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Short Report

Inductions of Germ Tube and Hyphal Formations are Controlled by mRNA Synthesis Inhibitor in *Candida albicans*

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Abstract

Candida albicans is a pathogenic dimorphic fungus. When yeast cells were pre-incubated in YPD medium at 25°C and released into HFM7 medium containing 4% serum at 37°C, germ tubes emerged within 0.5 h. To determine whether mRNA or protein synthesis was necessary for germ tube formation, we examined the effects of mRNA and protein syntheses inhibitors on this formation. In the presence of cycloheximide, cells were unbudded and no germ tube was observed. However, in the presence of actinomycin D, germ tube formation was observed while budding growth and true hyphae elongation were blocked. Next, we measured mRNA or protein accumulation during induction of germ tube formation in the presence of the inhibitors. In the presence of cycloheximide, protein was not synthesized, while in the presence of actinomycin D, mRNA synthesis decreased to 6.3% and protein synthesis to 37.7%. The condition we found which allows only germination but not budding or filamentation might be convenient to use in screening genes involved in the initial stage of morphological change in *C. albicans*.

Key words: *Candida albicans*, germ tube, serum, mRNA synthesis inhibitor

Introduction

The human pathogenic fungus, *Candida albicans* is capable of dimorphic transition from budding yeast growth form to hyphal growth form[1]. Its ability to switch between them is believed to be directly related to its virulence, because mutants defective in hyphal growth are less virulent in mouse models than their wild-type counterparts[2-4]. Hazan *et al.*[5] proposed that hyphae might be able to invade the host tissue, whereas the yeast form would be more easily disseminated within the host. Therefore, understanding of the mechanisms for this morphogenetic switch should provide insight into the pathogenicity of this organism.

In *C. albicans*, hyphal development is first observed by the induction of germ tube formation with serum[6]. To understand the mechanism of dimorphic transition, Land *et al.*[7] examined MIC (minimal inhibitory concentration) of seventeen kinds of antimetabolites including an mRNA synthesis inhibitor, actinomycin D, a DNA synthesis inhibitor hydroxyurea, and a protein synthesis inhibitor cycloheximide. They observed their effects on filamentous and budding growth of *C. albicans* when cells were cultured in Lee medium[8] containing each inhibitor at various temperatures to induce germ tube formation. They showed that both germ tube formation and yeast phase growth were inhibited by actinomycin D or hydroxyurea. On the basis of experiments using inhibitors of macromolecular biosyntheses, the accelerated growth kinetics may require *de novo* RNA and protein biosynthesis, but not DNA synthesis[9].

In this report, we used HFM7 (hyphal

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formation medium, pH 7) medium to induce germination of *C. albicans*. In this condition, the fungus readily shifted its morphology from yeast to filament. We found that the transition from yeast budding to filamentous growth required *de novo* synthesis of mRNA and protein. Moreover, we found a concentration of actinomycin D which allows cells to develop a germ tube but not to grow by yeast budding or true hyphae elongation. Our finding appears useful for gene analysis involved in germ tube formation which is important for the pathogenicity of *C. albicans*.

Materials and Methods

Strain and culture conditions

The *C. albicans* strain used in this study was a clinical isolate, IFM 40009 (ATCC 48130). Yeast cells were pre-cultured in 20 m*l* of YPD liquid medium (1% yeast extract, 2% glucose, 2% polypeptone), and incubated at 25°C with shaking (100 rpm) for about 30 h. The culture was diluted one hundred-fold into fresh YPD liquid medium and incubated at the same temperature with shaking. For germ tube formation, filter-sterilized HFM7 medium (5 g glucose, 0.26 g $Na_2HPO_4 \cdot 12H_2O$, 0.66 g KH_2PO_4, 0.08 g $MgSO_4 \cdot 7H_2O$, 3.3 g NH_4Cl per liter, 1.6×10^{-6}% biotin, 4% calf serum, pH 7.0) was used. The inoculated culture was incubated at 37°C. At various time points during the incubation, cell morphology was observed by an inverted phase - contrast microscope (Nikon TMD; Nippon Kogaku Inc.).

Inhibition of macromolecular biosyntheses

After yeast cells were pre-cultured in 20 m*l* of YPD medium, all cells were harvested and washed three times with Na-phosphate buffer (100 mM NaH_2PO_4, pH 6.5). In order to decay the mRNA initially present in the cells, the method of Santiago *et al.*[10] was employed. Briefly, the cells were resuspended in 20 m*l* of Na-phosphate buffer containing hydroxyurea (0.1 M), and actinomycin D (0.4 mg/m*l*), and incubated at 25°C with shaking (100 rpm) for 24 h. They were then washed three times with Na-phosphate buffer, and approximately 1×10^4 cells were transferred to 1 m*l* of the HFM7 containing actinomycin D (0.4 mg/m*l*), and incubated at 37°C. To inhibit protein synthesis, cycloheximide (25 mM) was used instead of actinomycin D. At various time points during the incubation, cell morphology was observed with an inverted phase-contrast microscope. The concentration of the inhibitors mentioned above was decided based on our observation that cells could not grow in YPD medium in the presence of the inhibitors (data not shown).

Analyses of mRNA and protein synthesis in cells treated with actinomycin D or cycloheximide

Cells were treated as described in the previous paragraph to decay the endogenous mRNA or protein molecules. To measure the amount of mRNA accumulation, 0.5 μCi of adenine [2,8-^{3}H] (NEN Life Science Products, Inc.) was added to 10 m*l* HFM7 containing 1×10^5 cells with or without 0.4 mg/m*l* actinomycin D, and the culture was incubated for 3 h at 37°C with shaking (100 rpm). Total RNA was extracted from *C. albicans* cells according to the hot phenol method[10]. mRNA was then purified with a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech) according to the manufacturer's instructions. Pellets of mRNA were suspended in 15 m*l* of Scintisol 500 and the radioactivity was measured by a liquid scintillation counter (LSC-5100; Aloka). For measurement of protein synthesis, the same procedure was employed except 0.5 μCi of ^{35}S-methionine (NEN Life Science Products, Inc.) and 25 mM cycloheximide were replaced with radiolabelled adenine and actinomycin D. Cells were washed twice with 0.05% tween 80 solution, then resuspended in 1 m*l* of the same solution. The suspension was added to 15 m*l* of Scintisol 500 and the radioactivity was measured in the liquid scintillation counter.

Results and Discussion

Germ tube formation was induced in HFM7 medium

In the human pathogen *C. albicans*, germ tube and hyphal formation are induced by many different environmental stimuli including serum, nitrogen level, pH and temperature, and the transition between yeast and hyphal forms is thought to be essential for its virulence[3, 11, 12]. In this study germ tube formation was induced in HFM7 medium. Cells of the stationary phase grown in YPD medium showed unbudded yeast form (Fig. 1a). The cells were then transferred to HFM7 medium, and incubated at 37°C for morphological observation. Germ tubes emerged at 0.5 h (Fig. 1b), elongation and septum formation were recognized at 3 h (Fig. 1c), and true hyphae grew at 9 h (Fig. 1d). We conclude here that the HFM7 medium we used in this study efficiently induces germ tube formation and filamentation in *C. albicans*.

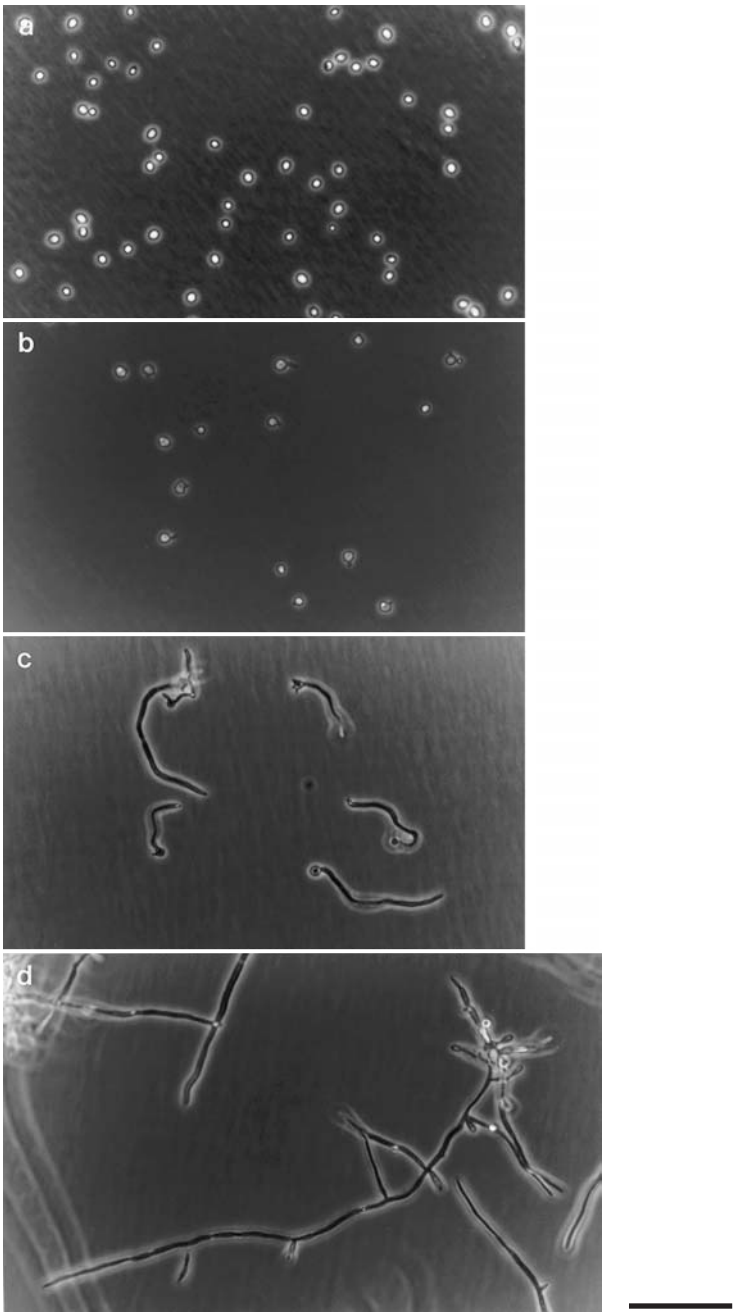

Fig. 1. Phase contrast micrographs of *C. albicans*

40009 cells grown in YPD at stationary phase (a), or grown in HFM7 at 0.5 h (b), 3 h (c), 9 h (d) after the inoculation. Bar = 50 μm.

The effects of mRNA or protein synthesis inhibitors to C. albicans

To clarify whether *de novo* mRNA or protein synthesis is essential for germ tube formation, the induction of a germ tube was carried out in the presence of various concentrations of actinomycin D or cycloheximide.

When up to 25 mM cycloheximide was added, both budding and filamentation were blocked, whereas without the inhibitor, germ tube formation was observed at 1.5 h in HFM7 medium (Fig. 2). The results suggested that *de novo* protein synthesis was essential for *C. albicans* to grow in either yeast or filamentous growth. Germ tube formation was not recognized until 1.5 h after induction in HFM7 medium without the inhibitor, which was delayed compared to the result shown in the previous section (see Fig. 1e and Fig. 2d). The delay might have been caused by our pre-treatment of the cells to decay the endogenous protein, and the fungus required an additional 1 h to recover the lost protein during pre-treatment. In pre-culture, hydroxyurea was also added to synchronize the

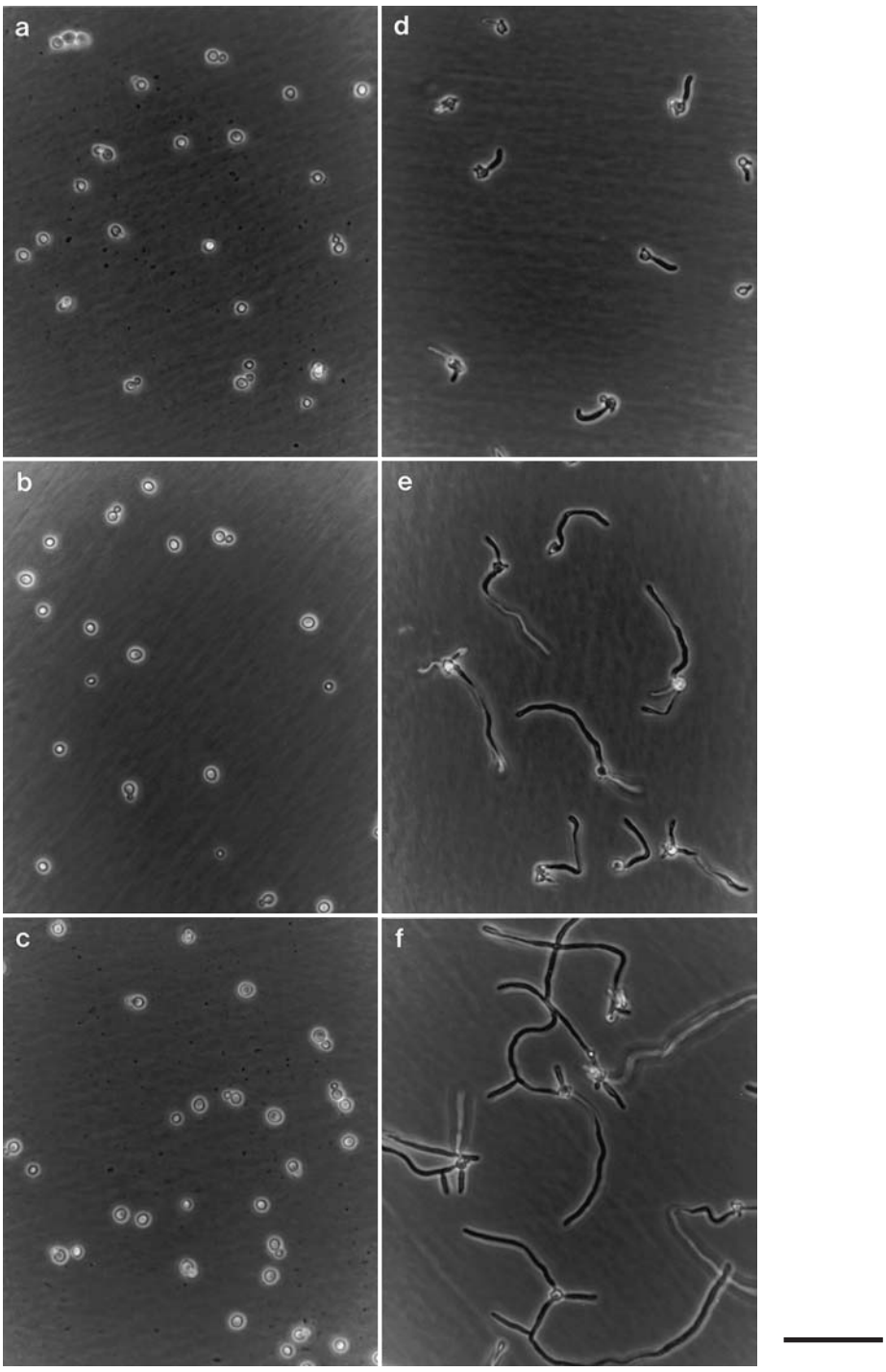

Fig. 2. Effect of the protein synthesis inhibitor, cycloheximide on the serum induced germ tube and filamentous formation.

Cells were inoculated in HFM7 containing cycloheximide and incubated for 1.5 h (a), 3 h (b), and 6 h (c) hours, or inoculated in HFM7 for 1.5 h (d), 3 h (e), and 6 h (f). Cells were preincubated for 24 h with actinomycin D (0.4 mg/m*l*) and hydroxyurea (0.1M). Bar = 50 μm.

cells. Hydroxyurea blocked DNA synthesis and prevented the dNTP pool expansion that occurs at G1/S[13]. Thus, not only protein synthesis inhibitor but also hydroxyurea might have affected the delay of germ tube induction.

When actinomycin D was added at the concentration of 0.4 mg/m*l*, yeast growth was completely blocked (data not shown) and germ tube formation proceeded (Fig. 3a, b), but filamentation was not observed (Fig. 3c). In this experiment, germ tube formation was also delayed 1.5 h after induction compared to the

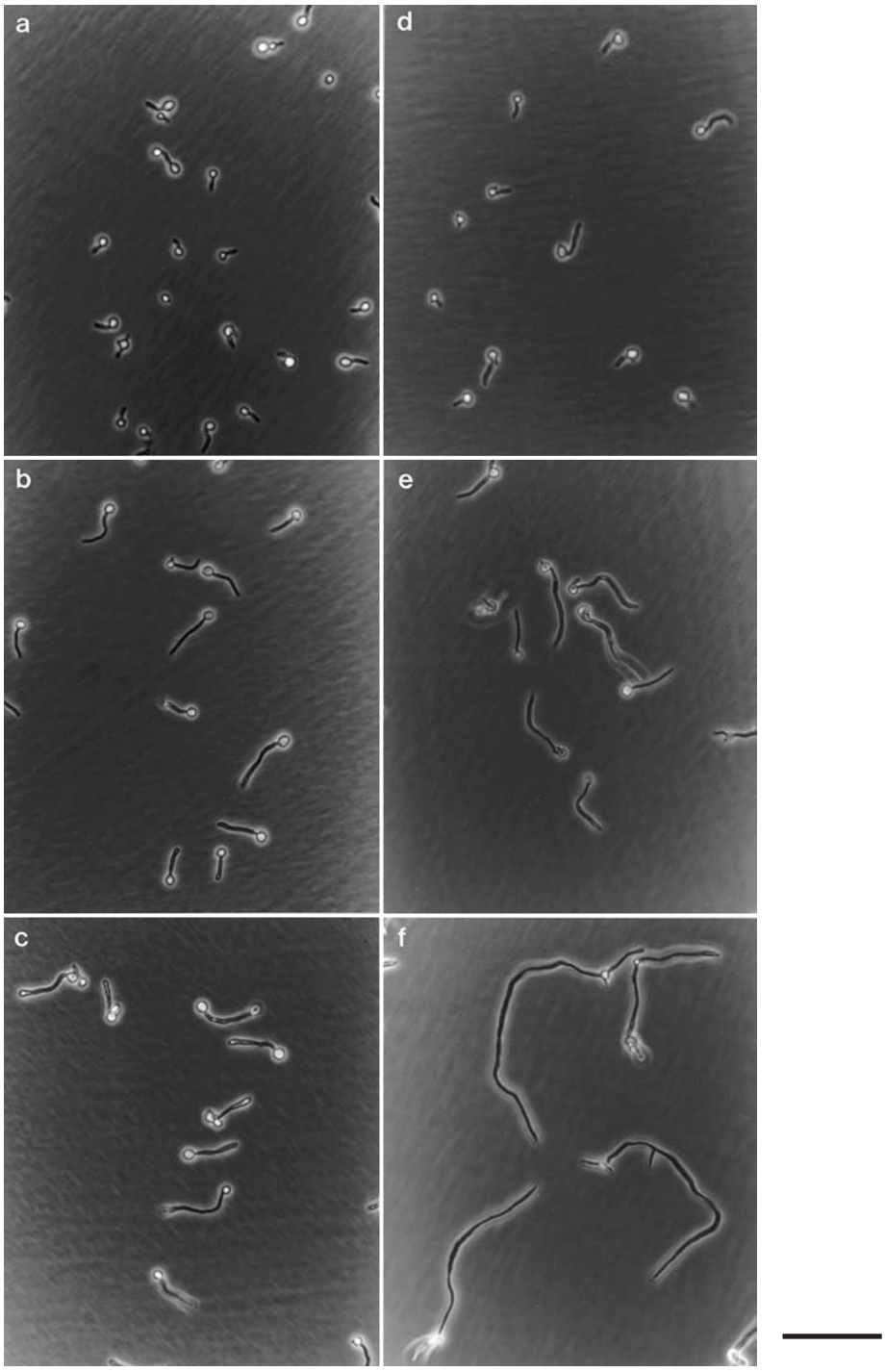

Fig. 3. Effect of mRNA synthesis inhibitor, actinomycin D, on the serum induced germ tube and filamentous formation.

Cells were inoculated in HFM7 containing actinomycin D and incubated for 1.5 h (a), 3 h (b), and 6 h (c) hours, or inoculated in HFM7 for 1.5 h (d), 3 h (e), and 6 h (f). Cells were preincubated for 24 h with actinomycin D (0.4 mg/m*l*) and hydroxyurea (0.1M). Bar = 50 μm.

result shown in Fig. 1. Thus, it may again be suggested that loss of the endogenous mRNA caused the delay in this formation. We also found that the actinomycin D (0.4 mg/m*l*) allowed germ tube formation but not true hyphae elongation. The length of a germ tube observed at 6 h was almost the same as that at 3 h, suggesting that elongation ceased after that time point (Fig. 3c). This observation indicates that a germ tube, but not true hyphae, can be formed in the presence of actinomycin D (0.4 mg/m*l*).

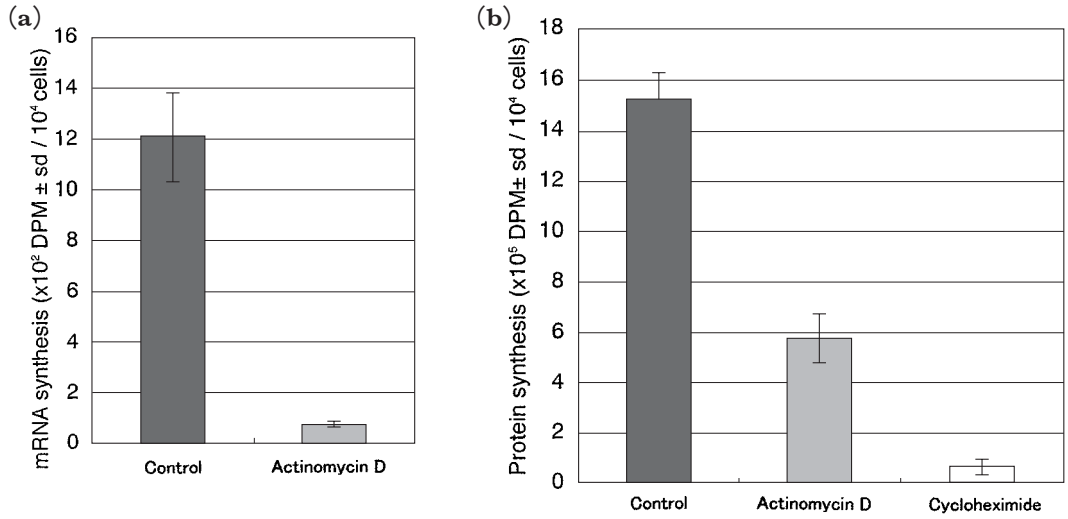

Fig. 4. mRNA and protein synthesis in the presence of inhibitors.

Cells were incubated with actinomycin D (shaded boxes) or cyclohexamide (open box). Control experiments (without inhibitors) are also indicated for comparison (closed boxes).

In the previous study, Land *et al.* indicated that both germ tube formation and yeast phase growth were inhibited by 0.1 mg/m*l* actinomycin D[7]. However, in our study, germ tube formation was observed in the presence of 0.4 mg/m*l* actinomycin D, even though our concentration was four times higher than that they used. In their study, cells were cultured in Lee medium at 37°C to induce germ tube formation, while we used HFM7 medium for the induction. Under our conditions, germ tube formation could be synchronized and almost all cells could form germ tubes. It may be possible that HFM7 medium induces germ tube formation and transcription of the mRNA required for this formation more strongly than Lee medium, which overcame the presence of actinomycin D, or that our condition somehow interfered with the uptake of the inhibitor into the cells, or that the serum might contain elements which inhibit the effects of actinomycin D. In addition, HFM7 with actinomycin D allowed only germ tube formation but not budding growth or filamentation, so that the condition would be useful for gene analysis specific for germ tube formation.

mRNA leaking from the inhibitor may be sufficient for germ tube formation

As cell morphology remarkably changed 3 h after the induction in HFM7 containing actinomycin D, we measured the amount of mRNA or protein accumulated during that 3 h after the shift (Fig. 4). In the presence of actinomycin D, mRNA synthesis was decreased to 6.3% and protein synthesis to 37.7% compared to those of the control cultures, and the cells formed germ tube elements as shown in Fig. 3. When cells were treated with cycloheximide, however, protein synthesis was decreased to 0.4% and germ tube formation was not observed. We assumed that essential and sufficient mRNA for germ tube formation, despite the expression level being much lower, must have been transcribed even in the presence of actinomycin D. *HYR1*, *CHS2* and *CHS3* have previously been identified as genes whose expression was induced during germ tube formation[14, 15]. These genes might have been transcribed for germ tube formation even in the presence of actinomycin D in this study.

We found here that *C. albicans* forms germ tubes in the presence of 0.4 mg/m*l* of actinomycin D which completely blocked yeast budding growth. Thus, this condition would be convenient for gene isolation and identification involved in morphological transition in *C. albicans*, especially for a transcript-based gene identification approach such as differential display. We are currently attempting to clone genes under the conditions described in this report, and will genetically characterize genes expressed during germ tube formation.

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