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β-Galactosidase of *Kluyveromyces lactis* (Lac4p) as reporter of gene expression in *Candida albicans* and *C. tropicalis*

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Summary. Vectors containing fusions of the *Candida albicans ACT* promoter to heterologous genes were constructed and transformed into a *C. albicans* host strain. *β*-Galactosidase (Lac4p) activity was detected in transformants carrying an *ACT* fusion to the *Kluyveromyces lactis LAC4* gene, while fusions to the *Escherichia coli lacZ* gene and to other heterologous genes were not expressed. Lac4p was also produced by *C. tropicalis* transformants carrying the *ACT*/*LAC4* fusion. Plasmids in transformed *C. albicans* strains were present either as free multimers in high copy number or, more frequently, integrated into the genome in low copy number yielding high and low *LAC4* mRNA and Lac4p expression levels, respectively. Lac4p-expressing transformants of *C. tropicalis*, but not of *C. albicans*, were able to utilize lactose as sole carbon source. An *ACT*/*LAC4* fusion was not differentially expressed during the yeast and hyphal growth phases of *C. albicans*, indicating that the *ACT* promoter is not regulated during morphogenesis. These results define the first reporter gene system for convenient monitoring of gene expression in *Candida* species.

Key words: *Candida albicans* – *Candida tropicalis* – Heterologous gene expression – Morphogenesis

Introduction

The related species *Candida albicans* and *C. tropicalis* are opportunistic pathogens causing mycoses in predisposed patients (Barns et al. 1991; Odds 1988). However, *C. tropicalis* is also of biotechnological interest, for example, being able to grow on alkanes as sole carbon source (Gmünder et al. 1981). Genetic analyses of both *Candida* species have been difficult since they are diploids that lack a sexual cycle. Recently, techniques for exploring the molecular genetics of *C. albicans* and *C. tropicalis* have been developed. Thus marked host strains, transformation vectors, and methods for transformation and gene disruption are now available (Kurtz et al. 1988; Ernst 1989; Haas et al. 1990). Several genes of *C. albicans* have been cloned, mostly by complementation of specific mutations in *Saccharomyces cerevisiae* and *Escherichia coli* (Kurtz et al. 1988). The analysis of the *URA3* gene revealed a close taxonomic relationship of *C. albicans* to non-pathogenic budding yeasts, especially to *Kluyveromyces lactis* (Losberger and Ernst 1989a).

Expression of heterologous genes in *Candida* species is desirable for several reasons. Firstly, some heterologous gene products that can be easily assayed may serve as reporters or probes, e.g. for gene expression, or protein localization studies. This is of special importance for *C. albicans* and *C. tropicalis*, which can undergo an inducible morphogenetic change from a yeast to a hyphal growth form (reviewed in Odds 1988). Secondly, antibiotic resistance genes may be used as selectable markers on transformation vectors, as in other fungi. Interestingly, with one exception (the *LEU2* gene of *S. cerevisiae*; Kelly et al. 1988), no expression of a heterologous gene in *C. albicans* or in *C. tropicalis* has been reported. While the lack of expression may have many causes, including transcriptional, translational, or post-translational processes, some evidence suggests that translation in *Candida* species may have peculiar features. UGA may be used as a translational sense codon in *C. albicans* (Tuite et al. 1986). CUG has been found to encode serine (not leucine) in another *Candida* species (Kawaguchi et al. 1989). Thus, lack of heterologous gene expression in *C. albicans* could be caused by translational misreading of heterologous mRNAs.

In the present study we have modified and used the promoter of the strongly expressed *C. albicans ACT* gene (Losberger and Ernst 1989b) in an attempt to obtain expression of heterologous genes in *C. albicans* and *C. tropicalis*. Surprisingly, only the *LAC4* gene of the taxonomically related yeast *K. lactis* (Gödecke et al. 1991) was found to be expressed. *β*-Galactosidase encoded by the *K. lactis LAC4* gene is the first heterologous reporter

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for convenient monitoring of gene expression in *C. albicans* and *C. tropicalis.*

Materials and methods

Strains and growth conditions. C. albicans SGY-243 (*ade2/ade2, Δura3::ADE2/ Δura3::ADE2 LEU2/LEU2*; Kelly et al. 1987), *C. tropicalis* Ha900 (*ade2/ade2*; Sanglard and Fiechter, submitted) and *S. cerevisiae* BJ1991 (Jones 1990) were used as yeast hosts for recombinant vectors. Yeasts were transformed by the spheroplast protocol and grown in SD minimal medium as described (Sherman et al. 1981). A *K. lactis* mutant constitutive for β-galactosidase expression (KB101) was used as reference strain (Zenke and Breunig, unpublished results). The induction protocol described by Holmes and Shepherd (1988) was modified to induce morphogenesis in *C. albicans*. Briefly, cells were grown first in SD medium to the exponential growth phase ($OD_{600nm} = 1.5$), followed by starvation of washed cells in distilled water for 2.5 h at 30° C. Starved cells were incubated at 37° C at a density of 5×10^8 cells/ml in yeast extract/peptone (YP) medium with, or without 3% horse serum. Plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) for the detection of β-galactosidase in yeasts or in *E. coli* were prepared as described (Maniatis et al. 1982; Ruby et al. 1983).

Oligonucleotide-directed mutagenesis. To insert a convenient *Eco*RI site upstream of the putative ATG translation initiation codon of the *ACT* gene (Losberger and Ernst 1989b) we first isolated the 1.5 kb *Cla*I promoter/intron fragment, filled in the 5′ ends with Klenow enzyme and inserted it into the *Hin*cII site of M13mp8. The oligonucleotide 5′-CATTTTGAATTCTTATATTTTTTT-3′ was used to mutagenize the resultant vector according to the method of Taylor et al. (1985).

Plasmid constructions. The expression vector for the *E. coli lacZ* gene, pCARS/AcZcyc (Fig. 2), contains the following segments: (i) the 1 kb *Bam*HI – *Eco*RI *ACT* promoter fragment from the M13 vector, mutated as described above, (ii) a 3.5 kb *Bam*HI fragment carrying the *lacZ* gene, which is derived from pMC1790 (Casadaban et al. 1983; the *Bal*I site 3′ to the *lacZ* gene had been changed to a *BamH*I site in a preceding construct); (iii) the following adapter sequence to join the *ACT* *Eco*RI and *lacZ Bam*HI sites, creating an ATG start codon (underlined)

5'-GAATTCC<u>ATG</u>GAGGATCC-3';
*Eco*RI *Nco*I *Bam*HI

(iv) the transcription terminator of the *S. cerevisiae CYC1* gene as a 300 bp *Bam*HI – *Hin*dIII fragment derived from plasmid pEX-2 (Ernst and Chan 1985); and (v) the large *Bam*HI – *Hin*dIII fragment of pCARS1 (Kelly et al. 1988), partially restricted with *Hin*dIII to avoid cutting the kanamycin resistance gene. The *ACT/lacZ/CYC1* fragment was also inserted into the *C. albicans* transformation vector pRC2312 (R. Cannon and M. Shepherd, unpublished results). This vector contains the *URA3* gene and origins of replication named CaARS1, 2 (Cannon et al. 1990) inserted into the cloning vector pUC9. The resulting plasmid was named pRC2312AcZcyc.

The *lacZ* reference vector for *S. cerevisiae*, pER1298, contains the *S. cerevisiae ACT* promoter (Ernst 1986) as a 0.5 kb *Bam*HI – *Nco*I fragment fused to the *lacZ* gene (the 3.5 kb *Nco*I – *Hin*dIII fragment of pCARSAcZcyc) inserted into the large *Bam*HI – *Eco*RI fragment of pJD-CEN6.

The expression vector for the *K. lactis LAC4* gene, pCL01, was constructed by first fusing the 1 kb *Bam*HI – *Eco*RI *C. albicans ACT* fragment (with the *Eco*RI site filled-in using Klenow enzyme) to a 4 kb *Sal*I (filled-in)-*Xba*I *LAC4* fragment derived from pK30 (obtained from K. Breunig), and then inserting the fusion into pUC19. The *Sal*I site in pK30 is derived from a linker inserted next to position −43 with respect to the ATG translation initiation codon. The resulting plasmid, pUC-19/LAC4, was cut with *Xba*I and the 5.6 kb fragment encompassing the *ACT/LAC4* fusion was inserted into the single *Nhe*I site of pCARS1 to generate pCL-01. The same fusion fragment was also inserted into the *Xba*I site of YEplac195 (Gietz and Sugino 1988) to generate pL-lac4. An expression vector for the entire *LAC4* gene (including the promoter sequences) of *K. lactis* was constructed by isolating a 4.8 kb *Xba*I fragment carrying *LAC4* from pKL-4 (obtained from K. Breunig) and inserting it into the *Nhe*I site of pCARS-1. The resulting plasmid was named pCL-09.

pCL07, an expression vector for *C. tropicalis* HA900, was constructed by inserting the 5.6 kb *Xba*I fragment of pUC19/LAC4 (containing the *ACT/LAC4* fusion) into one of the two *Nhe*I sites (within pBR322 sequences) of plasmid pMK16 (Kurtz et al. 1987). It has been observed previously that the CARS1 element in pMK16 can direct plasmid replication in *C. tropicalis* (Sanglard and Fiechter, submitted).

Other procedures. β-Galactosidase was determined in cell extracts as described (Ruby et al. 1983) and expressed as milliunits/mg protein (nmol/min per mg). Protein was determined by the Bradford (1976) procedure (Biorad). RNA was isolated from yeast cells as described (Schmitt et al. 1990). Formaldehyde-containing agarose gels (1.5%) and procedures for Southern and Northern analyses were according to the protocols of Maniatis et al. (1982).

Results

Construction of expression vectors

The *C. albicans ACT* gene had been isolated and sequenced previously (Losberger and Ernst 1989b). Sequence comparisons with actins from other species defined the translational initiation codon, which is preceded by a 1.024 kb region containing putative promoter sequences. A convenient *Eco*RI site was inserted imme-

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ACT        TATAATCATTCAAA[ATG]

ACT*       TATAAGAATTCAAA[ATG]
                EcoRI

                                  8   9
ACT/lacZ   TATAAGAATTCC[ATG]GAG GAT CCC GTC
               EcoRI           BamHI
                  NcoI

                                LAC4 --->
ACT/LAC4   TATAA GAATTTCGACG T(A)10TAAACACACATAC
                 EcoRI/SalI

                                   1   2   3
           TCATCGAGAACTGAAAGAT[ATG]TCT TGC
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Fig. 1. Fig. 1. Sequences preceding the translational start codon in *ACT* and *ACT*-fusions. The authentic sequence (ACT) was modified to insert an *Eco*RI site (ACT*) and fused to the *Escherichia coli lacZ* gene (ACT/lacZ), or to the *Kluyveromyces lactis LAC4* gene (ACT/LAC4). The ATG translational start codon is *boxed*

Fig. 2. Structure of β-galactosidase expression vectors. Sequences derived from *Candida albicans* are drawn as *black boxes*, while β-galactosidase genes (*lacZ*, or *LAC4*) are shown as *open boxes*. The *Saccharomyces cerevisiae CYC1* terminator (*CYC1-T*) is indicated by the *hatched box*. *Thin lines* represent pBR322 sequences. *ACT-P*, promoter of the *C. albicans ACT* gene; B, *Bam*HI; H, *Hin*dIII; S, *Sca*I

diately 5′ of the ATG sequence by site-directed mutagenesis (Fig. 1) to allow convenient use of the *ACT* promoter in expression vectors.

The *E. coli lacZ* gene encoding β-galactosidase has been used as a reporter of gene expression in numerous species including several fungal species. We therefore constructed pCARS/AcZcyc (Fig. 2), which contains the *lacZ* gene flanked by the *C. albicans ACT* promoter (junction sequence, Fig. 1) and the *S. cerevisiae CYC1* terminator. This plasmid also contains the *CARS1* region which allows plasmid replication (Kelly et al. 1988), as well as the *URA3* gene which permits selection of Ura^+ transformants in the *C. albicans ura3* host SGY-243 (Kelly et al. 1988). The same expression components are contained in plasmid pRC2312AcZcyc, which includes the CaARS elements for replication in *C. albicans* (Cannon et al. 1990). Since CaARS is functional in *S. cerevisiae*, pRC2312AcZcyc can be introduced into *C. albicans* as well as into *S. cerevisiae*.

An alternative β-galactosidase is produced by the lactose-utilizing yeast *K. lactis* (Gödecke et al. 1991), which appears to be taxonomically closely related to *C. albicans* (Losberger and Ernst 1989a). We joined the gene encoding the enzyme, *LAC4*, to the *ACT* promoter and inserted this unit into pCARS1 (Kelly et al. 1988) to construct pCL01 (Fig. 2). A defined transcription terminator was not added to *LAC4* in this vector; however, the inserted fragment contains 3′ untranslated sequences expected to contain the *LAC4* terminator. Note that a 49 bp region separates the end of the *ACT* promoter from the ATG initiation codon in this construct (Fig. 1). A second *LAC4* expression vector, pCL09, was constructed which is identical to pCL01 except that the *LAC4* promoter substitutes for the *ACT* promoter. The expression vector for *C. tropicalis*, pCL07, contains the *ACT/LAC4* fusion and the CARS1 element as in pCL01, but it includes the *C. albicans ADE2* gene to allow complementation of the *ade2/ade2 C. tropicalis* host strain.

β-Galactosidase expression

Expression vectors were transformed into fungal host strains and levels of β-galactosidase were determined. The results show that *LAC4* can be expressed in all three species *C. albicans*, *C. tropicalis* and *S. cerevisiae* (Table 1). In both *Candida* species two types of transformants were obtained containing either high or low levels of β-galactosidase. With *C. albicans* as host about 10% of the pCL01 transformants had high β-galactosidase activity (about 500 mU/mg), which formed blue colonies on X-Gal plates. About 90% of the transformants expressed low levels of β-galactosidase (about 0.5 mU/mg), which produced white colonies on X-Gal plates. Among this latter type, one unusual transformant was obtained (transformant "10"), which expressed somewhat elevated enzyme levels (6 mU/mg). Similar results were obtained for a *C. tropicalis* host strain using pCL07 as transformation vector (Table 1). With this species about 20% of all transformants expressed high levels and 80% expressed low levels of β-galactosidase.

While transcription in pCL01 and pCL07 is driven by the *C. albicans ACT* promoter, pCL09 contains the complete *LAC4* gene including the *LAC4* promoter region. Again, two classes of *C. albicans* transformants were obtained using pCL09: of six transformants, four contained no β-galactosidase activity and two contained low levels of enzyme that were not detectable on X-Gal plates. This result demonstrates that the heterologous *K. lactis* promoter is used in *C. albicans*; however, its activity is detectable only in a fraction of the transformants (presumably those containing the plasmid in high copy number, see below). We did not test whether the *LAC4* promoter is also active in *C. tropicalis*. Interestingly, the *C. albicans ACT* promoter appears to be functional in *S. cerevisiae* since a multicopy vector containing the *ACT/LAC4* fusion (pL-Lac4) directs high levels of β-galactosidase in *S. cerevisiae* transformants (Table 1).

C. tropicalis transformants expressing high levels of β-galactosidase encoded by pCL07 were able to grow on media containing lactose as sole carbon source. Transformants expressing low enzyme levels and the parental untransformed *C. tropicalis* strain, however, did not utilize lactose. Interestingly, *C. albicans* transfor-

Table 1. β-galactosidase expression of yeast strains

Protein	Host	Strain	Plasmid	Promoter	X-Gal plate[a]	β-Galactosidase (mU/mg)
LAC4p	*Candida albicans*	SGY-243	pCL09	LAC4	–	0
					–	8; 25
			pCL01	ACT	–	0.5–6
					+	574
	C. tropicalis	Ha900	pCL07	ACT	–	9; 20
					+	300
	Saccharomyces cerevisiae	BJ1991	pL–Lac4	ACT	+	20000
	Kluyveromyces lactis	KB101	–	LAC4	+	2500
LacZp	*C. albicans*	SGY-243	pCARS/AcZcyc	ACT	–	0
			pRC2312AcZcyc	ACT	–	0
	S. cerevisiae	BJ1991	pRC2312AcZcyc	ACT	+	2
			pER1298	S.c. ACT[b]	+	708
Control	*C. albicans*	SGY-243	pCARS	–	–	0
	C. tropicalis	Ha900	pMK16	–	–	0
	S. cerevisiae	BJ1991	YEplac195	–	–	0

[a] Blue coloration of colonies on X-Gal plates is indicated by (+)
[b] *ACT* promoter of *S. cerevisiae*

mants expressing Lac4p (high or low levels) did not grow in media containing lactose as sole carbon source. These results suggest that *C. tropicalis*, but not *C. albicans*, has a lactose uptake system allowing intracellular metabolism of lactose.

To examine plasmid stability in the two observed types of *C. albicans* transformants carrying pCL01, the percentage of Ura$^+$ (plasmid containing) and Ura$^-$ cells (lacking plasmid) was determined after nonselective growth in YPD medium. Transformants that initially expressed high levels of β-galactosidase had lost 70% of pCL01 after 20 generations of nonselective growth, indicating that in these cells pCL01 was present as an episome. Concomitantly with plasmid loss, β-galactosidase production declined to low levels. Interestingly, a loss of β-galactosidase activity was also observed during selective growth of the high-expression transformants: when a blue colony on X-Gal plates (lacking uridine) was restreaked onto X-Gal, 10–20% white Ura$^+$ colonies appeared. When such white Ura$^+$ colonies were restreaked, 5–10% of blue colonies reappeared. This result is consistent with the notion that in high-expression transformants the expression vector occurs in an oligomeric form (see below). Consequently, the observed variability in β-galactosidase activity can be explained by fluctuations in the number of plasmids, or plasmid repeat units. Free plasmids also appear to exist in transformant "10" (see above), since Ura$^-$ cells arose during nonselective growth of this strain (10% in 19 generations). In contrast, with transformants expressing low β-galactosidase levels, no Ura$^-$ cells were found and β-galactosidase activity remained stable during nonselective growth. In this type of transformant, the plasmid appears to be integrated into a chromosomal location. Thus, high β-galactosidase activity in *C. albicans* transformants is correlated with the episomal status of the expression plasmid; on the other hand, in most transformants expressing low levels of enzyme, the expression vector is integrated into a chromosomal site.

In contrast to the results obtained for *LAC4*, no expression of the *E. coli lacZ* gene could be obtained in *C. albicans* (Table 1). Negative expression results were obtained using vectors based on the CARS1 element (Kelly et al. 1988), or the CaARS1, 2 element (Cannon et al. 1990). The lack of expression was not due to defects in the constructs since pRC2312AcZcyc (which can be transformed into *S. cerevisiae* or *C. albicans*) directed expression of high levels of β-galactosidase in *S. cerevisiae* (Table 1). This result confirms the activity of the *C. albicans ACT* promoter in *S. cerevisiae* (see above); however, the *S. cerevisiae ACT* promoter is significantly more efficient in directing expression of *lacZ* in *S. cerevisiae* (expression vector pER1298, Table 1).

Southern and Northern analyses

The status of expression plasmids in *C. albicans* transformants expressing high and low levels of β-galactosidase was examined by Southern analyses using a *LAC4* segment as molecular probe (Fig. 3). In undigested DNA of all pCL01 transformants a single hybridizing band of about 60 kb was detected (Fig. 3A), representing the size of the chromosomal DNA preparation. Although this band was not found in DNA of the low-expression transformant after short autoradiographic exposure (Fig. 3A, lane 3), it was visible after extending exposure times (data not shown). This result is consistent with either chromosomal integration of pCL01, or its arrangement as a multimer; the presence of free monomeric plasmid in the transformants can be excluded. As has been reported previously (Kurtz et al. 1987) we consistently observed significant hybridization at the level of the wells (Fig. 3A, lane 1, lower band), indicating that the plasmid does not readily enter the gel, possibly because it is present in a large, closed-circular form. The most intense *LAC4* signal was detected in a transformant expressing high levels of β-galactosidase (Fig. 3A,

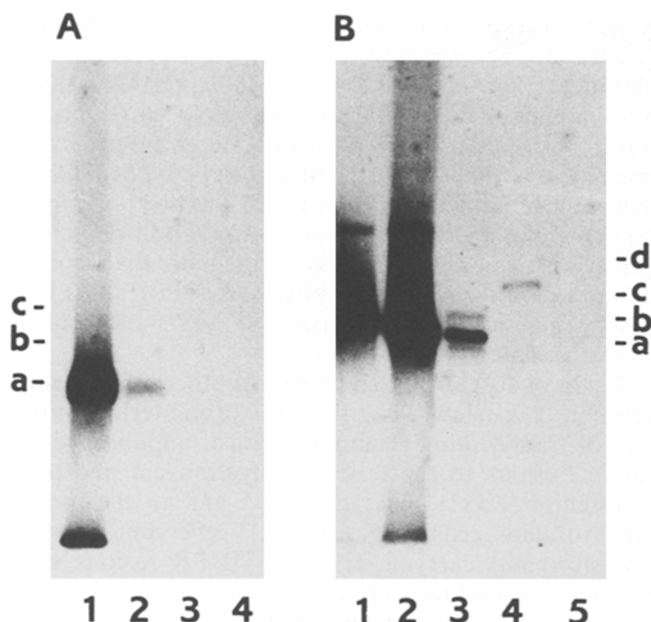

Fig. 3A, B. Detection of *LAC4* sequences in DNA of *C. albicans* transformants; 8 μg of genomic DNA was **A** not cut or **B** cut with *Sca*I, separated by agarose gel electrophoresis and transferred to nylon membranes. The 3.0 kb *Bam*HI fragment of pCL01 (Fig. 2) was used to detect *LAC4* sequences. Lanes A1–A3 and B2–B4, transformants carrying pCL01; lanes A4 and B5, transformant carrying pCARS; lane B1, pCL01 DNA. Lanes A1 and B2, high-expression transformant; lanes A2 and B3, transformant "10" (see text); lanes A3 and B4, low-expression transformant; lanes A4 and B5, transformant carrying pCARS. The migration of standards is indicated: a, 23.1 kb; b, 9.4 kb; c, 6.6 kb; d, 4.3 kb

lane 1), while weaker signals were found in low-expression transformants (Fig. 3A, lanes 2, 3). On the basis of the hybridization signals we estimate that the high-expression transformant contains at least 20 times more *LAC4* sequences relative to transformant "10" (see above), which, in turn, contains 5–10 times more *LAC4* DNA than transformants expressing low levels of β-galactosidase. Thus >100 copies of the *LAC4* expression plasmid appear to occur in the high-expression *C. albicans* transformants and plasmid copy number is directly related to β-galactosidase expression levels (cf. Table 1).

To check plasmid integrity, DNA of transformants was cut with *Sca*I and analysed in a Southern blot (Fig. 3B). *Sca*I cuts the expression vector into two fragments of 3.2 kb and 12 kb (Fig. 2). The 12 kb fragment and the 3.2 kb fragment (due to cross-hybridization with the *LAC4* probe) were detected in DNA of all transformants (Fig. 3B, lanes 2 and 3). The presence of unaltered vector in all transformants was confirmed by transforming *E. coli* MC1061 with the genomic DNA; intact pCL01 was discovered in the *E. coli* transformants. However in low-expression *C. albicans* transformants, an additional hybridizing fragment was detected which was the predominant band after short exposure times (Fig. 3B, lane 4). This result is consistent with previous results (Kurtz et al. 1987), in which a *CARS*-containing vector was shown to have integrated as an oligomer into a chromosomal site. The integration point appears not to be random since in 4 of 6 low-expression transformants the additional bands are identical in size (about 5 kb as shown in Fig. 3B, lane 4). In contrast, in transformants expressing high levels of β-galactosidase, only plasmid fragments are detected, indicating that the plasmid has not rearranged, or integrated into the genome (Fig. 3B, lane 2). Since the plasmid in these transformants comigrates with genomic DNA (Fig. 3A, lane 1) it appears to exist in tandem head-to-tail multimers composed of at least four monomeric plasmid units (size of pCL01: 13.4 kb). A similar consideration holds for transformant "10" (see above) (Fig. 3A, lane 2). The stability data (see above) confirm that the postulated multimers occur as free plasmids in the transformants. As determined by Northern analysis the different plasmid copy numbers in transformants are associated with corresponding variations in *LAC4* mRNA levels (data not shown).

Expression during morphogenesis

To investigate whether the morphogenetic change from a yeast to a hyphal growth form is associated with changes in *ACT* promoter expression, a standard protocol was used to induce morphogenesis of a *C. albicans* transformant (containing pCL01) expressing high levels

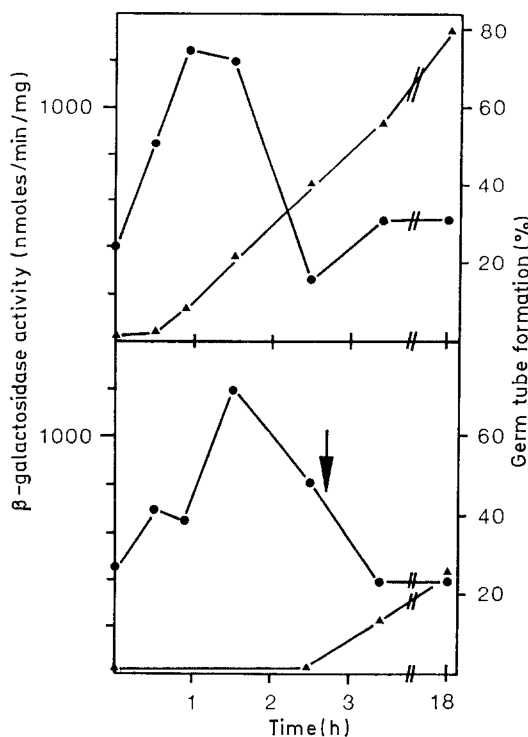

Fig. 4. Expression of *ACT*/*LAC4* fusion during morphogenesis. Starved cells of *C. albicans* SGY-243 [pCL01] (expressing high levels of β-galactosidase) were resuspended in induction medium containing 3% horse serum at 37° C (*upper* panel), or without serum at 30° C (*lower* panel). Levels of β-galactosidase (*circles*) and per cent hyphae (*triangles*) are indicated. Serum was at 3% added to the 30° C culture after 2.5 h of incubaton (*arrow*, lower panel)

of β-galactosidase. Following a period of starvation, germ tube formation was induced at 37° C in the presence of 3% serum, while at 30° C without serum the transformant grew in yeast form. As shown in Fig. 4, a transient increase in expression of the *LAC4* reporter was found under both growth conditions. If serum was added at 30° C hyphal induction occurred which was not associated with an increase in expression of the *ACT/LAC4* fusion (Fig. 4, lower panel). These results demonstrate that the *ACT* promoter is not differentially regulated during the yeast and hyphal growth modes of *C. albicans*. The reason for the observed transient increase of expression of the *ACT/LAC4* fusion remains to be determined.

Discussion

A comparison of genes encoding orotidine-5'-phosphate decarboxylase (Ura3p) among fungi previously revealed that *K. lactis* is closely related to *C. albicans* (Losberger and Ernst 1989). In the present study it is shown that a *K. lactis* gene, *LAC4*, but not the *E. coli lacZ* gene, can be expressed in *C. albicans* and *C. tropicalis* under transcriptional control of the *C. albicans ACT* promoter, thus establishing a first convenient reporter gene system for *Candida* species. Apart from *LAC4*, the *S. cerevisiae LEU2* gene is the only heterologous gene that has been expressed in *C. albicans* (Kelly et al. 1988). The results of the present study confirm a close relationship between *C. albicans* and *C. tropicalis*: (1) the *C. albicans* ACT promoter is also functional in *C. tropicalis*, (2) Lac4p activity is observed in both species and (3) *C. albicans* expression vectors are active in *C. tropicalis* (Sanglard and Fiechter, submitted). Since the *ACT* promoter is also active in *S. cerevisiae*, the *ACT/LAC4* fusion described here may be used as a reporter in the three species *C. albicans*, *C. tropicalis* and *S. cerevisiae*.

In agreement with previous results (Kurtz et al. 1987), the *LAC4* expression vectors exist in *C. albicans* as oligomers that are either free, or integrated into a chromosomal site. Integration in *C. albicans* may involve the *URA3* or *CARS* sequences present in pCL01; in *C. tropicalis*, integration of pCL07 could occur by recombination of the plasmid *ADE2* gene (derived from *C. albicans*) with the resident chromosomal *ADE2* gene. Since the two *Candida* species are closely related (Barns et al. 1991), the corresponding *ADE2* genes are expected to be highly homologous. In the present study we found that pCL01 transformants carried the plasmid predominantly in integrated form, while other *CARS*-containing plasmids have been reported to occur mostly as free plasmids in transformants (Kurtz et al. 1987). This difference may be due to toxicity of Lac4p at high expression levels, thus generating a bias towards the low-expression, integrated transformants. The fact that monomeric pCL01 can be recovered in *E. coli* using DNA of transformants carrying integrated pCL01 oligomers is reminiscent of findings in *Aspergillus nidulans* (Ballance and Turner 1985) and may be due to rare excisions of monomers, or oligomers, in *C. albicans*. Unexpectedly, in the present study we obtained evidence for heterogeneity among transformants carrying free oligomeric plasmids: plasmid levels (and Lac4p expression values) were found to differ at least tenfold in two types of transformants that interconvert at a high frequency. It remains to be determined whether the two types of transformants differ with respect to the number of repeat units per oligomeric plasmid or the total number of oligomeric plasmids. The reasons for the more than tenfold difference between copy numbers of pCL01 and other CARS-carrying vectors (Kurtz et al. 1987) also remain to be elucidated.

We have also attempted to express other heterologous genes in *C. albicans* using the *ACT* promoter for initiation of transcription (Hahn and Ernst, unpublished results). Fusions to the *lacZ* and hygromycin phosphotransferase (HPH) genes of *E. coli* and to the mouse dihydrofolate reductase (DHFR) gene were tested. Transformants carrying HPH or DHFR fusions were no more resistant to hygromycin B or methotrexate than the untransformed parental strains. In contrast to the *K. lactis LAC4* gene, the *E. coli lacZ* gene did not direct synthesis of β-galactosidase in transformants. It is conceivable that the difficulties encountered in heterologous gene expression in *C. albicans* are due to transcriptional problems. Thus we found that although considerable amounts of *lacZ* and HPH mRNA were produced in *C. albicans*, the corresponding proteins were not made, suggesting defects of translation. Overall codon usage in *C. albicans* is unusual (Brown et al. 1991) and individual codons like UGA and CUG may be translated differently than in other species (Tuite et al. 1986; Kawaguchi et al. 1989). However, if *Candida* genes contain such special codons they appear to be rarely used, since most *C. albicans* genes are readily expressed in *S. cerevisiae* (Kurtz et al. 1988). It is obvious that unusual codon usage would be a severe obstacle in the expression of heterologous genes in *Candida* species, which could be remedied only by specific codon adaptation or use of heterologous genes of related species, as described here.

Morphogenesis in *C. albicans*, which involves a switch from a yeast to a mycelial growth form, is a process of differentiation that may be associated with alterations in gene expression. Although we observed a transient increase in the expression of a *ACT/LAC4* fusion during morphogenesis, a comparison with non-induced cells revealed that this increase is unrelated to hyphal induction. Thus, the *ACT* promoter does not appear to be regulated during morphogenesis. It has been reported recently that expression of the *ACT* gene increases during morphogenesis (Paranjape and Datta 1991). In the context of our experiments this result could indicate that the *ACT* transcript is regulated post-transcriptionally by morphogenesis. However, we have observed recently that the observed increase in mRNA levels of *ACT* and other cellular genes is due mainly to starvation of cells prior to morphogenesis (Delbrück and Ernst, unpublished results).

Besides its potential use as reporter for regulated promoters the *LAC4* protein may be used as immunological tag, as a genetic marker, e.g. for gene disruption, or

in transformation experiments. Since *C. tropicalis* transformants expressing *LAC4* can grow with lactose as sole carbon source *LAC4* may serve as a dominant selectable marker in transformation vectors, allowing the use of genetically unmarked strains as hosts. Interestingly, *LAC4* expression is not sufficient to allow growth of *C. albicans* transformants, possibly because its membrane is not permeable to lactose. Experiments are currently in progress to investigate whether the secretion of *LAC4*, or its coexpression with *LAC12* (which encodes a lactose permease in *K. lactis*) will permit lactose growth of *C. albicans*.

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