson, 1994 The WD repeats of Tup1 interact with the homeo domain protein alpha 2. Genes Dev. **8:** 2857–2867.

Kupiec, M., B. Byers, R. E. Esposito and A. P. Mitchell, 1997 Meiosis and sporulation in Saccharomyces cerevisiae, pp. 889–1036 in *The Molecular and Cellular Biology of the Yeast Saccharomyces cerevisiae*, Vol. 3, edited by J. R. Broach, J. R. Pringle and E. W. Jones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Leberer, E., D. Harcus, I. D. Broadbent, K. L. Clark, D. Dignard *et al.*, 1996 Signal transduction through homologues of the Ste20p and Ste7p protein kinases can trigger hyphal formation in the pathogenic fungus *Candida albicans.* Proc. Natl. Acad. Sci. USA **93:** 13217–13222.

Liu, H., J. Kohler and G. R. Fink, 1994 Suppression of hyphal formation in Candida albicans by mutation of a STE12 homolog. Science **266:** 1723–1726.

Lo, H. J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti *et al.*, 1997 Nonfilamentous C. albicans mutants are avirulent. Cell **90:** 939–949.

Loeb, J. D., M. Sepulveda-Becerra, I. Hazan and H. Liu, 1999 A G1 cyclin is necessary for maintenance of filamentous growth in Candida albicans. Mol. Cell. Biol. **19:** 4019–4027.

Losberger, C., and J. F. Ernst, 1989 Sequence of the Candida albicans gene encoding actin. Nucleic Acids Res. **17:** 9488.

Madhani, H. D., and G. R. Fink, 1998a The control of filamentous differentiation and virulence in fungi [in process citation]. Trends Cell Biol. **8:** 348–353.

Madhani, H. D., and G. R. Fink, 1998b The riddle of MAP kinase signaling specificity. Trends Genet. **14:** 151–155.

McCreath, K. J., C. A. Specht and P. W. Robbins, 1995 Molecular cloning and characterization of chitinase genes from Candida albicans. Proc. Natl. Acad. Sci. USA **92:** 2544–2548.

Mitchell, A. P., 1998 Dimorphism and virulence in Candida albicans. Curr. Opin. Microbiol. **1:** 687–692.

Odds, F. C., 1988 *Candida and Candidosis.* Baillière Tindall, London.

Odds, F. C., 1994 *Candida* species and virulence. ASM News **60:** 313–318.

Perez-Martin, J., J. A. Uria and A. D. Johnson, 1999 Phenotypic switching in Candida albicans is controlled by a SIR2 gene. EMBO J. **18:** 2580–2592.

Pesti, M., M. Sipiczki and Y. Pinter, 1999 Scanning electron microscopy characterisation of colonies of Candida albicans morphological mutants. J. Med. Microbiol. **48:** 167–172.

Proft, M., and R. Serrano, 1999 Repressors and upstream repressing sequences of the stress-regulated ENA1 gene in saccharomyces cerevisiae: bZIP protein sko1p confers HOG-dependent osmotic regulation [in process citation]. Mol. Cell. Biol. **19:** 537–546.

Ptashne, M., 1992 *A Genetic Switch: Phage [Lambda] and Higher Organisms.* Cell Press/Blackwell Scientific Publications, Cambridge, MA.

Riggle, P. J., K. A. Andrutis, X. Chen, S. R. Tzipori and C. A. Kumamoto, 1999 Invasive lesions containing filamentous forms produced by a Candida albicans mutant that is defective in filamentous growth in culture. Infect. Immun. **67:** 3649–3652.

Ruis, H., and C. Schuller, 1995 Stress signaling in yeast. Bioessays **17:** 959–965.

Sentandreu, M., M. V. Elorza, R. Sentandreu and W. A. Fonzi, 1998 Cloning and characterization of PRA1, a gene encoding a novel pH-regulated antigen of Candida albicans. J. Bacteriol. **180:** 282–289.

Sharkey, L. L., M. D. McNemar, S. M. Saporito-Irwin, P. S. Sypherd and W. A. Fonzi, 1999 HWP1 functions in the morphological development of Candida albicans downstream of EFG1, TUP1, and RBF1. J. Bacteriol. **181:** 5273–5279.

Slutsky, B., J. Buffo and D. R. Soll, 1985 High-frequency switching of colony morphology in Candida albicans. Science **230:** 666–669.

Staab, J. F., C. A. Ferrer and P. Sundstrom, 1996 Developmental expression of a tandemly repeated, proline-and glutamine- rich amino acid motif on hyphal surfaces on Candida albicans. J. Biol. Chem. **271:** 6298–6305.

Stoldt, V. R., A. Sonneborn, C. E. Leuker and J. F. Ernst, 1997 Efg1p, an essential regulator of morphogenesis of the human pathogen Candida albicans, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J. **16:** 1982–1991.

Treitel, M. A., S. Kuchin and M. Carlson, 1998 Snf1 protein kinase regulates phosphorylation of the mig1 repressor in saccharomyces cerevisiae [in process citation]. Mol. Cell. Biol. **18:** 6273–6280.

Varanasi, U. S., M. Klis, P. B. Mikesell and R. J. Trumbly, 1996 The Cyc8 (Ssn6)-Tup1 corepressor complex is composed of one Cyc8 and four Tup1 subunits. Mol. Cell. Biol. **16:** 6707–6714.

Vivier, M. A., M. G. Lambrechts and I. S. Pretorius, 1997 Coregulation of starch degradation and dimorphism in the yeast Saccharomyces cerevisiae. Crit. Rev. Biochem. Mol. Biol. **32:** 405–435.

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