The dimorphic fungus Yarrowia lipolytica grows to form hyphae either in rich media or in media with GlcNAc as a carbon source. A visual screening, called FIL (filamentation minus), for Y. lipolytica yeast growth mutants has been developed. The FIL screen was used to identify three Y. lipolytica genes that abolish hypha formation in all media assayed. Y. lipolytica HOY1, a gene whose deletion prevents the yeast-hypha transition both in liquid and solid media, was characterized. HOY1 is predicted to encode a 509-amino-acid protein with a homeodomain homologous to that found in the chicken Hox4.8 gene. Analysis of the protein predicts a nuclear location. These observations suggest that Hoy1p may function as a transcriptional regulatory protein. In disrupted strains, reintroduction of HOY1 restored the capacity for hypha formation. Northern blot hybridization revealed the HOY1 transcript to be approximately 1.6 kb. Expression of this gene was detected when Y. lipolytica grew as a budding yeast, but an increase in its expression was observed by 1 h after cells had been induced to form hyphae. The possible functions of HOY1 in hyphal growth and the uses of the FIL screen to identify morphogenetic regulatory genes from heterologous organisms are discussed.

The yeast-to-hypha morphological transition (dimorphism) is typical of many pathogenic fungi (62). The dimorphic transition is a freely reversible process that can be induced by changes in many parameters (56). Much attention has been focused on Candida albicans as a model for analyzing dimorphism because it is the most frequently isolated fungal pathogen in humans. However, although several groups have reported the isolation of mutants which persist in either the yeast (10) or hyphal (21, 30) form, analysis of these mutants is hampered by the difficulties inherent to genetic manipulations and in its relationship to other yeasts (2).}

The second approach consists of cloning Candida homologs of Saccharomyces cerevisiae genes known to regulate pseudo-filamentous growth. Under conditions of nutrient limitation (i.e., nitrogen starvation), S. cerevisiae undergoes a dimorphic transition to growth of pseudohyphae, linear chains of elongated cells in which the daughters remain attached to the mothers (23, 24). In this context, several C. albicans genes that are members of a Candida map kinase (MAPK) cascade, i.e., CPH1 (40) or ACRP (43), HST7 (11), and CST20 (36, 39), have been isolated by complementation of the corresponding Saccharomyces mutants, i.e., STE12, STE7, and STE20, respectively (11, 36, 39, 40, 43). When C. albicans strains heterozygous and homozygous for null alleles of those genes were constructed, defects in hyphal formation were detected. For example, the CPH1 gene (cp/Cph1) shows suppressed hyphal formation on solid medium but not in liquid medium (40), and all mutant strains still form hyphae in response to serum (36, 39).

Homeobox-containing genes have been found in many species (15). In fungi, homeobox-containing genes occupy a key position in the determination of the mating type in both budding (1, 46) and fission (33) yeast cells and in the determination of filamentous versus yeast-like growth forms in Ustilago maydis (60); however, a gene, PHO2, with a homeodomain, which activates transcription of the regulated acid phosphatase gene PHO5 and possibly a phosphate permease, has also been reported for S. cerevisiae (9, 61).

Y. lipolytica is a dimorphic heterothallic yeast (3) that is unusual in both the structure of its genes coding for rRNA (17) and in its relationship to other yeasts (2). Y. lipolytica is amenable to genetic analysis (48), and DNA-mediated integration (12, 20) and autonomous transformation systems (18) have been developed. Mutations in the SEC14 gene (42) and deletion of XPR6 (16) have strong effects on the yeast-to-hypha transition. However, it is not known whether the proteins encoded by these genes are directly involved in the regulation of this transition. To gain insight into the developmental switch, we have developed an easy screen to obtain morphogenetic mutants unable to form hyphae. The screening is based on the simple system for inducing the yeast-hypha transition reported previously by us (54). In the present work, we describe characterization of the HOY1 gene. HOY1 encodes a putative nuclear protein with a homeodomain which is differentially regulated during the yeast-to-hypha transition. Deletion of HOY1 results in a defect in morphogenesis.

MATERIALS AND METHODS

Yeast strains, media, and microbiological techniques. The yeast strains used in the experiments are listed in Table 1. Our prototrophic standard strain, Y. lipolytica SA-1, was selected for its excellent ability to form mycelia in N-acetylglucosamine after sporulation of a diploid strain obtained from crossing CX 39-74B (MatB +/−1) with CX 39-72C (MatA +/−1) (48). Cells were grown in YED (1%...
TABLE 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference, source, or derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>W29</td>
<td>Mat A</td>
<td>C. Gaillardin*</td>
</tr>
<tr>
<td>SA-1</td>
<td>Mat B</td>
<td>A. Dominguez</td>
</tr>
<tr>
<td>21401-1</td>
<td>Mat A leu2-35 his8-11</td>
<td>C. Gaillardin</td>
</tr>
<tr>
<td>21501-13</td>
<td>Mat A leu2-35 his5-12 ura2-21</td>
<td>C. Gaillardin</td>
</tr>
<tr>
<td>21603-3</td>
<td>Mat B leu2-35 his1-13</td>
<td>C. Gaillardin</td>
</tr>
<tr>
<td>JM12</td>
<td>Mat B leu2-35 his5-12 ura3-18</td>
<td>C. Gaillardin</td>
</tr>
<tr>
<td>INAG23122</td>
<td>Mat A leu2-35 his2-2 xpl2 ade1</td>
<td>C. Gaillardin</td>
</tr>
<tr>
<td>Po1a</td>
<td>Mat A leu2-270 ura3-302</td>
<td>C. Gaillardin</td>
</tr>
<tr>
<td>JC8</td>
<td>Mat B fil A</td>
<td>This work</td>
</tr>
<tr>
<td>JC8-1</td>
<td>Mat B arg fil A</td>
<td>This work</td>
</tr>
<tr>
<td>JC20</td>
<td>Mat B fil A</td>
<td>This work</td>
</tr>
<tr>
<td>JC20-6</td>
<td>Mat B leu fil A</td>
<td>This work</td>
</tr>
<tr>
<td>JC90</td>
<td>Mat B trp fil B</td>
<td>This work</td>
</tr>
<tr>
<td>JC97</td>
<td>Mat B lys fil C</td>
<td>This work</td>
</tr>
<tr>
<td>JC80-1</td>
<td>Mat B leu2-35 arg fil A</td>
<td>This work</td>
</tr>
<tr>
<td>JC90-14</td>
<td>Mat A leu2-35 fil B</td>
<td>This work</td>
</tr>
<tr>
<td>JC97-1</td>
<td>Mat B leu2-35 lys fil C</td>
<td>This work</td>
</tr>
<tr>
<td>JC2113-1</td>
<td>Mat A leu2-35 his5-12 ura2-21 hoy1::LEU2</td>
<td>This work</td>
</tr>
<tr>
<td>JC33-1</td>
<td>Mat B leu2-35 his2-2 xpl2 ade1 hoy1::LEU2</td>
<td>This work</td>
</tr>
<tr>
<td>ADO16</td>
<td>Mat A leu2-270 ura3-302 hoy1::LEU2</td>
<td>This work</td>
</tr>
</tbody>
</table>

yeast extract, 1% glucose) or in appropriately supplemented minimal medium (MM; 0.67% yeast nitrogen base, 1% glucose). The induction of the yeast-hypha transition was carried out as described previously (54). Cells were grown on MM buffered with citric acid-sodium citrate (50 mM, pH 6.0) with glucose as a carbon source until they reached the exponential phase. They were then centrifuged, washed, and resuspended in the same medium (control cultures, growth in the yeast form) or in the same medium with N-acetylglucosamine at 1% as a carbon source (induction of the yeast-hypha transition). The media and procedures used for mating, sporulation, and transformation of Y. lipolytica have already been described (4, 67).

5. cerevisiae media and genetic manipulations were prepared and performed by standard methods described elsewhere (63).

Mutagens was carried out with ethyl methanesulfonate, as reported previously (38).

The Escherichia coli strains used for transformation and amplification of recombinant DNA were DH5α [supE44 lacZΔM15 proAB thi-1 endA1 galK5 Δ(uvrA6 thi-1 relA1) (25); MC1061 [hisD3-recA1 tetr]] (7); SV40 (Amersham), M15 (Stratagene), and a nested set of closely spaced Dharmacon (38).

The Northern analysis was performed as described by Sambrook et al. (55). In all Northern analysis experiments, the RNA concentration was normalized by hybridization with the LEU2 gene from Y. lipolytica DNA and protein sequences were analyzed with the DNAsis, PROSIS (Phar-macia-LKB; Hitachi), and PSORT (version 6.3; WWW) programs. The amino acid sequence of HOY1 was compared with the SWISS-PROT data bank by use of the FASTA program (50). Alignments of protein sequences were done with CLUSTAL programs (27).

Nucleotide sequence accession number. The sequence data reported here have been assigned EMBL accession number Z93956.

RESULTS

Isolation and characterization of morphological mutants. Normal Fil+ strains of Y. lipolytica (Fig. 2A) form rough-surfaced colonies on YED similar to those described for C. albicans for the irregular wrinkly type (64). Cells of Y. lipolytica SA-1 in the exponential phase (growing on YED) were mutagenized with ethyl methanesulfonate at a survival rate of 9% and plated onto YED agar. After 5 days of incubation at 28°C, 8,000 colonies were analyzed by visual screening, and 25 putative smooth nonfilamentous colonies were detected. However, only seven showed a clear phenotype that was maintained through the multiple propagations (Fig. 2B). The smooth colonies exhibited an unomitted, or unwrinkled, surface, with no aerial mycelia. Cells from smooth colonies were observed only in the budding form of growth. The frequency of mutants was 8.7 × 10−5, similar to those described for Y. lipolytica by other authors (18, 48).

The effects of the culture medium (YED or MM) and carbon sources (Glc or GlcNAc) on the growth and induction of the yeast-mycelium transition were analyzed in all seven mutants selected (Fig. 2B and D). One of them, JC3, showed a strong tendency to revert to the mycelial form. JC105 and JC109 displayed a lower growth rate than that of the wild type. Accordingly, these three mutants were discarded for further study. The other four mutants had similar growth rates to the wild type and were unable to form mycelia under any of the conditions tested. JC90 was auxotrophic for tryptophan and JC97 was auxotrophic for lysine. The other two mutants were prototrophic and were subjected to another round of mutagen-esis. Two auxotrophic mutants, JC20-6 and JC8-1, were selected.

Segregation of Fil− mutations. The Fil+ strains JC 20-6, JC 8-1, JC90, and JC97 were crossed with Y. lipolytica 21501-13 and 21401-1. The mutants formed stable diploids with both strains. All the diploids grew in the mycelial form, indicating that the Fil− mutations were recessive.

Diploids were sporulated on CSM medium (4). Since tetrad analysis is quite difficult for Y. lipolytica (67), segregation of the Fil− phenotype was analyzed in random spores. The results shown in Table 2 indicate a 2:2 pattern of segregation.

Complementation analysis. The four Fil− mutants were analyzed by genetic complementation. Table 3 shows the results of this analysis. The results point to the existence of three complementation groups.

The Y. lipolytica gene bank constructed in plasmid pINA62 contains the LEU2 gene of this yeast as a selectable marker. The unique sites AarI and NorI were localized outside the LEU2 gene (Fig. 1). Since the NorI sites are scattered at low frequencies in the yeast genome, for increased efficiency (67), we decided to linearize the gene bank by NorI digestion prior to the transformation experiments.

We transformed all the Fil− mutants with the gene bank, selected for Leu− colonies, and, after replicating the colonies on YED, searched for rough colonies. A total of 18,279 Leu− colonies of JC80-1 (filA), 4,361 of JC90-14 (filB), and 1,930 of JC97-1 (filC) were analyzed, and only one stable trans-
formant, called JC110-1 (obtained from JC80-1), with a rough phenotype similar on YED to that of the wild type was found.

**Isolation of the HOY1 gene.** To test whether homologous integration occurs at the Leu locus, as has been described previously (12, 67), and to obtain a physical map of the inserted DNA, we digested the DNAs of JC80-1 and JC110-1. After electrophoresis and Southern transfer, these DNAs were hybridized with a LEU2 probe. This probe hybridizes in the wild type after transformation with pINA62 with a BamHI-BamHI fragment of 11 kb or with a SphI-SphI fragment of 8 kb (see reference 67). The results indicate that the unique BamHI or SphI LEU2-specific chromosomal bands disappeared in the HOY1 transformant, and, hence, that integration of the transforming plasmid at the LEU2 locus had occurred (data not shown). The size of the plasmid was estimated to be about 15 kb. We rescued the HOY1 sequence from the DNA integrated at the LEU2 locus by a complete NotI cut of the total DNA of this transformant, followed by dilution, ligation, and transformation of E. coli DH5α and selection for ampicillin resistance. A resistant clone called pJCT3, which has only one NotI site over its entire 14.8-kb sequence, was isolated. The restriction map of the pJCT3 was established (Fig. 1). Since the restriction data obtained were in accordance with the molecular weights observed on the Southern blots (data not shown), we concluded that this plasmid had not undergone sequence rearrangements upon being subcloned into E. coli.

**Nucleotide sequence of the HOY1 gene and analysis of its flanking sequences.** The segment of pJCT3 that contains HOY1 was defined by subcloning fragments of the yeast segment into pINA62, transforming these constructs into JC80-1, and assaying their ability to undergo the yeast-hypha transition. The smallest segment that produced the morphological transition (a BamHI-BamHI 3.2-kb fragment) was sequenced after subcloning in pBluescript KS+ plasmid. An open reading frame was found, but no termination codon was detected and the sequence was extended. Finally, from 3,657 nucleotides sequenced, an open reading frame of 1,527 bp (Fig. 3), lacking introns and coding for a protein of 509 amino acids, was detected.

The first ATG codon of the HOY1 open reading frame has the conserved adenine residue at position –3 that is present in most of the *Y. lipolytica* genes (13a) and in 75% of *S. cerevisiae* initiator ATG codons (14). The region flanking the HOY1 gene shows a possible TATA box at position –227 (Fig. 3, upstream from the ATG codon). TATA boxes have been found in yeasts at positions ranging from –30 to –300 upstream from the translation start codon ATG (8, 34). The existence of a CAA (T/G) box is believed to be important for transcription. It is
normally located upstream from the TATA box. One sequence confirming this consensus was found upstream from the ORF at position −283 (Fig. 3).

Four putative targets for the general control of amino acid biosynthesis (29) were found in the 5′ sequence at positions −686, −447, −189, and −157 (Fig. 3). Two of them (−686 and −157) have the canonical sequence TGACTC, and the other two contain the highly conserved pentanucleotide sequence TGACT. Similar sequences were found upstream from the LYS5 (66) and LYS1 (119) genes of Y. lipolytica (52), and previous reports have shown that both LYS1 and LYS5 are under general amino acid control (19). The meaning of these sequences on the HOY1 promoter is unknown.

Also, four copies of the pentanucleotide CCCCT (named thermal stress responsive element C4T [35]) were found in the HOY1 promoter at positions −2486(−), −298(−), −287(+), and −216(+). In the 3′ region, typical terminator consensus sequences, i.e., TAA, TTT, and TAGT (68), were found, in common with other Y. lipolytica genes (57, 66).

**Hoylp is related to homeobox genes.** The deduced protein contains 509 amino acids corresponding to a molecular size of 55,920 Da, with an isoelectric point of 6.14. According to Bennezen and Hall (5), the codon bias value is 0.42, which corresponds to an intermediate protein abundance. Hydropathy analysis of the product inferred from the HOY1 nucleotide sequence, based on the calculations of Kyte and Doolittle (37), reveals a slightly hydrophilic polypeptide (data not shown).

Further examination of the polypeptide sequence was carried out with the PSORT program (see Materials and Methods) to determine the possible location of Hoylp in the cell. The results obtained, in agreement with the proposal of Rob-

<table>
<thead>
<tr>
<th>Cross</th>
<th>Total no. of colonies</th>
<th>% Rough colonies (Fil⁺)</th>
<th>% Smooth colonies (Fil⁻)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JC20-6 × 21501-13</td>
<td>1,176</td>
<td>48.9</td>
<td>51.1</td>
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<tr>
<td>JC8-1 × 21501-13</td>
<td>1,368</td>
<td>49.4</td>
<td>50.2</td>
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<tr>
<td>JC90 × 21501-13</td>
<td>1,246</td>
<td>45.7</td>
<td>54.3</td>
</tr>
<tr>
<td>JC97 × 21501-13</td>
<td>933</td>
<td>50.6</td>
<td>49.4</td>
</tr>
</tbody>
</table>

*Pairwise matings were done between all MatA and MatB haploid strains derived from random spore analysis (Table 2). The resulting diploid strains were scored for complementation or noncomplementation of hyphal formation. The mutations present in strains in noncomplementation pairs were placed in the same complementation group. S and R, smooth and rough phenotypes, respectively.*
FIG. 3. Nucleotide sequence of the yeast Y. lipolytica HOY1 gene region isolated from wild-type strain W29(pJCT3). The sequence of one DNA strand and the deduced amino acid sequence for the protein are shown. Nucleotides are numbered from the 5' end of the sequenced fragment to the 3' end. Amino acids are numbered from the first putative ATG in the large open reading frame at the left of the figure. A possible TATA box is indicated by bold type upstream from the ATG codon at position 222. A sequence confirming the existence of a CAA(T/G) box is indicated in bold type upstream of the open reading frame at position 283. Four putative targets for the general control of amino acid biosynthesis were found in the 5' sequence and are indicated by arrows. Underlining indicates four copies of the pentanucleotide CCCT found in the HOY1 promoter.
bins et al. for Xenopus nucleoplasmin (53), indicate the presence of two very clear nuclear targeting signals at positions 3 (KRX_{10}RNNKR_{9}) and 6 (KKX_{10}KRQRA) and point to the location of the enzyme in the nucleus.

Protein sequence analysis showed that Hoy1p has a homeobox at its N terminus. A potential DNA binding site is conferred by the 60-amino-acid homeodomain, which consists of a flexible stretch of nine residues, referred to as the N-terminal arm, followed by three \( \alpha \)-helices (15). All three appear in the \( \text{HOY1} \)-predicted protein (Fig. 4A) corresponding to the most conserved region of the C-terminal helix 3, as occurs with all the homeoboxes described to date (15). The best overall homology between the Hoy1p homeodomain and those described in the database is with the chicken \( \text{Hox4.8} \) homeodomain (31), where 25 of 60 amino acids are identical. In addition, the same region of Hoy1p is similar to several genes containing homeodomains (\text{Antp}, \text{en}, \text{ftz} [15], \text{bE2} [22], and \( \text{MAT} \alpha_2 [1] \), etc.) (data not shown). At the total amino acid sequence level, Hoy1p is most closely related to the \( S. \text{cerevisiae} \) transcriptional activator Pho2p (Fig. 4B).

![Sequence comparison of the homeodomain of \( Y. \text{lipolectica} \) Hoy1 with the homeodomain of the chicken Hox4.8p (Gg CHOX-4.8) (A) and with the Pho2p transcriptional activator of \( S. \text{cerevisiae} \) (Sc PHO2) (B). Identical residues (stars) and conservative amino acid substitutions (dots) are indicated; dashes represent gaps introduced to optimize alignments.](http://mcb.asm.org/)

FIG. 4. Sequence comparison of the homeodomain of \( Y. \text{lipolectica} \) Hoy1 (Yl HOY1) with the homeodomain of the chicken Hox4.8p (Gg CHOX-4.8) (A) and with the Pho2p transcriptional activator of \( S. \text{cerevisiae} \) (Sc PHO2) (B). Identical residues (stars) and conservative amino acid substitutions (dots) are indicated; dashes represent gaps introduced to optimize alignments.
three glutamine residues were detected in Hoy1p. Whether such differences are meaningful remains to be elucidated.

Analysis of the hybridization patterns of restriction fragments from different Y. lipolytica strains. DNA from four different strains of Y. lipolytica (SA-1, W29, 21501-13, and INAG33122) was digested with BamHI, SalI, or SalI-SphI, and the fragments obtained were hybridized with a radiolabeled SalI-ClaI fragment of 0.7 kb. The results indicate the existence of a single band and demonstrate that the restriction patterns of all four strains were identical (data not shown and reference 65).

Transcription of the HOY1 gene and expression during the dimorphic transition. Y. lipolytica SA-1 was grown in MM until the exponential phase was reached with glucose as a carbon source. The cells were then transferred to fresh medium under the same conditions (control) or to GlcNAc-containing medium (for inducing the yeast-hypha transition). At the times indicated, aliquots were collected, washed, and resuspended in the MM with GlcNAc as a carbon source. Samples of 15 μg of RNA taken after 0, 1, and 7 h (lanes 1, 2, and 3, respectively) after the yeast-hypha transition were hybridized with a HOY1 fragment as a probe (A) or with a LEU2 gene fragment (B).

FIG. 5. Northern blot analysis of Y. lipolytica HOY1 mRNA levels as a function of the yeast-hypha induction time. Total RNA was isolated from cells grown under induction conditions (cells in the exponential phase growing on glucose, in the yeast form, were collected, washed, and resuspended in the MM with GlcNAc as a carbon source). Samples of 15 μg of RNA taken after 0, 1, and 7 h (lanes 1, 2, and 3, respectively) after the yeast-hypha transition were hybridized with a HOY1 fragment as a probe (A) or with a LEU2 gene fragment (B).

source in both solid and liquid media). By Southern blotting followed by hybridization, we checked that the Leu" transformants indeed resulted from replacement of the chromosomal HOY1 gene by the disrupted LEU2 construct (Fig. 6B and C). Transformation of the disrupted strain with a LYSS autonomously replicating plasmid carrying HOY1 (plasmid pJTCT250) (Fig. 1) restored the rough phenotype. To further confirm these results, plasmid segregation experiments were carried out as described previously (56). In all cases (50 transformants selected randomly), loss of the Lys" character was accompanied by loss of the rough phenotype. The deleted strains JC2113-1 and ADO16 were crossed with filA strains (Table 1). Diploids were unable to form hyphae in normal inducing culture conditions (Table 3). Sporulation of the diploids shows a 4:0 segregation (smooth-rough colonies; only 2 of the 613 colonies analyzed were rough). Both results indicate that the HOY1 gene corresponds to the original filA mutation.

HOY1 enhanced hyphal growth. To examine the effect of HOY1 on filamentous growth, we transformed two wild-type and two mutant (filB and filC) Y. lipolytica strains (21501-13, P01a, JC90-14, and JC97-1, respectively) (Table 1) with the autonomous replicative plasmid pJTCT250 (Fig. 1). We selected six Leu" transformants from each strain and examined growth in liquid and solid media (in MM, yED with glucose, or both containing GlcNAc as a carbon source; for induction of the yeast-to-hypha transition). In all the media assayed, filB and filC mutants transformed with pJTCT250 gave rise to pseudohyphae which resembled those formed in C. albicans (Fig. 7A and B).

Integrative disruption of the HOY1 gene elicits the yeast morphology. To check the phenotype of a HOY1 null mutant in Y. lipolytica, gene disruption was performed. The LEU2 gene of Y. lipolytica was isolated as a SalI-Sall fragment from pINA62 (Fig. 1) and was inserted into the HOY1 gene between the Sall-SalI sites (see Fig. 6A). This replaced 1,043 bp of the HOY1 gene open reading frame with a 5.2-kb fragment containing the entire LEU2 gene. The construct was digested with BamHI-KpnI to release the entire LEU2 gene (Fig. 6A). This linear molecule was used to transform Y. lipolytica 21501-13 and INAG33122 to leucine prototrophy. Of 9 and 68 Leu" transformants obtained from strains 21501-13 and INAG33122, respectively, 3 and 3 exhibit a clear smooth phenotype in all the media tested (MM or YEPD with Glc or GlcNAc as a carbon source).
Pseudohyphal growth occurred in a more uniform way and was faster in all the media containing GlcNAc. Plasmid segregation experiments indicated that loss of the Leu+ character was accompanied by loss of the capacity for pseudohyphal formation.

Overexpression of HOY1 in the wild-type strains enhanced hyphal formation in all the media assayed. Again, hyphal formation was quicker and more vigorous in media with GlcNAc as a carbon source. In liquid medium, the differences were quantified with difficulty. Hyphal formation appeared retarded and hyphae appeared to be smaller in the transformant strain under noninducing conditions. The increase in hyphal formation appeared clearly in solid medium both in YED and in MM with GlcNAc as a carbon source. Figure 7 shows the behavior of the wild-type strain 21501-13, the transformed strains 21501-13 and JCT250, and the disrupted strain JC2113-1 (Δhoy1::LEU2). The HOY1 overexpression strains formed larger, well-defined hyphae (Fig. 7E and F), whereas the wild-type strain did not (Fig. 7C) and the deleted strain did not produce hyphae at all (Fig. 7D).

Expression of Y. lipolytica HOY1 in S. cerevisiae. When starved for nitrogen, diploid strains of S. cerevisiae switch from growth of the yeast form to growth of pseudohyphae (24). To check whether HOY1 is able to trigger the morphogenetic switch, a BamHI-BamHI fragment of 3.2 kb was inserted in the BamHI site of plasmids YEp352 (32) and YCP50 (28) (data not shown and reference 65).

We transformed S. cerevisiae CG25, CG41, CGX19, and CG67 with the four plasmids, and the Ura+ transformants were induced to form pseudohyphae (24). Only strains CGX19 and CG67 were able to form pseudohyphae, in agreement with results reported previously (24). No differences were found in the behaviors of the S. cerevisiae transformants carrying the plasmids containing the HOY1 gene. It is possible that the Y. lipolytica HOY1 gene might not be expressed in S. cerevisiae under the control of its own promoter. To circumvent this problem, we cloned the HOY1 gene under the control of the S. cerevisiae GAL1 promoter.

By designing specific oligonucleotides and using PCR amplification (65), we cloned a 1.5-kb band containing the entire HOY1 gene that was inserted in the XhoI-NotI site of pRS316-GAL1 (41), giving rise to pJC310 (Fig. 1). After transformation of the previously described S. cerevisiae strains, we selected the Ura+ transformants and grew all of them in either liquid or solid medium with glucose and galactose as carbon sources. No pseudohyphal formation was found in any of the cases assayed.

**DISCUSSION**

**FIL screen as a method to identify developmental pathways.** The FIL (filamentation minus) screen described in this work is a convenient method for identifying any gene involved in the yeast-hypha transition in Y. lipolytica because chemical and UV mutagenesis are standard procedures in many laboratories. Also, the FIL screen permits easy screening of a large number of transformants.

**Y. lipolytica** offers two main advantages compared to S. cerevisiae for analyzing the yeast-hypha transition. The first is that Y. lipolytica forms true septate hypha. The second is that Y. lipolytica is genetically closer to fungi than S. cerevisiae, as can be deduced by its tRNA (2) and by its codon bias (13a).

Moreover, some of the genes controlling development in Y. lipolytica are likely to be conserved in other fungi, plants, and animals, and the FIL screen could be used to identify these. Also, the same types of screening described for the morphological analysis of S. cerevisiae pseudohyphal formation (23) or transposon mutagenesis (47) can be carried out with Y. lipolytica.

HOY1 is related to transcriptional factors. With the FIL screen, we have been able to isolate several mutants of Y. lipolytica which grow perfectly only in the yeast form in all the media assayed. We have characterized at least three complementation groups (several more were obtained in a second screening and are currently being analyzed [52a]). By complementation of one of them, we isolated a gene, HOY1, which differs in several characteristics from the genes related to the yeast-hypha transition previously isolated in other yeast species (i.e., S. cerevisiae and C. albicans). From the structural point of view, although HOY1 contains a homeobox in its sequence, it does not present relevant homology either with the fungal genes involved in mating type functions (which also contain homeobox regions), with the genes involved in signal transduction (26), or with the genes controlling development in Aspergillus (44). A higher degree of homology was detected between HOY1 and the S. cerevisiae regulatory gene PHO2 (9, 61). One effect of HOY1 in phosphate metabolism cannot be ruled out. Thus, the transcription defect of hoy1 mutants would be a secondary metabolism defect. However, since no direct function in the transmission of P signals has been described for PHO2 (49), we are not in favor of such a hypothesis. In S. cerevisiae, Pho2p, also known as Bas2p or Grf10p, activates the transcription of numerous other genes, including HIS4, TRP4, CYC1, and genes for adenine biosynthesis. We are currently carrying out experiments to elucidate the effect of Pho2p on pseudohyphal formation in S. cerevisiae.

Along its whole sequence, HOY1 has characteristics similar to other Y. lipolytica genes, and only in its promoter are two aspects remarkable. One of them is the existence of several signals for general control of amino acid biosynthesis. Until now, in Y. lipolytica, we have been unable to establish a clear relationship between nitrogen starvation and filamentous growth, as has been described for S. cerevisiae (24). However, we cannot exclude this possibility, and, hence, the possible involvement of the TCACTC sequences in gene function is currently being investigated by directed mutagenesis.

The second interesting point is the existence of the pentanucleotide CCCCT, an essential component in stress response. It has been reported that any unfavorable circumstance that adversely affects growth can be understood as stress. Thus, the possibility exists that, at least partially, the yeast-hypha transition could be induced or regulated by a stress response. Experiments to test this hypothesis are also currently under way.

The results of Southern hybridization of genomic DNA digested with three restriction enzymes in four different strains of Y. lipolytica point to the existence of a single HOY1 gene, whose context appears to be relatively constant in the species. Northern blotting revealed a single transcript hybridization, and an increase in the amount of mRNA between two-
threefold was observed during the yeast-hypha transition. Since deletion of HOY1 is not lethal, our interpretation of the data is that in Y. lipolytica, hyphal formation is sensitive to the dosage of the HOY1 gene.

The Δhoy1 strains displayed suppressed hyphal formation in comparison to HOY1 strains, both in solid medium (YED) and in medium designed to induce hyphal formation (liquid MM with GlcNAc as a carbon source). The morphology of the colonies and of the cells in liquid medium (with either glucose or GlcNAc as a carbon source) of all the disrupted strains was similar to that obtained with the mutant strain JC80-1 (Fig. 1B and D). The flaw in hyphal formation is unlikely to result from a growth defect because all the strains grew with approximately equivalent doubling times. Several experiments indicated that defective hyphal formation was a direct consequence of the loss of HOY1 function. (i) Four independent strains showed the same defect in hyphal formation. (ii) Reintroduction of a wild-type HOY1 gene by integrative (with pJCT3 [Fig. 1]) or autonomous (with pJCT250 [Fig. 1]) transformation restored the ability of Δhoy1 strains to form hyphae. Because Δhoy1 strains were defective in hyphal formation in all media tested, our results indicate that HOY1 is an essential gene in the morphological transition. We transformed wild-type strains and the filB and filC Y. lipolytica mutants with the only replicative vector (containing the HOY1 gene) functional in this yeast and present at 1 to 3 copies per cell (3). The resulting phenotypes of this moderate HOY1 overexpression enhanced pseudohyphal growth in the Fil− mutants and hyphal formation in the wild-type in all the media tested. Pseudohyphal formation is unusual in Y. lipolytica, and more experiments are needed to further clarify this behavior. The induction of hyphal formation in the wild-type strains is consistent with the hypothesis that HOY1 is a transcription factor, and the phenotypes may be explained by assuming that HOY1 would activate genes responsible for the hyphal growth program.

Hox-4 homeobox genes are coordinately expressed during mouse limb and chicken wing development in striking temporal and spatial patterns. Furthermore, local application of retinoic acid, a putative endogenous morphogen, induces de novo transcription of Hox-4 genes (13, 31). Our results suggest that a similar hitherto-unknown mechanism may be involved in the yeast-hypha transition in fungi, this being the first time that a gene similar to the homeobox genes in higher eukaryotes and involved in development has been described for fungi. Alternatively, the possibility exists that HOY1 might interact with homologs to the STE7 or STE12 genes of S. cerevisiae in Y. lipolytica in such a way that the yeast-hypha transition would be triggered by some input signal(s), as it is in S. cerevisiae (26). Experiments to test both hypotheses are currently under way.

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