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Isolation and Molecular Analysis of *Umhda2* a Gene Encoding a Histone Deacetylase from *Ustilago maydis*

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By use of the polymerase chain reaction and synthetic oligonucleotides designed from conserved regions, we amplified a fragment of a gene from *Ustilago maydis* encoding a putative histone deacetylase. With this probe we isolated the full gene from a minigenomic library. The gene (designated as *Umhda2*) contains an open reading frame (ORF) of 1701 bp encoding a protein of 566 amino acids. Multiple comparison analysis with other histone deacetylases suggests that the *Umhda2* gene product belongs to the Rpd3-related family of proteins. The highest degree of homology with histone deacetylases from other organisms corresponded to Hda1p of *Schizosaccharomyces pombe* and Rpd3p of *Saccharomyces cerevisiae* with 64.2 and 62.2% of sequence similarity, respectively. It displayed a substantially lower similarity with another histone deacetylase from *U. maydis* (Hda1p, 52.4%). Semi-quantitative RTPCR results indicate that the gene is transcriptionally up-regulated during the *in vitro* yeast-to-mycelium dimorphic transition.

Keywords: Histone deacetylase; Chromatin structure; Nucleosomes; *Ustilago maydis*

Chromatin structure in eukaryotic organisms depends on the interaction of DNA with the histones that make up the nucleosomes. It may be altered by covalent modifications of histones, that affect the regulation of transcription. One of these modifications is the acetylation of nucleosomal histones. The lysine residues present in the relatively short unstructured tail of core histones are the sites of their reversible acetylation catalyzed two types of enzymes, histone acetyltransferases and histone deacetylases. Acetylated lysine residues characterize transcriptionally active chromatin (Allfrey *et al.*, 1964). Acetylation of histones reduces their interactions with DNA and allows the access of transcription factors to nucleosomal DNA, facilitating transcription (Lee *et al.*, 1993; Garcia-Ramirez *et al.*, 1995). On the other hand elimination of the acetyl residues from histones is correlated with transcriptional silencing (Braunstein *et al.*, 1993; Kadosh and Struhl, 1998; Suka *et al.*, 2001; Wu *et al.*, 2001). Nevertheless, it has been shown that a histone deacetylase from the Rpd3 family, Hos2p from *Saccharomyces cerevisiae*, is required for efficient gene transcription of several genes (Wang *et al.*, 2002). Strong evidence demonstrated that histone acetylation and deacetylation also play important roles in cell-cycle progression and differentiation in eukaryotic cells (Kouzarides, 1999).

We are interested in the study of cell differentiation and pathogenesis of *Ustilago maydis*, the causal agent of corn smut (Ruiz-Herrera and Martinez-Espinoza, 1998). *U. maydis* belongs to the Basidiomycetes, a group of fungi that includes common mushrooms and many plant pathogens. *U. maydis* is a dimorphic fungus; one of the forms

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is haploid, yeast-like and saprophytic; whereas the other one is filamentous, dikaryotic and pathogenic (reviewed by Banuett, 1992; Ruiz-Herrera and Martinez-Espinoza, 1998). Among the possible mechanisms for regulating differentiation and pathogenicity of the fungus, chromatin structure appears to be a likely candidate, motivating us to isolate genes encoding histone acetyltransferases and deacetylases from the fungus. This hypothesis was supported by a recent report showing that a gene encoding a histone deacetylase from *U. maydis* (*hda1*) was essential for the formation of teliospores (Reichmann *et al.*, 2002).

In this paper we report the cloning and molecular analysis of the gene encoding a second histone deacetylase from *U. maydis*, *Umhda2.* DNA sequence of *Umhda2* has been submitted to the EMBL databank under Accession Number AJ133752.

MATERIALS AND METHODS

Strains, Culture Media and Plasmids

U. maydis strain FB1 (*a1b1*) was obtained from Flora Banuett, maintained at −70°C in 50% glycerol, and propagated in YPD (1% yeast extract, 2% peptone and 2% glucose) medium. Dimorphic transition was acomplished in minimal medium (Holliday, 1974) as previously described (Ruiz-Herrera *et al.*, 1995). *Escherichia coli* strain DH5α was used for DNA transformation and amplification. The bacterium was grown in LB broth or 2x YT medium supplemented with ampicillin for selection when necessary. Bluescript KS^+(Stratagene) was used as cloning vector. Topo TA cloning Kit (pCRII topo, Invitrogene) was used to clone the amplified PCR product.

DNA and RNA Purification, cDNA Preparation and Blotting

Fungal DNA and RNA were purified as described by Sambrook *et al.* (1989) and Chomczynski and Sacchi (1987) respectively. Concentration and purity were analyzed respectively by spectrophotometry and agarose gel electrophoresis. Plasmid DNA was extracted from *E. coli* cells with Midiprep kits (Qiagen) or Wizard plus SV minipreps DNA purification system (Promega) according to the manufacturer's instructions. cDNA was prepared using genomic DNA-free RNA as described by Huang *et al.* (1996). Southern and Northern blottings were performed as described by Sambrook *et al.* (1989). Hybridization was made with a 498 bp specific probe of the *Umhda2* gene obtained by PCR (see below), radioactively labeled with the Rediprime II labeling system kit (Amersham Pharmacia Biotech).

PCR Amplifications and RTPCR

PCR experiments were performed using Promega Taq DNA polymerase as recommended by the supplier. A 498 bp fragment of *Umhda2* was amplified with oligonucleotides 1 (forward) (5′ TAYTAYTAYGGNCARGGNCAYCCNATC 3′), and 2 (reverse) (5′ RAANGCYTCYTCNACNCCRT-CNCCRTG 3′). PCR conditions for amplifications were as follows: 100 ng of total DNA were mixed with 50 pmoles of each primer in a final reaction volume of 50 μl. The template was initially denatured at 95°C for 5 min. This was followed by 30 amplification cycles (95°C 1 min; 55°C 1 min; 72°C 1 min) and final extension at 72°C for 7 min. PCR products were extracted from agarose gel using the Wizard PCR preps DNA purification system Kit (Promega) or Geneclean kit (BIO 101). DNA ligation was performed using the Topo TA cloning kit and T4 DNA ligase (Promega). Selection of transformants was performed by standard protocols (Sambrook *et al.*, 1989).

Semi-quantitative RTPCR was performed following the conditions described by Dang *et al.* (2000). Yeasts and mycelial forms of the fungus were obtained after 18 h of growth as described above. RNA was isolated and used to prepare cDNA. This was PCR-amplified with the following primers: 1645 (forward) (5′ GCGAGTGGTTTCTGCTAC 3′), and 1644 (reverse) (5′ GACCGTTGACAGCACTTC), using the same conditions described above. The actin gene transcript was used as internal control. PCR was performed with the following oligonucleotides: forward (5′ CCAAGGCCAACCGCGAGAAGA-TGAC 3′); reverse (5′AGGGTACATGGTGGTGCCG-CCAGAC3′). Controls without reverse transcriptase were included. The products were subjected to ethidium bromide-containing agarose electrophoresis, scanned and digitalized with an IS-1000 digital Imaging System.

Minigenomic Library Construction

Genomic DNA from *U. maydis* was digested with several restriction endonucleases. Products were separated by agarose gel electrophoresis, and hybridized with the radiolabeled PCR specific fragment (see above). With the data obtained, we selected *Eco* RI for a preparative digestion. Fragments from 4 to 5 kb were extracted from the gels, ligated into pBS, and used to transform *E. coli* cells. Positive clones were identified with the radioactive probe by high stringency hybridization after transfer to positively charged nylon membranes (Hybond N^+).

DNA Sequencing and Analysis

DNA sequencing was done with double-stranded templates in an ABI PRISM 377 DNA automatic sequencer (Perkin Elmer). Search of sequence homology was performed using the Fasta3 searches of EMBL, SWISS-PROT and SWALL databases. Analyses of nucleotide sequences were done with the DNASTAR, DNAStrider 1.1 and Transfac (the transcription factor databases) programs.

RESULTS

We synthesized a fragment of a *U. maydis* gene encoding a putative histone deacetylase by PCR using two degenerate primers designed from the conserved regions of several histone deacetylases. A single PCR product of 498 bp was amplified. DNA sequencing of this fragment revealed a high degree of homology with histone deacetylases. The PCR product was used as a probe in Southern blot analysis of genomic DNA from *U. maydis* cells. This fragment hybridized as a single band with a 4 kb fragment present in *Hin*dIII digests, a 5 kb *Bam* HI fragment, and a 4.5 kb *Eco* RI fragment (Fig. 1).

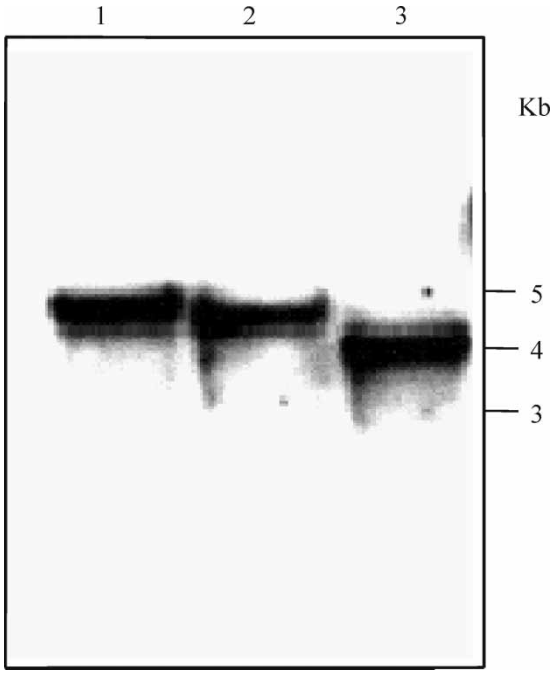

FIGURE 1 Southern-blot analysis of *U. maydis* DNA with the *Umhda2* probe. Genomic DNA from *U. maydis* FB1 strain was digested with *BamH* I (lane 1), *EcoR* I (lane 2) or *Hind* III (lane 3), separated by electrophoresis, blotted and hybridized with the 498 bp *Umhda* probe obtained by PCR.

To clone the full-length gene we constructed a minigenomic library (see Materials and Methods), which was screened with the PCR product. Positive clones that hybridized were isolated and one of the transformants was selected to sequence the gene on both strands. A 4.45 kb *Eco* RI fragment was found to contain the whole coding region of the gene. By its sequence similarity with other genes encoding histone deacetylases, and considering the existence of another *U. maydis* gene coding for a histone deacetylase (*hda1*, Reichmann *et al.*, 2002), the gene was named *Umhda2*. The gene contains a single uninterrupted ORF of 1701 bp. It has a GC content of 57.26% and exhibits the codon usage bias for G/C-containing triplets (Gonzalez-Prieto and Ruiz-Herrera, 2002). At the 5′ upstream region of *Umhda2*, up to −700 bp, no potential TATA box was found, unlike most yeast genes. At position -3 relative to the putative initiation ATG codon of this gene, an A residue was found, in agreement with the observations made by Kozak (1989) concerning yeast translation initiation sites. At position -372 relative to the ATG initiation codon, a tcgTGATTca sequence was found. This is a potential binding site for the GCN4p activator of genes involved in protein and purine biosynthesis. The 3′ end has the consensus tripartite sequence TAG…T rich…. TAGT…TTT involved in transcription termination in yeasts (Sherman, 1982). Sequence data reveals that *Umhda2* encodes a protein made of 566 amino acids with a molecular weight of 62.85 kDa, and a pI of 5.75. The presence in Umhda2p of the conserved histidine and aspartic residues assumed to be involved in catalysis by histone deacetylases (Hassig *et al.*, 1998; Kadosh and Struhl, 1997) supports its presumed physiological function. These residues were the following: H124, H125, H162, D158 and D175. The corresponding sequence is shown in Fig. 2 (GeneBank accession number AJ133752).

Sequence comparison of the deduced protein with other histone deacetylases was carried out using the Fasta3 program. From the group of fungal sequences, the two best scores were obtained with Hda1p from *S. pombe* (Olsson *et al.*, 1998, 64.21% identity), and the *S. cerevisiae* Rpd3 protein (62.6% identity). Similarity with the Hda1p from *U. maydis*was lower, 52.4%. Alignment with these proteins is shown in Fig. 3. Analysis of the sequence dat by the CLUSTAL method produced the dendrogram shown in Fig. 4. According to the results Umhda2p belongs to the Rpd3 group of histone deacetylases.

The transcript of *Umhda2* was analyzed by Northern blot. A single band of ca. 2.2 kb was observed only in mycelial cells. Even with the highest amount of RNA loaded (40 μg), no

FIGURE 2 Nucleotide sequence of the *Umhda2* gene and its predicted amino acid sequence.

hybridization signal was obtained when RNA from yeast cells was analyzed (Fig. 5). By use of semi-quantitative RT-PCR we analyzed gene expression in the yeast and mycelial forms of the fungus. Results revealed significantly higher levels of transcript in the mycelial form of the fungus (Fig. 6), in agreement with the results obtained by Southern analysis.

DISCUSSION

Histone deacetylases are highly conserved proteins. Enzymes from such divergent organisms as human, *Arabidopsis thaliana* and budding yeasts are approximately 50–70% identical at the amino acid sequence level. A recent proposal for their classification (Trojer *et al.*, 2003) suggests

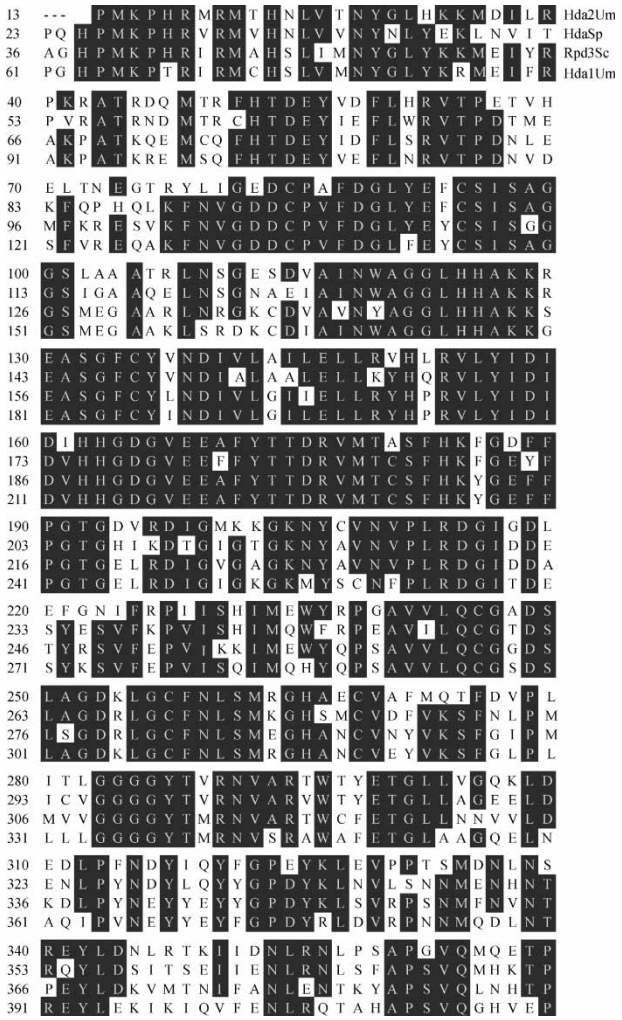

FIGURE 3 Multiple amino acid sequences alignment of the most conserved fragments of UmHda1p and other fungal histone deacetylases. Um, *Ustilago maydis*: Sp. *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*.

the existence of three classes: (i) histone deacetylases divided in subclasses with strong similarities to Rpd3, Hda1 and Hos3; (ii) NAD+ dependent sirtuines with subclasses Sir2 and Hst1-4; (ii) plant-specific Hda2-related enzymes. According to the results obtained, the histone deacetylase gene isolated in this study from *U. maydis*(*Umhda2*) encodes an enzyme belonging to the Rpd3p subclass of the first group, same as described for *U.maydis* hda1p (Reichmann *et al.*, 2002). Umhda2p displays a lower degree of homology with members of the second histone deacetylase subclass, such as Hos1p, Hos2p and Hda1p from yeast, and with one member of the eubacterial acetoin utilization proteins (acuC, with 34.4% identity). It has been reported that these latter proteins have a significant similarity with histone deacetylases and with acetylpolyamine amidohydrolases (APH), showing evidence for the evolution of histone deacetylases (Leipe and Landsman, 1997).

It is worth noticing that no putative TATA box was identified in *Umhda2*. This is not rare in *U. maydis*, where other reported genes apparently lack a TATA box (Guevara-Olvera *et al.*, 1997; Xoconostle-Cazares *et al.*, 1997). It is worthwhile to mention also that with the exception of a putative Gcn4-binding site, the upstream region of *Umhda2* does not display common regulatory motifs.

The observation that *Umhda2* is transcriptionally up-regulated in the mycelial form of *U. maydis* deserves a comment. Histone deacetylases are normally involved in gene silencing (but see Wang *et al.*, 2002). Furthermore, Reichmann *et al.* (2002) described that haploid *hda1* mutants expressed several genes that are normally expressed only in the dikaryon form of the fungus, suggesting that histone deacetylase activity is required to repress their transcription. The observation that *Umhda2* is expressed at higher levels in the filamentous form points to a role in the repression of yeast-specific genes, and suggests that the normal pattern of growth of *U. maydis* is the budding yeast.

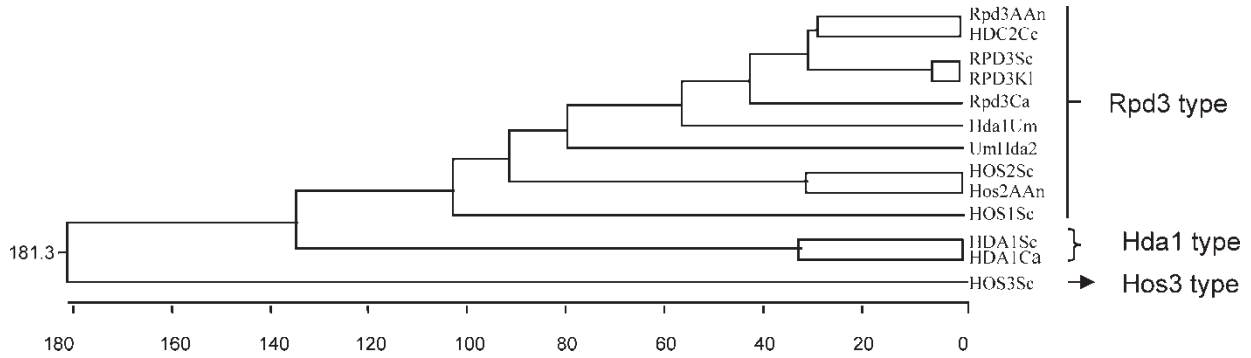

FIGURE 4 Maximal parsimony dendrogram showing grouping of selected histone deacetylases. The tree was calculated using Clustal analysis. An, *Aspergillus nidulans*; Cc, *Cochliobolus carbonum*; Sc, *Saccharomyces cerevisiae*; Kl, *Kluyveromyces lactis*; Ca, *Candida albicans*; Um, *Ustilago maydis*.

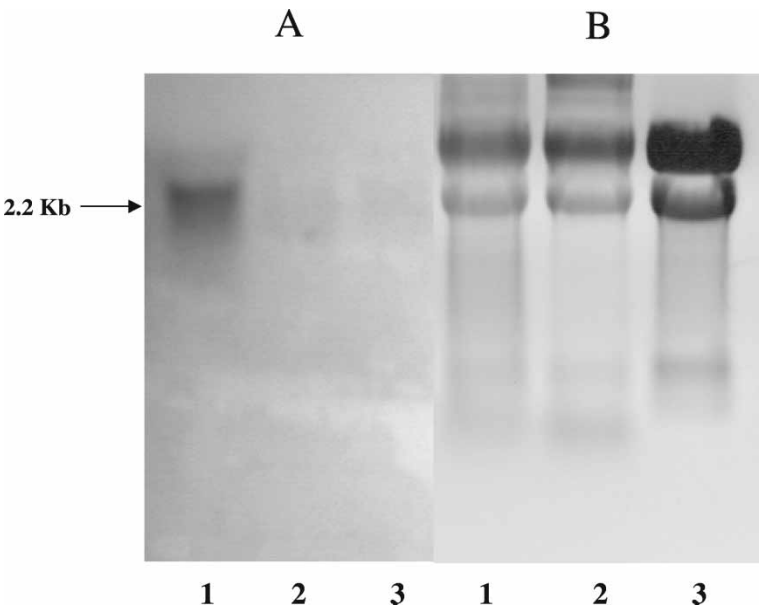

FIGURE 5 Northern blot analysis of the transcript of the *Umhda1* gene. RNA was obtained from the mycelial and yeast-like forms of the fungus, subjected to denaturing RNA electrophoresis, transferred to nylon membranes and hybridized with the specific probe of the gene. (A) Lane 1, RNA (20 μg) from mycelial cells; lanes 2 and 3 RNA from yeast cells (20 and 40 μg respectively). (B) Photograph of the ethidium bromide-stained gel to show ribosomal RNAs as an indicator of the amount of loaded RNA. Lane numbers as in A.

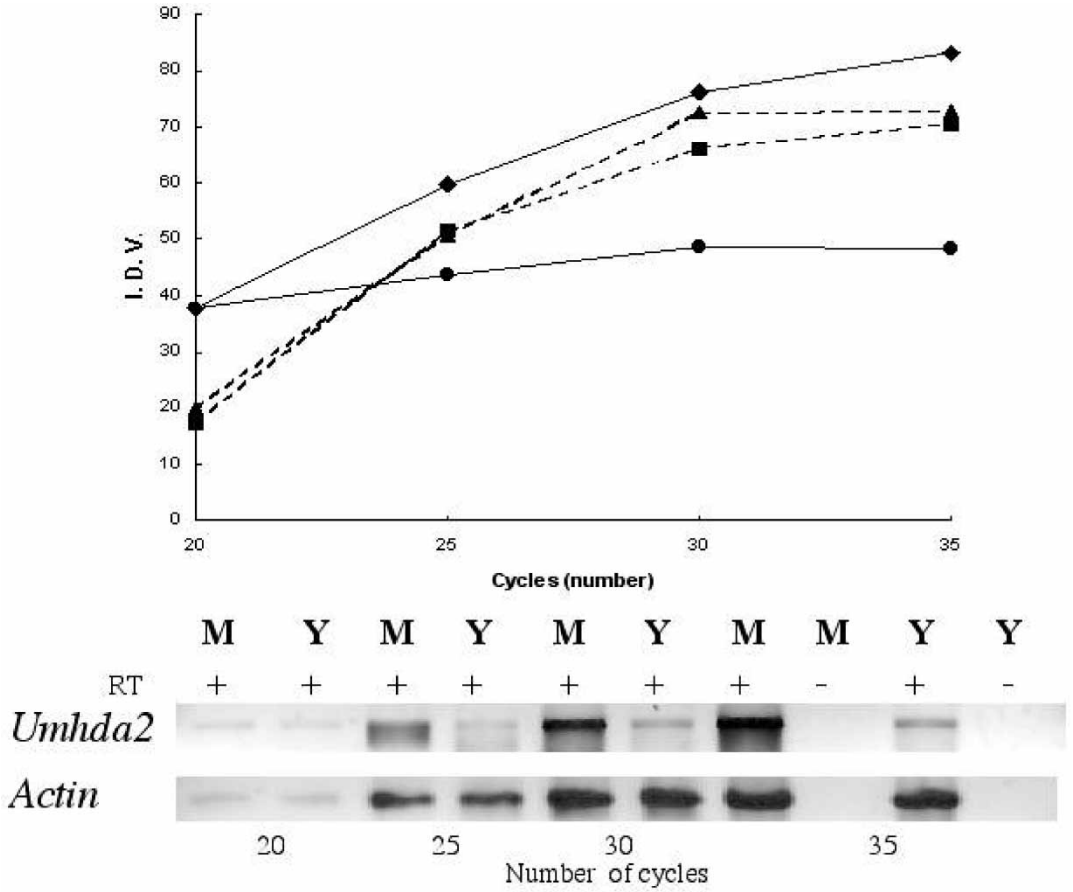

FIGURE 6 Expression of *Umhda2* gene. Data of semiquantitative RTPCR showing the relative amounts of *Umhda2* transcript in the yeast and mycelial forms of *U. maydis*. Actin gene transcript was used as internal control. Diamonds, cDNA from mycelial cells amplified with *Umhda*-specific oligonucleotides; circles, *ibid* from yeastlike cells; triangles, cDNA from mycelial cells amplified with the actin gene transcript-specific oligonucleotides; squares, *ibid* from yeast-like cells. (C) Photographs of the amplified fragments separated by gel electrophoresis. M, mycelium; Y, yeast-like cells; RT, reverse transcriptase, + added, - not added.

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