INTRODUCTION

A virulence characteristic of the human fungal pathogen *Candida albicans* is its ability to grow in various morphological forms. The conversion between yeasts, elongated yeasts (pseudohyphae) and filamentous forms in response to environmental cues is accompanied by differential expression of several genes regulated at the transcriptional level (reviewed by Calderone & Fonzi, 2001; Ernst, 2000; Liu, 2001). The expression of the Hyphal Wall Protein 1 (HWP1) gene is tightly controlled at the transcriptional level; HWP1 mRNA and protein are absent in yeasts and pseudohyphae (Staab et al., 1996, and unpublished results). The transcriptional regulation of HWP1 and other genes regulated by the bud–hypha transition, such as *HVR1, RBT1* and *ECE1* (Braun & Johnson, 2000), suggests that *cis*-elements in the promoter regions respond to signals that control morphology. Several signalling pathways and transcriptional factors have been described that promote the formation of germ tubes and hyphae but common promoter elements in hypha-specific genes proven to be essential for expression have yet to be uncovered. Functional analysis of the HWP1 promoter will provide insights into common regulatory elements of other genes controlled by the bud–hypha transition. With this aim, we constructed a reporter plasmid that would permit easy visualization of HWP1 promoter activity by using yeast-enhanced green fluorescent protein (Cormack et al., 1997) as a readout. Green fluorescent protein (GFP) has been widely used in cell biology to monitor gene expression and cellular localization of proteins (Chalfie et al., 1994; Gerami-Nejad et al., 2001; Niedenthal et al., 1996). We engineered the reporter plasmid with the selectable marker gene *URA3* within the targeting DNA sequences to direct insertion at the *ENO1* locus of a *Ura–* strain. As a counterpart to the developmentally expressed yEGFP3 construct, we replaced the HWP1 promoter with the *ENO1* promoter to express relatively high amounts of GFP in a constitutive manner.

To demonstrate the use of the plasmids in expressing genes other than GFP, we replaced the yEGFP3 gene with the putative *C. albicans BCY1* (SRA1, regulatory subunit of cAMP-dependent protein kinase A, PKA), a member of the cAMP signalling pathway. The cAMP signalling pathway is known to be involved in germ tube formation (Bahn & Sundstrom, 2001; Chattaway et al., 1981; Niimi, 1996; Niimi et al., 1980; Zelada et al., 1996), and perturbations of cAMP levels or PKA activity can induce or inhibit germ

Abbreviations: GFP, green fluorescent protein; PKA, protein kinase A.
tube formation (Bahn & Sundstrom, 2001; Castilla et al., 1998; Chattaway et al., 1981). Signals that increase cAMP levels or addition of exogenous cAMP or dibutyryl cAMP activate the cAMP pathway and promote germ tube formation (Bahn & Sundstrom, 2001; Castilla et al., 1998; Chattaway et al., 1981). If, on the other hand, the release of active subunits of PKA is blocked, the cAMP signalling pathway is deactivated, and germ tube induction is suppressed. Overexpression of the regulatory subunit of PKA should prevent the release of active PKA subunits and abrogate the activation of genes involved in germ tube formation. Thus, the expected phenotype of strains overproducing Bcy1p is a reduction in germ tube formation.

The plasmids described here offer several uses as tools for molecular genetic research in C. albicans. The developmental expression of GFP by the HWP1 promoter was maintained even when the construct was integrated ectopically at the ENO1 locus. Constitutive expression of yEGFP3 from the ENO1 promoter permitted visualization of GFP in all cell types, and served as a control for a non-developmentally regulated promoter. Lastly, the versatility of the constructs was tested by substituting yEGFP3 for Bcy1, a member of the cAMP signalling pathway, to determine the effect of mis-expression or overexpression of Bcy1 on filamentation.

**METHODS**

Construction of an HWP1 promoter expression plasmid. All enzymes were from Gibco-BRL Life Technologies or Promega Biotech and were used according to standard techniques (Sambrook et al., 1989). Polymerase chain reactions (PCR) were performed to amplify components of the expression vector. TaqPlus DNA polymerase (Gibco-BRL) was used, unless otherwise specified, according to the manufacturer’s recommendations. The −1410 to +67 region upstream of the HWP1 open reading frame was amplified with oligonucleotides 5′GGGCCGGGTATTTTTTTTCTTTCC3′ and 5′GGAGCCTATTTGACCAAACTAAAGAC3′ engineered with Smal and HindIII sites, respectively (underlined nucleotides) using a genomic C. albicans DNA plasmid clone isolated from an SC5314 (Gillum et al., 1984) genomic library (Birse et al., 1993) harbouring a BglII fragment encompassing nucleotides −1410 to +120. The 1–46 kb PCR product was ligated to the HindII and HindIII sites of pBluescript SK+ (Stratagene) to create pBS5. The codon-optimized Aequorea victoria GFP gene, yEGFP3, was released from pYGFP3 (Cormack et al., 1997) by digestion with HindIII and PstI, and the gene fragment was cloned downstream of the HWP1 promoter between the HindIII and PstI sites of pBS5 to generate pBS147GFP. The untranslated 3′ region of HWP1 was amplified with oligonucleotides 5′GGTATTTGCTGATTTCTGATTCATTG3′ and 5′GGAGCCTATTTGACCAAACTATTAAGGAACTGAATATAAGGAACTATTAAGGAACT/TAAGGAACTATTAAGGAACTGATTCATTG3′. A. albicans DNA plasmid clone having a 3 kbp BamHI fragment with C-terminal and downstream HWP1 sequences was used as template (pGB23; Staab & Sundstrom, 1998) with Pfu DNA polymerase (Stratagene) to generate a blunt-ended DNA amplification product. The right oligonucleotide was chosen to amplify the 3′ region of HWP1 ending at the unique SacI site (double-underlined nucleotides) and across a HindIII site that was eliminated by changing a T to an A (nucleotide in bold). The 342 bp PCR product was digested with SacI and ligated to the Smal and SacI sites of p147GFP to create p147GFP3. A URA3-disrupted ENO1 DNA fragment was used as a selectable marker and targeting sequences for integration at the chromosomal ENO1 locus. The entire ENO1 ORF was amplified with oligonucleotides engineered with XbaI (5′ primer, underlined) and Xhol sites (3′ primer, double-underlined): 5′GGGTCTAGACGAAATATTACAACTGTCTTACGC3′ and 5′GGGTCTAGACGAAATATTACAACTGTCTTACGC3′. The amplified product was cloned into pbLueScript SK+ (Stratagene) at the XbaI and Xhol sites to generate pENO1. The 9′ HindIII site and the unique Clal in ENO1 were mutagenized by site-directed mutagenesis (Promega GeneEditor) to eliminate the HindIII site and to prevent methylation adjacent to the Clal site (Clal is site-specific methylation sensitive at the dam site; McClelland et al., 1994). The 125 bp between the two remaining HindIII sites in ENO1 was replaced with the URA3 ORF found in the 1–44 kb RsaI fragment (Kelly et al., 1988) in p5921 (Fonzi & Irwin, 1993) after creating blunt ends at the HindIII sites with Klenow fragment DNA polymerase (Sambrook et al., 1989).

The 9′ HindIII construct was subsequently amplified by PCR with oligonucleotides having KpnI sites at the ends (underlined): 5′GGGGTACCATGTCTTACGCACAAATATCCAC3′ and 5′GGGTACCCCCGCTAGATAGCTTCAGAACCT3′. The 26 bp PCR product was diluted with KpnI and cloned into the unique KpnI site of p147GFP3 to generate pHWP1GFP3. Each construct was analysed for proper cloning at the DNA level by sequencing across ligation junctions (automated cycle sequencing, ABI Prism, model 377 and 373, Perkin-Elmer). A promoterless construct was created by digesting pHWP1GFP3 with Xhol and HindIII, purifying the vector away from the 1–47 bp 5′ HWP1 fragment, and incubating with Klenow fragment DNA polymerase to produce blunt DNA ends (Sambrook et al., 1989). Self-ligation of the blunt-ended vector produced p0GFP3.

Substitution of the HWP1 promoter in pHWp1GFP3 with the ENO1 promoter. The constitutive expression of yEGFP3 was achieved by replacing the HWP1p with the ENO1p region. The contig encoding ENO1 was identified (ORF6.6269 on Contig 2451) at Stanford’s Candida albicans Sequencing Project Assembly 6 (see URL below), and the DNA sequence information used to amplify the promoter region from −900 to +36 with oligonucleotides engineered with Xhol (underlined) and HindIII (double-underlined), respectively: 5′CCCATCGATTTGATTAAGGTAAGGTCTTCATATTCTA3′ and 5′CCCATCGATTTGATTAAGGTAAGGTCTTCATATTCTA3′. Wild-type genomic DNA from SC5314 served as template. The 955 bp PCR product was digested with Xhol and HindIII and cloned into pbLueScript SK+ (Stratagene) at the Xhol and HindIII sites. Once the ENO1p region was verified by DNA sequencing, the insert was excised with Xhol and HindIII and used to replace the HWP1p in pHWP1GFP3 cloned between the Xhol and HindIII sites. The new recombinant plasmid was named pENO1GFP3.

Substitution of yEGFP3 with Bcy1 (SRA1). The amino acid sequence of Saccharomyces cerevisiae Bcy1p obtained at the Saccharomyces Genome Database (http://www.yeastgenome.org/) was used to search the C. albicans genome at Stanford’s Candida albicans Sequencing Project Assembly 6 (http://sequence-www.stanford.edu/group/candida/index.html) for a homologous gene product. ORF 6.2117, named SRA1, coded for a 459 amino acid protein with 47% identity to S. cerevisiae Bcy1p (Sra1p). Because the preferred gene name at the Saccharomyces Genome Database is BCY1, the C. albicans putative homologue was also designated BCY1 (CaBCY1). The entire C. albicans BCY1 ORF was generated by PCR using Pfu polymerase, SC5314 genomic DNA as template and two oligonucleotides, 5′CCCAAGCTTTGATTAAGGTAAGGTCTTCATATTCTA3′ and 5′GGGTACCCCCGCTAGATAGCTTCAGAACCT3′, engineered with HindIII (underlined) and PstI (double-underlined) sites. The yEGFP3 gene fragment in pENO1GFP3 was replaced with the
1–37 kbp BCY1 PCR product digested with HindIII and PstI, to generate pENO1BCY1. The authenticity of BCY1 was confirmed by automated cycle sequencing as above.

Transformation of C. albicans with GFP and BCY1 plasmids, and verification of plasmid integration at ENO1. Plasmid constructs were targeted to the chromosomal ENO1 locus by digesting the plasmids at the unique ClaI site prior to transformation of the ura3 C. albicans strain CAI4 (Fonzi & Irwin, 1993). Strain CAI4 was transformed with 5 μg linearized DNA using the protoplasting method (Kurtz et al., 1986), and stable transformants were staked for isolation onto yeast nitrogen base plates (YNB, 50 mM glucose). Single-copy integrations of the plasmid constructs were verified by Southern blotting of genomic DNA digested with BglII probed with cENO1 (Postlethwait & Sundstrom, 1995) directly labelled with horseradish peroxidase (Amersham Pharmacia) and developed with chemiluminescence reagents (Pierce).

Induction of GFP expression in C. albicans transformants. Yeast strains grown to stationary phase on YNB plates or in liquid medium at 30 °C were used as inoculum for 30 °C yeast peptone dextrose [glucose] (YPD), 37 °C YPD plus 10% bovine calf serum (Sigma) (Braun & Johnson, 2000), or 37 °C Medium 199 (M199, Life Technologies) as before (Bahn & Sundstrom, 2001; Staab et al., 1996). The cells were allowed to germinate at 37 °C or grow as budding yeasts at 30 °C for 2–3 h before microscopic examination by epifluorescence using a fluorescein isothiocyanate (470–490 nm emission) cube. Expression of yEGFP3 regulated by the HWPI promoter was also assessed by growing yeasts to exponential phase in modified Lee’s media (Brummel & Soll, 1982; Staab et al., 1996; Sundstrom & Aliaga, 1994; Sundstrom et al., 1990). Cells were photographed at 400X magnification with an Olympus BX60 microscope fitted with a MagnaFire S99806 camera. Images were manipulated with Adobe PhotoShop 5.0.

yEGFP3 expression was also induced in agar-containing media (Lo et al., 1997). Stationary-phase yeasts grown in YNB were mixed (100 cells in 25 ml) with liquefied 2% agar containing 4% bovine calf serum and poured into plates. The hardened plates were incubated at 37 °C for up to 7 days. Colonies were photographed under epifluorescence at 20X magnification as above.

Analysis of filamentation in solid and liquid media. Strains transformed with pHWP1GFP3 (HWP1GFP3), pENO1GFP3 (EGFP3) and pENO1BCY1 (BCY1) were induced to form agar-embedded filamentous colonies in Spider medium (Liu et al., 1994) and 2% agar with 4% bovine calf serum plates as described above. Stationary-phase cells grown in YNB were mixed (200 cells in 25 ml) with liquefied 2% agar containing 4% bovine calf serum and poured into petri dishes. The hardened plates were incubated at 37 °C for 7–10 days. Colonies were photographed at 1X magnification with a stereoscope (Olympus SZX12) fitted with a MagnaFire S99806 camera. Germ tube formation was also assessed by growth in liquid M199 at 37 °C as before (Bahn & Sundstrom, 2001). Images were manipulated with Adobe PhotoShop 5.0.

Northern blot analysis. Total RNA was prepared (Schmitt et al., 1990) from CAI4 transformed with pENO1GFP3 (EGFP3, control strain) and pENO1BCY1 (BCY1). RNA was isolated from exponential-phase cells growing in modified Lee’s media at pH 4.5 at 25 °C (yeasts) and pH 6.5 at 37 °C (germ tubes), and analysed in standard formaldehyde gels (10 μg RNA per lane) followed by blotting onto nitrocellulose membranes as before (Staab et al., 1996). The membranes were probed with 32P-labelled BCY1 ORF used to construct pENO1BCY1 (see above), and with a probe for 18S rRNA (Bahn & Sundstrom, 2001).

RESULTS AND DISCUSSION

Plasmid constructs integrated at the ENO1 locus retain promoter regulation

Our goals were to construct plasmids for the study of the hypha-specific gene promoter, HWPI, which would permit easy visualization of promoter activity combined with simple replacement of promoter and reporter gene fragments. The availability of a codon-optimized yEGFP3 gene fragment (Cormack et al., 1997) was ideal in that it allowed for easy visualization of gene expression. The expression plasmid included 1.47 kbp of upstream HWPI DNA and 352 bp of 3’ untranslated HWPI (Staab & Sundstrom, 1998) (Fig. 1). A URA3-disrupted ENO1 fragment was a convenient way to include a selectable marker within the targeting sequences. Previous studies in our laboratory have shown that disruption of one ENO1 homologue is not detrimental to cell growth on glucose or pyruvate, and does not affect virulence as measured in the murine systemic.

Fig. 1. Schematic map of the HWPIGFP3 integration plasmid. The HWPI promoter (open region) controls expression of yEGFP3 (closely hatched segment), followed by the HWPI 3’ untranslated region (black segment). The targeting/selection sequences, eno1::URA3, are shown as widely hatched segments (ENO1 sequences) interrupted by a closely double-hatched region (URA3). The thick and thin arrows represent the direction of transcription of yEGFP3 and URA3, respectively. The plasmid was integrated at the ENO1 locus after digestion with ClaI prior to transforming the Ura- strain, CAI4. Digestion with BstEII also directs integration of the construct to the ENO1 locus (data not shown). To create pENO1GFP3, the HWPI1 promoter was replaced with the unique XhoI and HindIII sites with 0.9 kbp 5’ ENO1 sequences. The expression plasmids were constructed in the vector pBluescript SK- (Stratagene) (thin black arc). Single restriction enzyme sites and gene segment descriptions are shown on the periphery of the plasmid.
Fig. 2. Integration of the GFP expression plasmids into the chromosome of *C. albicans* at the *ENO1* locus. (a) Restriction map of the *ENO1* locus with the predicted Southern blot hybridization fragments shown above as double-headed arrows. *BglII* digestion of genomic DNA generates two fragments, of 1·3 and 3·4 kbp, which hybridize to an *ENO1* probe comprising the entire *ENO1* ORF (cENO, black arrow below the *ENO1* locus; the probe has limited homology to the wild-type 3·4 kbp fragment). (b) Diagrams of the *HWP1*, *ENO1* and no promoter constructs (top to bottom, respectively) integrated at the *ENO1* locus. Double-headed arrows above the gene maps indicate the sizes of the *BglII* fragments hybridized by the cENO probe. Thin black arrows below the gene maps show the direction of transcription of yEGFP3 (represented by rectangles filled with thin parallel lines). Restriction sites are shown above the gene maps. Scale bar, 500 bp. (c) Southern blot analysis of *BglII*-digested genomic DNA from strains transformed with the yEGFP3 plasmids probed with cENO (arrow below *ENO1* locus in a). Lane 1, strain HGFP3 (CAI4 transformed with pHWP1GFP3) contains the expected two new fragments of 6·8 and 2·6 kbp (arrows at right) in addition to the *ENO1* gene fragments from intact homologues (dashed lines at right, see lane P). Lane 2, strain EGFP3 (CAI4 transformed with pENO1GFP3) contains the expected two new fragments of 5·3 and 2·6 kbp (arrows at right) in addition to the two fragments from intact homologues (dashed lines at right). The cENO probe does not recognize the central *BglII* fragment within the *ENO1* promoter (see b). Lane 3, strain 0GFP3 (CAI4 transformed with the promoterless plasmid) contains two new fragments of 5·3 and 2·6 kbp (arrows at right) in addition to the two fragments from the intact *ENO1* homologues (dashed lines at right). Lane P, parental strain CAI4.
A candidiasis model (Postlethwait & Sundstrom, 1995; Sundstrom et al., 2002). Initially we used the eno1::URA3 fragment previously generated in our laboratory (Postlethwait & Sundstrom, 1995), but we noticed a tendency for multiple tandem copies of the plasmid to integrate into the chromosome (data not shown), an observation reported by others using a different chromosomal locus (Srikantha et al., 1995). Multiple tandem integrations of transforming DNA have also been well documented in the yeast *Pichia pastoris* (Clare et al., 1991). In an attempt to minimize plasmid copy number and etopic integrations, a longer region of homology to the 5′ region of ENO1 was incorporated into a new eno1::URA3 fragment (see Methods). Fewer plasmid integrations occurred with the new construct, and the C. albicans transformation efficiency increased by 10–100-fold (data not shown). Subsequently, two derivative plasmids were constructed by substituting the *HWP1*p for the constitutive ENO1p to generate pENO1GFP3, and by deleting promoter sequences to create p0GFP3 (Fig. 2b). Southern blot analysis of Ura+ transformants confirmed site-specific integration of the plasmid constructs at the ENO1 locus (Fig. 2c). Phosphor-Imager analysis of genomic DNA probed for ACT1 (actin) and ENO1 sequences (Postlethwait & Sundstrom, 1995) revealed single plasmid integrations at two or three out of the four ENO1 homologues (data not shown). The low copy number is similar to that conferred by CEN episomal vectors in *S. cerevisiae* which are maintained in one to two copies per cell (Bloom et al., 1983). Analyses of multiple independent transformants of each construct did not reveal growth or germination defects (data not shown).

A critical factor validating the *HWP1*p construct was maintaining developmental regulation of the *HWP1* locus. Epigenetic regulation of *HWP1* could prevent use of the construct if developmental yEGFP3 expression was lost at the ENO1 locus. Analysis of yEGFP3 expression in yeasts, pseudohyphae and hyphae by growing cells in all four modified Lee’s media (Brummel & Soll, 1982; Staab et al., 1996) confirmed the developmental regulation of the reporter gene (Fig. 3a). GFP was only observed in true hyphae, paralleling the expression pattern of *HWP1* (Staab et al., 1996); pseudohyphae (arrows in Fig. 3a) and yeasts were negative for green fluorescence. Thus, the construct contained all the necessary *cis* elements for developmental expression, and ectopic placement of the *HWP1* promoter did not change its regulation.

![Fig. 3. Expression of GFP in *C. albicans*.](http://mic.sgmjournals.org)

(a) Strain HGFP3 expresses GFP in a hyphae-specific manner, paralleling the expression pattern of *HWP1*. Mid-exponential-phase cells were grown in all four Lee’s media for 3 h prior to microscopic examination. White arrows point to GFP-negative cells forming pseudohyphae in Lee’s pH 4.5 at 37°C. Scale bar, 10 μm. (b) Strains expressing GFP controlled by the *HWP1*, ENO1 and no promoter constructs. Stationary-phase yeast cells were diluted into fresh YPD or YPD plus 5% bovine calf serum, and grown at 30 °C or 37 °C, respectively, for 3 h prior to microscopic examination. The ENO1 promoter (EGFP3) drives constitutive expression of GFP regardless of cell type (middle column), while the promoterless construct is negative for GFP (right column). The *HWP1* promoter (left column) maintained developmental regulation of GFP. Scale bars, 10 μm.
Constitutive expression of yEGFP3 was achieved when the \textit{HWP1}\textsubscript{p} was replaced with the \textit{ENO1}\textsubscript{p} (Fig. 3b). All cell types of EGFP3 grown in the four modified Lee’s media were also brightly fluorescent (data not shown). Cells transformed with the promoterless construct were negative for GFP as expected (Fig. 3b).

Filamentation and yEGFP3 expression were examined in solid serum plates (Fig. 4). Embedded colonies expressed GFP when either the \textit{HWP1} or the \textit{ENO1} promoter controlled expression of yEGFP3. Closer examination of budding branches near the ends of HGFP3 filaments revealed GFP-negative buds and pseudohyphae (Fig. 4, arrows) suggesting that developmental regulation of yEGFP3 expression was maintained by the \textit{HWP1} promoter in solid medium. GFP was observed in all cell types in strain EGFP3 as expected.

Constitutive expression of \textit{BCY1} diminishes germ tube formation

The versatility of the constructs was tested by substituting yEGFP3 with \textit{CaBCY1} (\textit{SRA1}), a gene encoding the regulatory subunit of cAMP-dependent PKA, a component of the cAMP signalling pathway. Since the increase in intracellular cAMP levels positively affects germ tube formation (Bahn & Sundstrom, 2001; Castilla \textit{et al.}, 1998; Chattaway \textit{et al.}, 1981), degradation of cAMP should diminish or inhibit filamentation. In \textit{S. cerevisiae}, increasing the expression of \textit{BCY1} shifts the equilibrium of association/dissociation of PKA from subunits towards the associated (inactive) state even in the presence of cAMP (Portela \textit{et al.}, 2001), thus blunting downstream effects of PKA on gene activation. Therefore, if PKA activity is regulated by an analogous mechanism in \textit{C. albicans}, we expected that over-expression of \textit{BCY1} should reduce germ tube formation and...
filamentation. Constitutive overexpression of \textit{BCY1} by the \textit{ENO1}p indeed produced strains deficient in filamentation. Strain EBCY1 was deficient in germ tube formation and filamentation in liquid and solid media, respectively (Fig. 5b, c). \textit{BCY1} mRNA was overexpressed in both EBCY1 yeasts and germ tubes relative to the control strain EGFP3 (Fig. 5a), consistent with the morphology-independent expression of the \textit{ENO1} gene (Postlethwait & Sundstrom, 1995; Staab et al., 1996). EBCY1 continued to form elongated yeasts when placed in M199 at 37°C (Fig. 5b, white arrow) without forming true hyphae (Fig. 5b, black arrow). No change in the doubling times of EBCY1 yeasts relative to EGFP3 or to another control strain, UnoPP-1 (Postlethwait & Sundstrom, 1995), was noted (data not shown), indicating that a continuous abundance of Bcy1p did not affect growth and perhaps indirectly interfere with germ tube formation. The inhibition of true hyphae formation in strain EBCY1 suggests that overexpression of \textit{BCY1} leads to titration of free and active PKA subunits into bound and inactive molecules unable to signal downstream gene targets involved in germ tube formation. The filamentation defect of EBCY1 was also seen in colonies embedded in Spider and serum agar media (Fig. 5c). The morphology of EBCY1 cells at the periphery of the colonies in both solid media was mostly yeasts or very short germ tubes. Constitutive expression of yEGFP3 in the control strain, EGFP3, did not affect normal filamentation in either medium. Copious hyphae (in serum plates) and hyphae with branching yeasts (in Spider plates) were seen at the periphery of embedded colonies of EGFP3, phenotypes that are associated with filamentation-competent strains (Bahn & Sundstrom, 2001; Liu et al., 1994; Lo et al., 1997). The data suggest that induction of germ tube formation or filamentation by different environmental signals in EBCY1 was not enough to surpass the effect of constitutive overexpression of \textit{BCY1} by the \textit{ENO1}p. The results confirmed the expected filamentation-defective phenotype of overexpressing \textit{BCY1} and subsequent inactivation of

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Overexpression of \textit{BCY1} inhibits germ tube formation and filamentation. (a) Overexpression of \textit{BCY1} by the \textit{ENO1} promoter. Northern blot analysis of \textit{BCY1} mRNA expression driven by the \textit{ENO1} promoter in yeasts (lanes 1 and 2) or germ tubes (lanes 3 and 4). \textit{BCY1} mRNA levels are higher in EBCY1 (lanes 2 and 4) than in the wild-type strain SC5314 (lanes 1 and 3). A probe for 18S RNA (see Methods) served as control for total RNA loaded per lane (bottom row). (b) Inhibition of germ tube formation by overexpression of \textit{BCY1}. Control (EGFP3) and EBCY1 yeasts were induced to form germ tubes in liquid M199 at 37°C for 3 h. After incubation, the control strain (EGFP3) formed germ tubes (black arrow) whereas EBCY1 grew mostly as yeasts or elongated yeasts (white arrow). (c) Filamentation in solid media. EBCY1 and the control strain (EGFP3) yeast cells embedded in Spider (left two columns) and serum (right two columns) media were grown at 37°C for several days. EGFP3 produced typical filamentous colonies in both media (top row) while the EBCY1 strain formed more compact colonies lacking filamentous projections. Closer examination of the periphery of the colonies of EGFP3 revealed filamentous cells with branching buds in Spider medium (first column, second row) absent in EBCY1 colonies (second column, second row). Serum medium produced EGFP3 colonies with copious hyphae (third column, second row) not seen at the periphery of EBCY1 colonies (fourth column, second row). White and black scale bars represent 0.5 mm and 150 μm, respectively.}
\end{figure}
PKA. The data also imply that regulation of the cAMP signalling pathways in S. cerevisiae and C. albicans occur through similar mechanisms involving titration of free cAMP concentrations.

We envision several uses for the plasmid constructs. The main feature of the pHWP1GFP3 construct is its utility as a reporter of germ tube induction and true hyphae formation. Because HWP1 expression is coordinately controlled with true hyphae formation (Staab et al., 1996), HWP1 mRNA has been used as a marker of germ tube formation (Braun & Johnson, 2000; Braun et al., 2001; Davis et al., 2002; Kadosh & Johnson, 2001; Lane et al., 2001a, b; Liu, 2001; Murad et al., 2001). The pHWP1GFP3 construct allows for the easy visualization of HWP1p activity in cells expressing true hyphae without having to prepare RNA for analysis. The customary method of HWP1p mRNA analysis by Northern blotting only examines HWP1 expression in a culture of cells without taking into account the percentage of germinating cells. The abundance of HWP1 message in germinating cells (Staab et al., 1996) makes its detection possible even if a small percentage of cells have germinated (data not shown). pHWP1GFP3-transformed strains permit easy determination of the percentage of germ tube formation in a mixed population of cells during the course of an experiment. In addition, GFP expression in true hyphae eliminates the guesswork of enumerating cells with very short germ tubes or cells that are forming pseudohyphae (negative for GFP).

A convenient feature of the plasmids is the ability to substitute yEGFP3 with heterologous genes for expression either concomitantly with germ tube induction or constitutively in all cell types. As an example we used the ENO1p plasmid to test the predicted germ-tube-defective phenotype resulting from overexpression of a member of the cAMP signalling pathway gene, BCY1, the regulatory subunit of PKA. The highly active ENO1p effectively over-expressed BCY1 mRNA several fold relative to that driven by the native BCY1p (Fig. 5a). These results suggest that the pENO1GFP3 construct is amenable to other genetic studies such as epistatic analyses to determine the functional relationship of genes among signalling pathways. Alternatively, the HWP1p may be utilized for expressing genes in conjunction with germ tube formation. One caveat regarding the HWP1 promoter is that it may not be the best choice for studying genes directly involved in dimorphism. Intermediate phenotypes may confuse the interpretation of results if the HWP1p promoter is used to induce genes within filamentation signalling pathways that ultimately regulate HWP1 expression.

The plasmids described here expand the molecular genetic tools for studying gene expression and functional relationships between gene products in C. albicans. Although the chromosomal integration of plasmids or DNA constructs for gene expression analyses in C. albicans have been described before (Backen et al., 2000; Morschhauser et al., 1998; Srikantha et al., 1996; Uhl & Johnson, 2001), the visualization of GFP expression at the cellular level in tight association with morphology has not been reported. This makes the HWP1p construct attractive for studies examining true hyphae formation and filamentation, while the ENO1p permits constitutive overexpression of genes in all cell morphologies. Although it is difficult to assess the relative strengths of each promoter, both HWP1 and ENO1 express their cognate mRNAs at relatively high levels (Staab et al., 1996, 1999). The brighter appearance of EGFP3 yeasts and germ tubes relative to HGFP3 germ tubes (data not shown) is most likely a result of the continuous accumulation of the stable GFP (Chalfie et al., 1994; Li et al., 1998) in EGFP3 cells. yEGFP3 mRNA is also detected in larger amounts in EGFP3 cells relative to HGFP3 germ tubes (data not shown), consistent with the constitutive expression of yEGFP3 by the ENO1 promoter and apparent stability of the message. Nonetheless, both promoters express high amounts of GFP in C. albicans readily visible by epifluorescence. Both plasmids produce transformants with stable, low-copy integrations into the chromosome at a known genomic locus.

ACKNOWLEDGEMENTS

We thank B. Cormack for generously providing pYGFP3. Support for this research was provided by a grant from the National Institute of Allergy and Infectious Diseases (R01 AI46608). P. Sundstrom is a recipient of a Scholar Award from the Burroughs Wellcome Fund.

REFERENCES


S. cerevisiae ura3(YEGFP): a reporter of gene expression in Microbiology fragment C in Pichia pastoris integrations of the gene.

http://mic.sgmjournals.org 2985


