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Abstract
A method for determining mRNA half-lives in the polymorphic fungus *Candida albicans* is described. It employs growth in a defined medium, the inhibition of transcription with thiolutin (10–20 μg/mL), and quantitative Northern blotting. The method is effective for the A72, SC5314, and CAI-4 strains of *C. albicans*, and for mRNAs that have a wide variety of decay rates and steady-state abundances. The range of half-lives detected (from 4–168 min) shows that this method is effective for mRNAs with widely varying half-lives. The mRNA decay rates obtained are compared with those for orthologous mRNAs from *Saccharomyces cerevisiae*. This procedure should work for a broad range of *C. albicans* strains and can be adapted to other fungal species.

Keywords: comparative mRNA stability, *ACT1*, *ADH1*, *EFG1*, *PGK1*, *18S* rRNA, mRNA decay

Résumé
Une méthode pour déterminer les demi-vies d'ARNm dans le champignon polymorphe *Candida albicans* est décrite. Il utilise la croissance dans un milieu défini, l'inhibition de la transcription avec la thiolutine (10–20 μg / mL) et le Northern

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blot quantitatif. La méthode est efficace pour les souches A72, SC5314 et CAI-4 de *C. albicans*, et pour les ARNm qui ont une grande variété de taux de désintégration et d'abondances à l'état d'équilibre. La gamme de demi-vies détectées (de 4 à 168 min) montre que cette méthode est efficace pour les ARNm avec des demi-vies très variables. Les taux de désintégration des ARNm obtenus sont comparés à ceux des ARNm orthologues de *Saccharomyces cerevisiae*. Cette procédure devrait fonctionner pour une large gamme de souches de *C. albicans* et peut être adaptée à d'autres espèces fongiques.

Mots-clés: stabilité comparative de l'ARNm, ACT1, ADH1, EFG1, PGK1, ARNr 18S, désintégration de l'ARNm

Introduction

The polymorphic fungus *Candida albicans* is a commensal, found in many different locations of the human body, that can become a major fungal pathogen in immunocompromised individuals. Because of its pathogenicity, *C. albicans* has become the 4th most common nosocomial infection in the United States. However, current antifungal treatments are only partly effective. Consequently, a significant number of patients with nosocomial *C. albicans* infections who are treated with current antifungals will die (Gudlaugsson et al. 2003; Macphail et al. 2002). *Candida albicans* is eukaryotic, sharing many biological processes with humans. Therefore, it is important to understand the regulation of gene expression of *C. albicans* to develop or improve treatment methods (Berman and Sudbery 2002).

The amount of protein that is synthesized by a cell depends on the rate of synthesis of the protein, which in turn depends on the steady-state level of the corresponding mRNA. The steady-state levels of mRNAs are determined by 2 factors: the rate of synthesis of the specific mRNA, and the rate of decay of that mRNA. Thus, mRNA stability is an important aspect of the regulation of gene expression. Eukaryotic mRNA half-lives can vary from a few minutes in *Saccharomyces cerevisiae* to several hours in mammalian cells (Ross 1995). Herrick et al. (1990) reported that for *S. cerevisiae*, most of the mRNA half-lives are in the 3–45 min range.

However, half-lives of individual *C. albicans* mRNAs have not been reported. Bulk half-lives for total poly(A)–containing RNA have been determined using the pulse– chase technique, and were found to be about 60 min (Bhattacharya and Datta 1975). This value is significantly

longer than that reported for *S. cerevisiae* (Herrick et al. 1990). In this paper, we describe the determination of mRNA half-lives of select *C. albicans* mRNAs that represent both stable and unstable mRNAs. The method works by shutting off the transcription of new mRNAs, using thiolutin, and then looking at the turnover of the already present messages via quantitative Northern blotting. The method is effective for all 3 of the *C. albicans* strains tested and for mRNAs that are present in both low and high abundance.

Materials and methods

Candida albicans strains and growth media

Three strains of *Candida albicans* were used: A72, SC5314, and CAI-4 (*ura3::imm434/ura3::imm434*) (Fonzi and Irwin 1993). A72 and SC5314 are clinical isolates and SC5314 is the reference strain for the *Candida* genome sequence (Arnaud et al. 2005). SC5314 and CAI-4 were obtained from Alexander Johnson, University of California at San Francisco. A72 was obtained from Patrick Sullivan, University of Otago, Dunedin, New Zealand, and A72 has been deposited with the American Type Culture Collection (Rockville, MD) as strain MYA-2430.

All cultures were grown in either YPD or the defined glucose–phosphate–proline (GPP) (pH 4.8), which was supplemented with 50 μg/mL uridine for CAI-4. YPD contains 1% yeast extract, 0.5% peptone, and 2% glucose. GPP contains, per liter: 20 g glucose, 1.15 g L-proline, 0.55 g *N*-acetylglucosamine, 4 g KH_2PO_4, 3.2 g NaH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 mg $CuSO_4 \cdot 5H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $MnCl_2$, 1 mg $FeSO_4$, 20 μg biotin, 200 μg pyridoxine·HCl, and 200 mg thiamine·HCl (pH 4.8). The glucose (20% *w*/*v*) and L-proline (100 mmol/L) were autoclaved separately and added aseptically to the GPP to give a final volume of 1 L.

Growth curves in the presence or absence of thiolutin

Thiolutin (Pfizer compound CP-4,092) was kindly provided by Edward Pagani, Manager Research and Development Operations, Central Research Division, Pfizer Inc. (Groton, Conn.). Thiolutin was solubilized to a final concentration of 10 mg/mL in dimethyl sulfoxide

(DMSO). All growth conditions were at 30 °C with shaking at 200 r/min, using a G10 gyrotory shaker (New Brunswick Scientific Inc., New Brunswick, N.J.). An overnight culture of *C. albicans* was prepared in 5 mL YPD or GPP. From that overnight culture, 1 mL was added to 40 mL of prewarmed YPD or GPP in a 250 mL nephaloflask, and turbidity readings were taken with the Klett–Summerson colorimeter (Klett Manufacturing Company Inc., New York), using a red filter, until the culture reached 250 Klett units (roughly 0.5 OD_{600}). Then, 10 mL of culture was added to each of 3 prewarmed nephaloflasks. Thiolutin in DMSO was added to 2 separate flasks, at a final concentration of 10 or 20 μg/mL, respectively. An equivalent amount of DMSO alone was added to a third flask (DMSO-only control). The remaining 10 mL in the original flask served as an untreated control. Klett readings were taken every 5 to 10 min for the first hour, and as needed thereafter. Viability counts were determined using a hemocytometer and methylene blue staining.

Uridine incorporation

^{3}H-uridine incorporation followed the protocol described by Bini et al. (2002), with modifications. *Candida albicans* CAI-4 was grown in GPP, supplemented with 0.175 μg/mL of nonradioactive uridine to an OD_{600} of ca. 0.32. Portions (1.7 mL) of this culture were transferred to sterile 25 mL Erlenmeyer flasks and equilibrated for 5 min at 30 °C with shaking at 200 r/min. Radiolabeled uridine (5,6-^{3}H, 37 Ci/mmol; MP Biomedicals Inc., Irvin, Calif.) was added to make 0.013 μCi/mL. Samples (10 μL) were collected in duplicate at 0, 10, 20, 40, and 60 min, and transferred to capped eppendorf tubes containing 490 μL of unlabelled carrier cells (ca. 4×10^{8} cells/mL), added to promote centrifugal recovery of trichloroacetic acid (TCA) precipitates. A total of 150 μL of cold 50% (*w*/*v*) TCA was added to each tube, to give a final concentration of 11.5% (*w*/*v*) TCA. Samples were vortexed, allowed to precipitate on ice for at least 5 min, and centrifuged at 15 000*g*, whereupon the supernatants were discarded. Pellets were resuspended in 25 μL of Tris–EDTA (pH. 8.0), placed in scintillation vials containing 2 mL of scintisafe F scintillation fluid (Fisher Scientific, Pittsburg, Penn.), and counted on an LS1701 liquid scintillation counter (Beckman Instruments, Fullerton, Calif.).

To assess thiolutin as a transcription inhibitor, after 60 min the culture was split into 3 flasks, each containing 500 µL of culture with, respectively, thiolutin 10 µg/mL in DMSO (0.1%), DMSO (0.1%), and no further additions. Samples (10 µL) were taken from each flask after 65, 70, 75, 80, 85, 90, 95, and 100 min and processed, as described above.

mRNA half-lives

Candida albicans CAI-4 cells were grown overnight at 30 °C in YPD or GPP, supplemented with 50 µg/mL uridine to an OD_{600} of 0.4–0.6. Thiolutin (10 mg/mL in DMSO) was added to a final concentration of 10 µg/mL. Cells were then harvested at different time points after thiolutin was added, and total RNA was extracted using the hot phenol method (Kebaara et al. 2003). Equal amounts of RNA (15 µg) were resolved on 1.0% agarose–formaldehyde gels, and the RNA was transferred to GeneScreen Plus (NEN Life Science Products Inc., Boston, Mass.), using the capillary blot transfer protocol recommended by the manufacturer. The Northern blots were probed sequentially with *ACT1, ADH1, EFG1, PGK1*, and *18S* DNA probes. The probes were pre pared using PCR with SC5314 genomic DNA; the primers are listed in Table 1. The probes were labeled with ^{32}P using an oligolabelling kit (RadPrime DNA labeling system; Invitrogen, Life Technologies, Carlsbad, Calif.). Northern blots were phosphorimaged using a Storm (Amersham Pharmacia Biotech Inc., Piscataway, N.J.). Half-lives were determined by plotting $\log_{10}$ of the percent mRNA remaining against time, using SigmaPlot 2000, v. 6.10 (SPSS Science, Chicago, Ill.). Half-life measurements were done in triplicate.

Table 1. Primers used to generate probe fragments, and sizes of the probe fragments.

Gene	*5′ primer*	*3′ primer*	*Probe fragment size (bp)*
ACT1	AGTTATCGATAACGGTTCTG	AGATTTCCAGAATTTCACTC	1124
ADH1	CAAAAAGGATAATCCCGCAC	CTGGAGCAGTGACTTTAGCGTG	1145
EFG1	TTGACTACCAAGAATATAACC	CACTGGTAGCAGATATACTG	1128
PGK1	CAATCAAGCGATTATCAATTG	ATGTTACCATTTTCAGCAGA	1141
18S rRNA	TCAGTTATCGTTTATTTGAT	TTCCTCTAAATGACCAAGTTTG	1621

Results

Thiolutin inhibits C. albicans growth

A series of known transcription inhibitors were tested for their ability to inhibit the growth of *C. albicans*. Thiolutin inhibited the growth of *C. albicans* strains CAI-4 (Fig. 1), SC5314, and A72 when added at 10–20 μg/mL. It also inhibits the growth of the yeast *S. cerevisiae* strain Y166 (Jimenez et al. 1973) and strain DBY747 (Herrick et al. 1990) when present at 2–4 μg/mL, by reversibly inhibiting RNA synthesis. In contrast, actinomycin D, at concentrations up to 2 mg/mL, did not inhibit the growth of *C. albicans*. This inactivity by actinomycin D is in accord with the generalization that most fungi are impermeable to actinomycin D.

Before conducting the *C. albicans* mRNA half-life experiments, it was important to determine the concentration of thiolutin needed for that particular strain and set of growth conditions. When grown in YPD at 30 °C, 10 μg/mL thiolutin was required to inhibit *C. albicans* CAI-4 (Fig. 1A); a higher concentration (20 μg/mL) was required for *C. albicans* A72 (data not shown). For CAI-4 in YPD (Fig. 1A), after the addition of 10 μg/mL thiolutin, there was a lag of about 25 min before the cells stopped growing. In contrast, when *C. albicans* CAI-4 was grown in the defined GPP with 50 μg/mL of uridine, the cells did not exhibit a lag period between thiolutin addition and growth inhibition, but they required 20 μg/mL of thiolutin to inhibit growth (Fig 1C and D). Similar observations were made with *C. albicans* SC5314 (20 μg/mL thiolutin) and *C. albicans* A72 (20 μg/mL of thiolutin); there was a 20–25 min lag period when grown in YPD but no lag period with GPP. Viability of *C. albicans* cells in the presence of thiolutin was ≥90% but in its absence was 100%. Viability of *C. albicans* cells was determined using a hemocytometer with methylene blue staining.

Thiolutin treatment arrests transcription

To determine whether the growth arrest following thiolutin treatment is accompanied by transcription inhibition, we measured the incorporation of ^{3}H-uridine into TCA-precipitable RNA, as described elsewhere (Bini et al. 2002). *C. albicans* CAI-4 incorporates ^{3}H-uridine

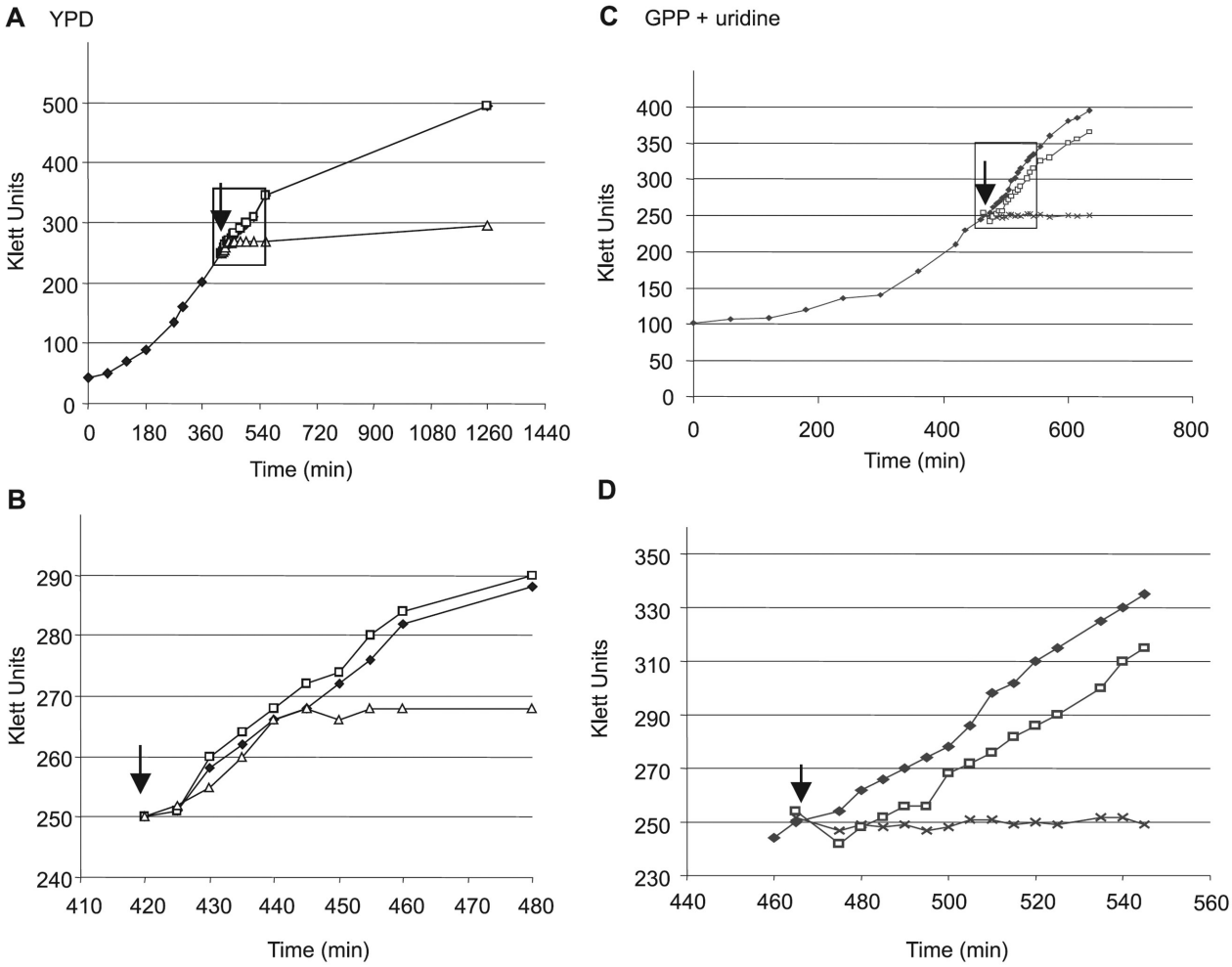

Fig. 1. Effect of thiolutin on the growth of *Candida albicans* CAI-4. *C. albicans* CAI-4 grown in YPD (A and B) or glucosephosphate-proline (GPP) supplemented with uridine (C and D) in the presence or absence of thiolutin. The bottom panels (B and D) are expansions of the boxes shown in the upper panels. ♦, control; □, dimethyl sulfoxide (DMSO); Δ, thiolutin (10 μg/mL); ×, thiolutin (20 μg/mL); GPP, glucosephosphate-proline. Thiolutin curves are an average of 3 trials.

when grown in GPP supplemented with as little as 0.175 μg/mL of unlabelled uridine (Fig. 2), and the addition of thiolutin (10 μg/mL) inhibited ^{3}H-uridine incorporation. A 10–15 min lag period was observed before inhibition was complete (Fig. 2), after which the level of TCA-precipitable RNA remained fairly constant. As expected, cells grown in the absence of thiolutin continued to incorporate ^{3}H-uridine (Fig. 2).

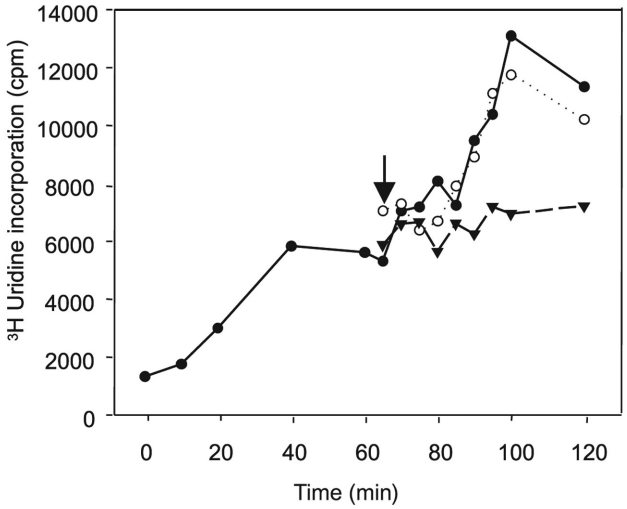

Fig. 2. Effect of thiolutin on RNA synthesis. Measurement of 3Huridine incorporation into *C. albicans* CAI-4 (a uridine auxotroph). Thiolutin (10 μg/mL in DMSO, ▼) or DMSO (○), both added at 60 min (indicated by arrow). Untreated control (●).

mRNA half-lives

mRNA half-lives of select transcripts were determined for *C. albicans* CAI-4 grown in both YPD and GPP supplemented with uridine (Table 2; Fig. 3). The transcripts were selected to include mRNAs expected to be stable (*ACT1* mRNA, which encodes actin, and *PGK1* mRNA, which encodes phosphoglycerate kinase), unstable (*EFG1* mRNA, which encodes enhanced filamentous growth factor, a transcription regulator), and of intermediate stability (*ADH1* mRNA, which encodes alcohol dehydrogenase). The half-life of *18S* rRNA was also determined because it is an RNA polymerase I transcript and it has been reported that thiolutin inhibits all 3 RNA polymerases in *S. cerevisiae* (Jimenez et al. 1973). The mRNA half-lives were measured for 60 min for *ADH1* and *EFG1* mRNAs, and for 120 min for *ACT1, ADH1*, and *PGK1* mRNAs. Figure 3 shows representative phosphorimages of *PGK1* mRNA at different times after the inhibition of transcription. mRNA half-lives were determined from the slopes of the lines plotting percent mRNA remaining against time after transcription arrest. The lag in growth inhibition observed when thiolutin was added to *C. albicans* CAI-4 in YPD was also observed when determining mRNA half-lives (Fig. 3A), although the lag was shorter (~10 min). There was also a short lag

Table 2. Comparison of *Candida albicans* mRNA half-lives with previously determined *Saccharomyces cerevisiae* mRNA half-lives.

Transcript	*C. albicans mRNA half-lives*[a] *(min)* 30 °C	*S. cerevisiae mRNA half-lives (min)* 30 °C	36 °C	*rpb1–1*[b]	Reference
ACT1 mRNA	34.6	135	30	40	Li et al. 1999
ADH1 mRNA	33.3	—	—	33	Wang et al. 2002
EFG1/SOK2 mRNA	4.3	—	—	21	Wang et al. 2002
PGK1 mRNA	43.3	>100	—	45	Herrick et al. 1990
18S rRNA	168	—	—	—	—

a. *C. albicans* half-lives were done in YPD at 30 °C.
b. *rpb1–1* is a temperature sensitive allele of RNA polymerase II (Nonet et al. 1987).

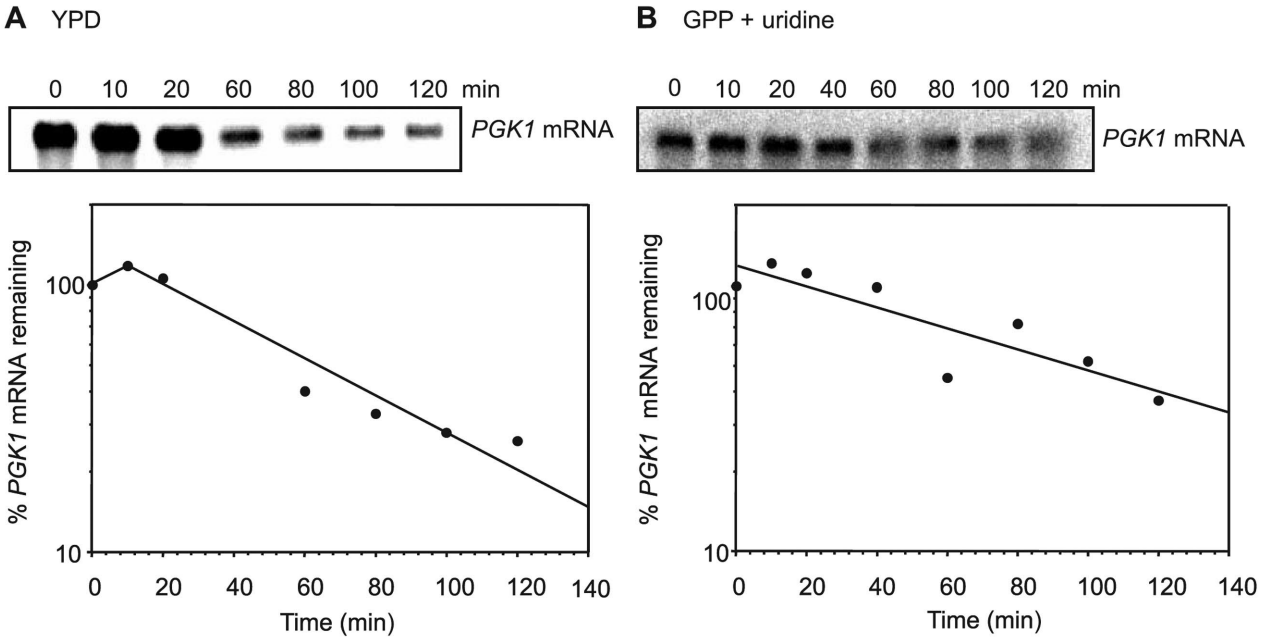

Fig. 3. *PGK1* mRNA half-life in YPD (A) and in GPP supplemented with uridine (B). The phosphorimages show the *PGK1* mRNA at different time points after the arrest of transcription using thiolutin. The time points are indicated above the phosphorimages. The percent mRNA remaining was calculated by dividing the volume of a particular band by the volume of the 0 min time point. The percent mRNA remaining vs. time was plotted using SigmaPlot.

period observed for mRNA half-lives in GPP (Fig. 3B), even though there was no lag period for growth inhibition under those conditions (Fig. 1C and 1D). Transcript half-lives in YPD at 30 °C were determined to be 34.6 ± 5 min for *ACT1* mRNA, 43.3 ± 0.6 min for *PGK1* mRNA, 33.3 ± 3 min for *ADH1* mRNA, 4.3 ± 1.2 min for *EFG1* mRNA, and 168 ± 25 min for *18S* rRNA.

The half-lives of *ADH1* and *PGK1* mRNAs were also determined for CAI-4 grown in GPP supplemented with uridine. In this case, the *ADH1* mRNA half-life in GPP was 33 ± 4.7 min, consistent with the 33.3 ± 3 min half-life determined in YPD. *PGK1* mRNA half-life in GPP supplemented with uridine was 58 ± 12 min (Fig. 3B), whereas it was only 43.3 ± 0.6 min in YPD (Fig. 3A).

Discussion

We have devised an effective method for determining mRNA half-lives in the dimorphic fungus *C. albicans*. No method for measuring *Candida* mRNA half-lives has been reported to date. Because mRNA half-life is an important gene regulatory mechanism, this method will be an essential new tool for the study of gene regulation in *C. albicans*. This method prevents new mRNA synthesis with the transcription inhibitor thiolutin. This procedure, adapted from one previously used for *S. cerevisiae* (Herrick et al. 1990), had to be validated for *C. albicans* because of the distinctive features of *C. albicans*: unlike *S. cerevisiae*, it cannot uptake uracil; its genome contains ca. 2000 more genes than does that of *S. cerevisiae*; strains of *C. albicans* can behave very different physiologically; and *C. albicans* exhibits a temperature-regulated dimorphism, in that it usually grows as budding yeasts at 25–30 °C and as mycelia at ≥37 °C (Odds 1988). For this last reason, the most common method for determining mRNA half-lives in *S. cerevisiae* (based on a 24–36 °C shift in a strain containing a thermolabile RNA polymerase II) would have limited utility in *C. albicans*, even if the corresponding temperature-sensitive mutant was available. The mRNA half-life method used here involves inhibiting transcription with thiolutin and then measuring mRNA accumulation at subsequent time points.

Thiolutin is a purine analog and the inability of *C. albicans* to take up uracil, unlike *S. cerevisiae*, suggests that there is a fundamental

difference in nucleoside uptake between *C. albicans* and *S. cerevisiae*. We found that thiolutin inhibited the growth of 3 *C. albicans* strains: CAI-4 (Fig. 1), SC5314, and A72. Further, for *C. albicans* CAI-4, we showed that growth inhibition was accompanied by transcription inhibition (Fig. 2). These strains were chosen because CAI-4 is routinely used for molecular genetic and genomic studies (Magee et al. 2003), SC5314 is the reference strain for the *Candida* genome sequence (Arnaud et al. 2005), and A72 is a well-characterized farnesol-producing and farnesol-responsive strain. Thus, the use of thiolutin to inhibit transcription will be of broad utility to *Candida* researchers. Further, our method should be adaptable to other fungal species sensitive to thiolutin.

Unstable mRNAs tend to be less abundant than stable mRNAs; thus, mRNA half-lives are important in determining both the abundance of a specific message and the way a particular gene is controlled. Thiolutin has been used successfully to measure mRNA half-lives in *S. cerevisiae*, and the decay measurements made with thiolutin were comparable to those done with other methods (Herrick et al. 1990). The method described here can be used to determine mRNA half-lives in all strains of *C. albicans* tested, with cells grown at many different temperatures in both minimal and rich media. However, in all cases, it is important to tailor the thiolutin concentration to the strain of *C. albicans* and the growth conditions being studied. The choice of growth conditions might also determine the length of the lag period observed after thiolutin is added. For *C. albicans* CAI-4 grown in YPD, there was a lag period of 25 min before growth inhibition (Fig. 1B), but the lag period was only ~10 min before the inhibition of transcription (Fig. 3A). In contrast, for *C. albicans* CAI-4 grown in the defined GPP medium, there was no detectable lag before growth inhibition (Fig. 1C and 1D) and a very short lag before transcription inhibition (Fig. 3B). Similar mRNA half-life measurements in *S. cerevisiae* have shown a lag period between thiolutin addition and transcription inhibition of about 10 min (Herrick et al. 1990).

We have determined the half-lives of 5 transcripts that have varying stabilities. Based on their known stabilities in *S. cerevisiae* (Herrick et al. 1990), *ACT1* and *PGK1* mRNAs were expected to be stable; they were found to be long lived in *C. albicans* as well (Table 2). Interestingly, the half-lives of *PGK1* and *ADH1* mRNAs were 43.3 and 33.3 min, respectively, in *C. albicans*, compared with 45 and 33 min,

respectively, in *S. cerevisiae* (Herrick et al. 1990; Wang et al. 2002). However, the *PGK1* mRNA half-life of 43.3 min in YPD was increased to 58 min when the cells were grown in GPP. This shows that mRNAs of the same transcript can have different half-lives, depending on the growth conditions. The *ADH1* and *ACT1* mRNA half-lives were also similar to those for *S. cerevisiae* (Table 2). *ADH1* mRNA showed intermediate stability, whereas *EFG1* mRNA, which encodes a transcription factor, was unstable, as was expected because of its regulatory function (Stoldt et al. 1997). This range of half-lives, from 4 to 168 min (Table 2), shows that this method can be used to determine the stability of mRNAs with widely varying half-lives. It also allows the tentative conclusion that mRNA half-lives are generally equivalent for *S. cerevisiae* and *C. albicans*. Finally, the method of inhibiting transcription with thiolutin can also be used with other RNA detection methods, such as RNase protection or quantitative reverse tran scription (RT)-PCR. In both cases, thiolutin would be added to growing cells, RNA would be extracted, and the amount of RNA remaining would be determined at successive time points. Quantitative Northern blotting is currently the method of choice, at least initially, because the size of the RNA and the potential presence of multiple bands can be detected on Northern blots. mRNA size is one indicator that the correct mRNA is being detected. Multiple bands could arise from RNA isoforms or cross hybridization to related or similar RNAs. It is more difficult to discriminate between different RNA isoforms with RT-PCR or RNase protection.

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