Regulation of the gene encoding translation elongation factor 3 during growth and morphogenesis in *Candida albicans*

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The level of the *TEF3* mRNA, which encodes the fungal-specific translation elongation factor 3 (EF-3), was measured during the yeast-to-hyphal transition in *Candida albicans*. In contrast to a previous report, *TEF3* mRNA levels were shown to change during dilution into fresh medium, increasing only transiently when dimorphism was induced by either (i) an increase in growth temperature (from 25 °C to 37 °C) combined with the addition of 10% (v/v) bovine calf serum to the medium, or (ii) an increase in growth temperature (from 25 °C to 37 °C) combined with an increase in the pH of the medium (from pH 4.5 to 6.5). *TEF3* mRNA levels also increased in control cultures under conditions where germ tubes were not formed, but they remained elevated in contrast to cultures undergoing morphological changes. *TEF3* mRNA levels were not significantly affected by heat-shock, but were tightly regulated during batch growth of the yeast form, reaching maximal levels in exponential phase. Therefore, the changes in *TEF3* expression that accompany the dimorphic transition in *C. albicans* appear to reflect the underlying physiological changes that occur during morphogenesis and are not a response to morphogenesis *per se*. For this reason *TEF3* mRNA measurement cannot be used as a loading control in Northern analyses of dimorphic gene regulation. Comparison of *TEF3* mRNA levels with the abundance of the EF-3 polypeptide indicated that the synthesis of this essential translation factor might be subject to post-transcriptional regulation.

Keywords: *Candida albicans*, translation elongation factors, *TEF3* regulation, dimorphism, heat-shock

INTRODUCTION

Candida albicans most frequently infects the mucous epithelial tissues of the oral and urogenital tracts, but in the immunocompromised host it can cause deep-seated and systemic infections (Odds, 1988). A number of factors are thought to influence the pathogenicity of this fungus (for reviews see Cutler, 1991; Odds, 1988), one of which is its ability to undergo a dimorphic switch from a budding yeast to a hyphal form (for reviews see Gow & Gooday, 1987; Odds, 1985; Soll, 1986). Although both yeast and hyphal cells can be observed in infected tissue (Odds, 1988), and the yeast form is pathogenic under some experimental conditions (Ryley & Ryley, 1990), *C. albicans* hyphae appear better adapted to penetrate epithelia

(Sherwood *et al.*, 1992), suggesting that dimorphism is an important aspect of pathogenicity *in vivo*.

Translational elongation in eukaryotic organisms is dependent upon the two soluble elongation factors EF-1 and EF-2, which are functionally analogous to the prokaryotic factors EF-Tu and EF-G, respectively (for reviews see Hinnebusch & Liebman, 1991; Merrick, 1992; Nygard & Nilsson, 1990; Tuite, 1989). However, a third elongation factor (EF-3) is required for translation in fungi (for review see Belfield & Tuite, 1993). EF-3, which was first identified in *Saccharomyces cerevisiae* (Skogerson & Wakatama, 1976), has now been observed in numerous fungi including *Schizosaccharomyces pombe* (Uritani & Miyazaki, 1988), *Pneumocystis carinii* (Ypma-Wong *et al.*, 1992), and several *Candida* species (Chakraborty & Kamath, 1988; Colthurst *et al.*, 1991). Although the precise function of EF-3 has not been

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Fig. 1. *TEF3* mRNA levels during the yeast-to-hyphal transition. (a) An overnight starter culture of *C. albicans* (S) grown in YPD at 25 °C was used to inoculate flasks containing either YPD at 25 °C, YPD containing serum at 25 °C, YPD at 37 °C, or YPD containing serum at 37 °C, and the proportion of cells forming germ tubes in each culture was estimated by light microscopy. The levels of the *TEF3* and *ACT1* mRNAs were measured at various times (min) thereafter by quantitative Northern analysis. (b) An overnight starter culture of *C. albicans* (S) grown in Soll's Medium at pH 4.5 and 25 °C was used to inoculate flasks containing Soll's Medium either at pH 4.5 and 25 °C, pH 4.5 and 37 °C, pH 6.5 and 25 °C, or pH 6.5 and 37 °C. Cell morphology and *TEF3* and *ACT1* mRNA levels were then determined as described in (a). The radioactive signals obtained on the filters shown in (a) and (b) were quantified directly by 2D-radioimaging and the data are presented in (c) and (d), respectively.

established, it displays ribosome-dependent GTPase and ATPase activities (Dasmahapatra & Chakraborty, 1981; Miyazaki *et al.*, 1988; Skogerson & Wakatama, 1976) and it might play a role in maintaining the accuracy of mRNA decoding at the ribosomal A-site (Belfield & Tuite, 1993). EF-3 represents an attractive antifungal target because it is essential for translation in fungi, but not in mammalian systems.

A previous analysis of *TEF3* expression in *C. albicans* suggested that there is no significant difference in the level of the *TEF3* mRNA in the yeast and hyphal forms (DiDomenico *et al.*, 1992). This suggested that *TEF3* mRNA levels may provide a useful loading control in Northern analysis of other mRNAs during the dimorphic transition. Actin mRNA (*ACT1*) levels which are normally used in other systems, change during morphogenesis (Lasker & Riggsby, 1992; Paranjape & Datta, 1991; Delbrück & Ernst, 1993). Therefore, in this study we have sought to clarify the influence of dimorphism on *TEF3* gene expression.

METHODS

Strains and culture conditions. *Candida albicans* Robin (Berkhout) strain 3153 from the London Mycological Reference Laboratory was used throughout. The dimorphic transition was induced using two different experimental conditions. *C. albicans* was grown in the yeast form to late-exponential growth phase at 25 °C with shaking at 200 r.p.m. in YPD [2% (w/v) glucose, 2% (w/v) bacteriological peptone, 1% (w/v) yeast extract]. To induce hyphal growth, 10 ml of this culture was then used to inoculate (approximately 2×10^7 cells ml⁻¹) 100 ml of YPD containing 10% (v/v) bovine calf serum at 37 °C and samples were removed at various times up to 120 min. Control cultures were grown in fresh YPD at 25 °C without serum, 25 °C with serum, and 37 °C without serum. Alternatively, *C. albicans* was grown in the yeast form to late-exponential growth phase in Soll's Medium at 25 °C and pH 4.5 with shaking at 200 r.p.m. and hyphal growth was induced by inoculating 10 ml of this culture (approximately 2×10^7 cells ml⁻¹) into 100 ml Soll's Medium at 37 °C and pH 6.5 (Soll, 1986) and samples were removed at various times up to 180 min. Control cultures were grown in fresh Soll's Medium at 25 °C and pH 4.5, 25 °C and pH 6.5, and 37 °C and pH 4.5. Soll's Medium is the defined

medium of Lee *et al.* (1975) modified according to Buffo *et al.* (1984). Cell numbers and morphology were monitored by light microscopy. Cell numbers were measured by light microscopy using an improved Neubauer haemocytometer. At least 200 cells were counted at each time point (errors < 10%).

RNA isolation and Northern blotting. Total RNA was prepared from *C. albicans* using the procedures of Lindquist (1981). Northern analysis was performed as described previously (Moore *et al.*, 1991) using radiolabelled probes prepared by random-priming of purified DNA restriction fragments (Feinberg & Vogelstein, 1983). A 0.7 kbp fragment representing coordinates 126–829 bp of the *C. albicans* *TEF3* coding region (cloned in pUC19; Colthurst *et al.*, 1992) and the 1.5 kbp *Bam*HI–*Hind*III fragment of the *S. cerevisiae* *ACT1* gene (cloned in pSP65; Bettany *et al.*, 1989) were used as probes. Hybridizations were performed under conditions of excess probe, and signals were quantified directly by 2D-radioimaging using an AMBIS Radioanalytic System (LabLogic). No internal mRNA loading control is available for *C. albicans*. Therefore, mRNA levels were measured relative to the ribosomal RNAs by loading approximately equal amounts of total RNA in each lane of the gels. Bound probes were removed by washing the filters in boiling 0.1% (w/v) SDS, and the filters were probed sequentially for the *TEF3* and *ACT1* mRNAs to allow direct comparison of expression patterns. Other DNA and RNA manipulations were performed according to standard procedures (Sambrook *et al.*, 1989).

Western blot analysis. Protein extracts (Hostetter *et al.*, 1990) were electrophoresed on SDS-10% (w/v) polyacrylamide gels (Laemmli, 1970), electroblotted onto nitrocellulose membranes, and the EF-3 polypeptide detected using an anti-EF-3 polyclonal antibody (Colthurst *et al.*, 1991). The secondary antibody was alkaline-phosphatase-conjugated anti-rabbit IgG (Sigma).

RESULTS

TEF3 mRNA levels during morphogenesis

TEF3 mRNA levels were measured during the yeast-to-hyphal transition under two different sets of growth conditions (Fig. 1). Hyphal growth was induced using (i) an increase in growth temperature (25 to 37 °C) combined with the addition of bovine calf serum to the medium, or (ii) an increase in growth temperature (25 to 37 °C) combined with an increase in the pH of the medium (pH 4.5 to 6.5). Under each set of conditions several parameters were altered to induce the transition, and therefore additional cultures were included to control for the effects of temperature, serum or pH (Fig. 1). In both experimental protocols an increase in temperature alone induced a low level of germ tube formation (Fig. 1a, b), but these levels never reached those achieved under optimal conditions using (i) a temperature increase plus serum or (ii) a temperature increase plus a pH change. The levels of the *TEF3* mRNA were relatively low in the late-exponential cells in the starter culture, but they increased rapidly upon dilution of these cells into fresh medium. This increase occurred over the first 20–60 min depending upon the growth conditions. During growth at 25 °C *TEF3* mRNA levels remained at relatively high levels over the time period analysed, whereas at 37 °C, *TEF3* mRNA levels decreased after about 20–30 min (Fig. 1c, d). The decline was faster in cultures which showed a higher induction of germ tubes or hyphae. This pattern, which

was reproducible, was not influenced significantly by the use of a medium that was rich or defined, at pH 4.5 versus pH 6.5, or whether it contained serum or not. Therefore, *TEF3* mRNA levels changed during the dimorphic transition under the experimental conditions analysed, but did not correlate absolutely with morphogenesis *per se*.

The *ACT1* mRNA is frequently used as a control in *S. cerevisiae* (e.g. Moore *et al.*, 1991). Therefore, we measured the levels of the *ACT1* mRNA under our experimental conditions (Fig. 1c, d), but *ACT1* mRNA levels have

Fig. 2. Effect of heat-shock on *TEF3* mRNA levels. An early-exponential culture of *C. albicans* grown in YPD at 25 °C was diluted fourfold in fresh YPD at 25 °C (■), 37 °C (▲) or 45 °C (+); the cultures were held at these temperatures for 10 min, and then incubated at 25 °C (time = 0 min). RNA was prepared from each culture at various times thereafter, and the level of the *TEF3* mRNA determined by Northern analysis using 2D-radioimaging to quantify signals directly on the filter. Cells grew in the yeast form throughout the experiment.

Fig. 3. *TEF3* mRNA levels during batch growth of the yeast form in YPD at 25 °C. An overnight starter culture of *C. albicans* was used to inoculate fresh culture of YPD (arrow). The culture was incubated at 25 °C with shaking (200 r.p.m.) and growth (▲) monitored by light microscopy. *TEF3* mRNA levels (■) were measured by quantitative Northern analysis. Points at time = 0 represent cell counts and *TEF3* mRNA levels immediately before dilution into fresh medium.

Fig. 4. Comparison of EF-3 and *TEF3* mRNA levels under different growth conditions. The yeast-to-hyphal transition was induced by an increase in culture temperature and the addition of bovine calf serum, and control cultures set up as described for Fig. 1(a). EF-3 was detected by Western blotting and compared with *TEF3* mRNA levels under similar growth conditions (Northern data taken from Fig. 1a). S, Starter culture.

been shown to change during morphogenesis in *C. albicans* (Lasker & Riggsby, 1992; Paranjape & Datta, 1991; Delbrück & Ernst, 1993). Consistent with previous reports, *ACT1* mRNA levels increased upon dilution of late-exponential cells into fresh medium, thus validating the observed increase in *TEF3* mRNA levels. However, the patterns of *ACT1* and *TEF3* expression differed in that the rate of increase in the *ACT1* mRNA level following dilution into fresh medium was slower in the cultures at 25 °C compared with 37 °C. The ratio of the *TEF3* to *ACT1* mRNA level did not remain constant under the conditions studied.

The effect of heat-shock on *TEF3* mRNA levels

To separate the influence of the temperature increase from that of the dimorphic transition, cells were heat-shocked under conditions which do not induce the dimorphic switch. Heat-shock at 37 °C or 45 °C did not affect *TEF3* mRNA levels to any great extent (Fig. 2).

TEF3 mRNA levels during batch growth

The patterns of expression observed for *TEF3* in Fig. 1 indicated that this gene might be responding to changes in growth. To test this, cells from a late-exponential culture were diluted 50-fold into fresh YPD at 25 °C, and *TEF3* mRNA levels measured at various times during the growth of the yeast form (Fig. 3). *TEF3* mRNA levels increased rapidly upon dilution into fresh medium, reached their maximal levels during the exponential growth phase, and declined to low levels during the transition and stationary phases. Therefore, *TEF3* expression is regulated during batch growth, and might be related to cell growth rate.

Comparison of EF-3 and *TEF3* mRNA levels

Some *C. albicans* genes appear to be subject to post-transcriptional regulation (Mason *et al.*, 1993). Therefore, the level of the EF-3 polypeptide was analysed by Western

blotting and compared with *TEF3* mRNA levels under the same conditions (Fig. 4). The EF-3 polypeptide of molecular mass about 130 kDa was detected by Western blot analysis under all the conditions tested, although the levels were lower during growth at 37 °C compared with 25 °C. This was reasonably consistent with the observed *TEF3* mRNA levels. However, in contrast to the *TEF3* mRNA, the EF-3 polypeptide was relatively abundant in late-exponential cells (in the starter culture), and EF-3 levels appeared to decrease when these cells were diluted into fresh media under some conditions (Fig. 4). This difference in *TEF3* mRNA and EF-3 levels was reproducible. Therefore, *TEF3* expression appears to be modulated at a post-transcriptional level(s).

DISCUSSION

A previous analysis of *TEF3* gene expression in *C. albicans* suggested that the abundance of its mRNA was similar in the yeast and hyphal forms when measured relative to the *ACT1* mRNA (DiDomenico *et al.*, 1992). However, *ACT1* mRNA levels are known to change during the yeast-to-hyphal transition in *C. albicans* (Lasker & Riggsby, 1992; Paranjape & Datta, 1991; Delbrück & Ernst, 1993), and therefore in this study we investigated *TEF3* expression in more detail with a view to testing the possibility of using this mRNA as a loading control in studies of dimorphic gene regulation. We measured *TEF3* mRNA levels relative to the ribosomal RNAs as a loading control in the Northern blots. *TEF3* mRNA levels changed during the dimorphic transitions under the two different experimental conditions analysed, but there was no strict correlation between *TEF3* mRNA levels and the rate of germ tube formation (Fig. 1). It should be noted, however, that there may be a potential influence of hyphal development on *TEF3* expression since the rate of decrease in *TEF3* mRNA levels after 20–30 min was more rapid in those cultures forming germ tubes (Fig. 1).

TEF3 mRNA levels were not significantly affected by the presence or absence of serum in the medium, or by the pH of the medium (Fig. 1). Also, *TEF3* mRNA levels were

not dramatically affected by heat-shock under conditions which did not permit germ tube formation (Fig. 2). However, the expression of the gene was tightly regulated during batch growth of the yeast form, with maximal *TEF3* mRNA levels being observed during the early exponential growth phase (Fig. 3). Therefore, the fluctuations in *TEF3* mRNA levels appear to reflect the underlying changes in cell cytology and physiology that accompany morphogenesis (Gow *et al.*, 1986; Gow & Gooday, 1987; Odds, 1988; Stewart *et al.*, 1988) and not necessarily morphogenesis *per se*. The fact that the levels of the actin, chitin synthase 1, chitin synthase 2, pyruvate kinase and alcohol dehydrogenase mRNAs also change during the dimorphic transition (Chen-Wu *et al.*, 1992; Lasker & Riggsby, 1992; Paranjape & Datta, 1991; Delbrück & Ernst, 1993; Swoboda *et al.*, 1993) would seem to support this view. Interestingly, the levels of the *TEF1* and *TEF2* mRNAs, which encode identical EF-1 α polypeptides, appear to be similar in yeast and hyphal cells (Sundstrom *et al.*, 1991). However, no detailed kinetic analysis of these mRNAs was performed during the dimorphic transition, and therefore it is possible that these mRNAs behave in a similar fashion to the *TEF3* mRNA. This would present a technical problem in that, to our knowledge, none of the *C. albicans* mRNAs studied to date have been shown to remain at constant levels during the dimorphic transition and hence no internal mRNA loading control is available for Northern analysis under these conditions. This study shows that the *TEF3* mRNA is not a suitable loading control for such studies.

The abundance of the EF-3 polypeptide did not always mirror the *TEF3* mRNA level under the conditions tested (Fig. 4). For example, EF-3 appeared to be relatively abundant in late-exponential cells when the *TEF3* mRNA was at low levels, and the polypeptide was not abundant during growth at 37 °C when *TEF3* mRNA levels were elevated. This might indicate that *TEF3* expression is subject to post-transcriptional regulation. Differential rates of *TEF3* mRNA translation, modulation of EF-3 stability, or combination of these factors could account for this observation. Interestingly the *C. albicans* enolase gene might also be regulated at a post-transcriptional level (Mason *et al.*, 1993). However, it should be noted that the observed changes in the abundance of the EF-3 polypeptide might not reflect the activity *in vivo* of this essential, fungal-specific translation factor under all experimental conditions.

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