



Yeast Functional Analysis Report

Cassettes for the PCR-mediated construction of regulatable alleles in *Candida albicans*

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Abstract

The recent availability of genome sequence information for the opportunistic pathogen *Candida albicans* has greatly facilitated the ability to perform genetic manipulations in this organism. Two important molecular tools for studying gene function are regulatable promoters for generating conditional mutants and fluorescent proteins for determining the subcellular localization of fusion gene products. We describe a set of plasmids containing promoter–GFP cassettes (P_{MET3} –GFP, P_{GALI} –GFP, and P_{PCK1} –GFP), linked to a selectable nutritional marker gene (*URA3*). PCR-mediated gene modification generates gene-specific promoter, or gene-specific promoter–GFP, fusions at the 5'-end of the gene of interest. One set of primers can be used to generate three strains expressing a native protein of interest, or an amino-terminal GFP-tagged version, from three different regulatable promoters. Thus, these promoter cassette plasmids facilitate construction of conditional mutant strains, over-expression alleles and/or inducible amino-terminal GFP fusion proteins. Copyright © 2004 John Wiley & Sons, Ltd.

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Introduction

Candida albicans is a leading cause of serious mucosal and systemic fungal infections in immunocompromised patients (Hajjeh, 2002; Odds, 1994). Over the past 10 years, the adaptation of genetic, molecular and genomic approaches to the study of fungi have led to rapid advances in our understanding of *C. albicans* biology (Magee *et al.*, 2003). In particular, the use of PCR-mediated strategies has allowed researchers to quickly and efficiently construct strains with modified or disrupted genes (Gale *et al.*, 2001; Wilson *et al.*, 1999).

Regulated promoters are useful tools for monitoring the consequences of expression, or lack of expression, of a gene. This is especially important in the case of genes that are essential for

cell viability. *C. albicans* promoters that regulate expression in response to nutritional conditions include *GALI* (Gorman *et al.*, 1991), *MAL1* (Backen *et al.*, 2000), *MAL2* (Umeyama *et al.*, 2002), *PCK1* (Leuker *et al.*, 1997), and *MET3* (Care *et al.*, 1999). Conventional cloning techniques have been used with these promoters to generate strains expressing very little to no gene product. The constitutive *ADHI* promoter, as well as the *MAL2* and *MET3* promoters, also have been used to express gene sequences that have been tagged at the 3'-end with the FLAG epitope and integrated at the *RP10* locus (Umeyama *et al.*, 2002).

Recently, we constructed a set of plasmids useful for the PCR-mediated construction of carboxy-terminal fluorescent fusion proteins in *C. albicans* (Gerami-Nejad *et al.*, 2001). To tag genomic

sequences of interest, PCR modules that encode selectable nutritional markers and a fluorescent protein sequence (either *GFP*, *YFP* or *CFP*, codon-optimized for expression in *C. albicans*) are amplified from the plasmids using primers designed to include gene-specific sequences that target integration of the cassette to the 3'-end of the gene. Successful integrations result in expression of the protein of interest tagged with a fluorescent protein at its carboxy-terminus.

To expand the repertoire of tools available for PCR-mediated gene modification in *C. albicans*, we report here a new set of plasmids useful for the introduction of regulatable promoters, with or without a fluorescent protein sequence, to any genomic locus of interest. Because of the ease and speed with which PCR-mediated gene modification can be performed, these plasmids facilitate the construction of conditional mutants, overexpression alleles, and amino-terminally tagged fluorescent protein fusions in *C. albicans*.

Materials and methods

Strains, growth conditions and DNA methods

All strains constructed for this study were derived from BWP17 (*ura3::imm434/ura3::imm434 his1::hisG/his1::hisG arg4::hisG/larg4::hisG*) (Wilson et al., 1999) and were grown at 28 °C in synthetic medium containing 80 µg/ml uridine (SM; Sherman, 1991) and 2% glucose, unless otherwise noted. To induce expression from P_{GAL1} , P_{MET3} , and P_{PCK1} , strains were grown overnight in non-inducing conditions (SM), washed twice in non-inducing media, and then diluted 1:10 into fresh inducing media (SM + 2% galactose + 2% raffinose, SM + 2% glucose

and lacking methionine and cysteine, and SM + 2% succinate, respectively). Plasmid constructions were done using *Escherichia coli* strain XL1-blue (Stratagene, La Jolla, CA) and standard media and methods (Ausubel et al., 1995). Yeast genomic DNA was isolated according to the method of Hoffman and Winston (1987). Polyacrylamide gel electrophoresis-purified oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). PCR amplification of the promoter cassettes, *C. albicans* transformation, and screening of yeast transformants was performed essentially as described (Gerami-Nejad et al., 2001). Briefly, promoter cassettes were generated by PCR using the Qiagen Taq DNA polymerase kit (Qiagen, Valencia, CA). The products from 10 50 µl reactions were pooled, the DNA was precipitated (using 1/10 volume of 3 M sodium acetate and 2× volume of 95% ethanol), the products were resuspended in 20 µl water, and then used directly for transformation of *C. albicans* strain BWP17 (Wilson et al., 1999). Transformants were selected by plating the transformation mix on SM medium lacking uracil and uridine. Transformants were screened by PCR using oligonucleotide primers that annealed to the target gene locus outside of the altered region.

Plasmid constructions

The *MET3* promoter was isolated as a *HindIII*–*PvuII* fragment from pCaDis (Care et al., 1999) and ligated to *HindIII*–*EcoRV*–digested pGFP–*URA3* (Gerami-Nejad et al., 2001), which contains the *C. albicans URA3* gene, to generate pMG1876. The *C. albicans* codon-optimized green fluorescent protein (GFP) sequence was amplified using pMG871 (Gerami-Nejad et al., 2001) as the template, forward primer 686 (designed to include a *BamHI* site; italicized, Table 1), and reverse

Table 1. PCR primers for construction of plasmids^a

Primer	Sequence
686	5'-CGCGGATCCGCGTTTATTAATAATGTCTAAAGGTGAA-3'
687	5'-CCCAAGCTTGGGCTGCAGTTATTTGTACAATTCATC-3'
912	5'-GGGCCGCTCGAGCCTGTACAGTGAGGGAAGATCTGATATTGACGAAGAGG-3'
913	5'-CCCCGCGGATCCCTCTTTCTTATAAAAAATCGGTTTGTATGTGTATATGTGG-3'
945	5'-CAAACAATCCTCTACCAACA-3'
1092	5'-CCCCGCGGATCCGATTGATAATTATTTG-3'

^a Use of the primers in plasmid constructions is described in Materials and methods. Restriction enzyme sites are noted in italics.

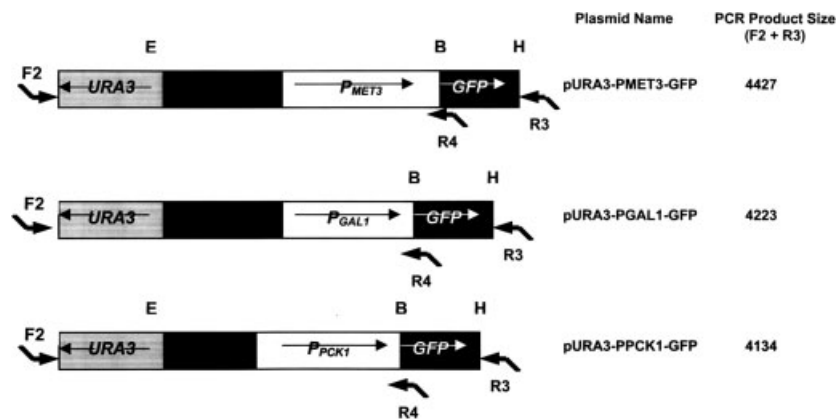


Figure 1. Diagram of the *C. albicans* promoter–GFP modules. Arrows within the boxes indicate the direction of transcription, and numbers and arrows outside of the boxes indicate the primer used (see Table 2) and the direction of replication, respectively. The plasmid names and the expected sizes of the PCR products (using primers F2 and R3), including 70 nucleotides of flanking sequence for each primer, are indicated. For consistency, numbering and nomenclature used previously by our group (Gerami-Nejad, *et al.*, 2001) have been continued. E, *EcoRI*; B, *BamHI*, H, *HindIII*

primer 687 (designed to include a *HindIII* site; italicized, Table 1). The resulting PCR product was digested with *BamHI* and *HindIII* and ligated to *BamHI*–*HindIII* cut pMG1876 to generate pURA3–PMET3–GFP (Figure 1).

The *GAL1* promoter sequence, identified by comparison of *S. cerevisiae* P_{GAL1} to the *C. albicans* genome sequence (<http://sequence-www.stanford.edu/group/candida/search.html>), was amplified using forward primer 912 (designed to include an *Xho1* site; italicized, Table 1), reverse primer 913 (designed to include a *BamHI* site; italicized, Table 1) and genomic DNA isolated from BWP17 as the template. The resulting PCR product was digested with *Xho1* and *BamHI* and ligated to *BamHI*–*Xho1*–cut pURA3–PMET3–GFP, effectively replacing P_{MET3} with P_{GAL1} , to generate pURA3–PGAL1–GFP (Figure 1).

The P_{PCK1} promoter sequence was amplified using pCAHUH (Stoldt *et al.*, 1997) as the template, forward primer 1092 (designed to contain a *BamHI* site; italicized, Table 1), and reverse primer 945. The resulting PCR product was digested with *BamHI* and *PstI* and ligated to *BamHI*–*NsiI*–cut pURA3–PGAL1–GFP, effectively replacing P_{GAL1} with P_{PCK1} , to generate pURA3–PPCK1–GFP (Figure 1).

The DNA sequences of the *URA3*–promoter–GFP sequences in each plasmid were verified (University of Minnesota Microchemical Facility) to ensure that no mutations were introduced during the cloning process.

Primer design for integration of promoter cassettes

To integrate P_{MET3} –GFP, P_{GAL1} –GFP, and P_{PCK1} –GFP upstream of *INT1*, a forward primer (FINT1, Table 2) was designed to include 70 nucleotides of *INT1* 5′-upstream sequence (sequence immediately preceding nucleotide –15 from the *INT1* start codon) followed by primer sequence F2 (underlined sequence within FINT1, Table 2). A reverse primer (RINT1, Table 2) was designed by joining the reverse complement of nucleotides 4–74 of the *INT1* ORF to primer R3 (underlined sequence within RINT1, Table 2). Because the F2 and R3 sequences are present in all three *URA3*–promoter–GFP plasmids, the same primer pair was used to amplify all three *URA3*–promoter–GFP fusions and to insert the three promoters upstream of the *C. albicans* *INT1* gene.

Morphological observations

Differential interference contrast and epifluorescence microscopy were performed using a Nikon Eclipse E600 photomicroscope equipped with a 100 W mercury lamp, and epifluorescence illumination with an Endow GFP filter set (excitation 450–490 nm, emission 500–550 nm) (Chroma Technology Corp., Brattleboro, VT). Digital images were collected using a Photometrics CoolSNAP HQ camera and Metamorph software.

Table 2. PCR primers for amplification of the promoter modules

Primer	Purpose	Sequence
F2	Promoter introduction	5'-(Gene-specific sequence) TCTAGAAGGACCACCTTTGATTG-3' ^a
R3	Promoter-GFP	5'-(Gene-specific sequence) TTT GTA CAA TTC ATC CAT AC-3' ^b
R4	Promoter-no tag	5'-(Gene-specific sequence) CAT TTTAATAAACGCGGATCC-3' ^c
FINT1	Promoter introduction to <i>INT1</i> locus	5'-TAAAATAAAAAACAAAACAAAACAAAAG TACTAACAAATTATTGAACTTTT AATTTT TAATAAAGAATCAGTAGATTCTAGAAGGACCACCTTTGATTG-3' ^d
RINT1	Promoter-GFP to <i>INT1</i> locus	5'-GA GGA CGA TTG AGG CTG TAA <u>TTG TAA ATG AGA ATGTTT ATC TAT CGG</u> TAA TAA TTT ACT TGG AGT TGA GTT <u>TTT GTA CAA TTC ATC CAT AC-3'</u> ^e

Primer design for integrating promoter and promoter-GFP cassettes. The position of the forward (F) and reverse (R) primers are also depicted in Figure 1. The reading frames of *GFP* and *INT1* are indicated by spacing, and the reverse complement of the start codon is in bold.

^a The gene-specific sequences for this primer can be chosen immediately upstream of the target gene (see e.g. FINT1). The sequences shown here are the complements of the 5'-*URA3* sequences contained in the template plasmids.

^b The gene-specific sequences of these primers are chosen to correspond to the reverse complements of the N-terminal codons of the target gene. The sequences shown here are the reverse complement of the 3'-*GFP* sequence contained in the template plasmids.

^c The gene-specific sequences of these primers are the reverse complements of the nucleotides immediately downstream of the target site (see e.g. RINT1). The start codon is encoded by the cassette, and sequences of the target gene should be chosen according to this reading frame.

^d See Materials and methods. The underlined sequence corresponds to the complement of the 5'-*URA3* sequence contained in the template plasmids.

^e See Materials and methods. The underlined sequence corresponds to the reverse complement of the 3'-*GFP* sequence contained in the template plasmids.

Protein detection

C. albicans strains were grown for approximately 8 h under inducing conditions, as described above, to induce expression of GFP-tagged Int1p. Cells were washed once with 10 ml water, resuspended in 0.5 ml 15% glycerol, and stored at -70 °C. On the day of use, the cells were thawed and equal A₆₀₀ units of cells (2.5 U) for each strain were resuspended in 50 µl Thorner buffer (40 mM Tris, pH 6.8, 5% SDS, 8 M urea and 100 µM EDTA) and disrupted by vortexing for 2 h at 4 °C with an equal volume of 500 µm glass beads. The lysate was diluted 1 : 4 with water and SDS-PAGE reducing buffer (20 mM Tris, pH 6.8, 10% glycerol, 0.005% bromophenol blue, 2% SDS and 5% β-mercaptoethanol; 200 µl) and the resulting samples were heated for 60 min at 95 °C. Protein concentration was determined (estimated by A₂₈₀ of each sample) and equal amounts of protein from each strain (0.15 U) were loaded and then separated on a 7.5% SDS-polyacrylamide gel. Proteins were transferred to a PVDF membrane, blocked for 1 h with 2% skimmed milk in TBST (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween-20) and incubated with mouse anti-GFP antibodies (Roche, Indianapolis, IN), diluted 1 : 2000 in 0.1% skimmed milk in TBST, for 1 h. The blot was subsequently washed in TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse

IgG (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1 : 50 000 in 0.1% skimmed milk in TBST, for 1 h. The blot was washed again in TBST and proteins were then detected using the Supersignal Fempto Chemiluminescent reagent (Pierce, Rockford, IL). The amount of protein detected (signal intensity) was quantitated using a Alpha-Innotech Chemi-Imager (San Leandro, CA) and the Image-Quant image analysis software program. To further control for differences in protein loading, signals were standardized according to the total amount of protein in each lane, as detected by India ink staining of the blot (Figure 3B), followed by quantitation of protein signal using Image-Quant.

Plasmid requests

Plasmids can be obtained by contacting Cheryl Gale via e-mail at galex012@umn.edu. Electronic sequence files of the cassettes can be found on the Berman laboratory web page (<http://www.cbs.umn.edu/labs/berman/>) under the 'Candida fluorescent protein cassettes' link.

Results and discussion

Construction and validation of the promoter cassette system

To construct promoter-GFP module templates for the PCR-mediated introduction of regulatable

amino-terminal GFP fusions, we modified the GFP-tagging vector, pGFP-URA3 (Gerami-Nejad *et al.*, 2001), by inserting the *C. albicans* *MET3* promoter sequence (nucleotides -1353 to -7 of *MET3*) followed by the *C. albicans* codon-optimized *GFP* sequence (Cormack *et al.*, 1997) to generate pURA3-PMET3-GFP. Subsequently, the *MET3* promoter sequence was replaced with the *GALI* promoter (nucleotides -1223 to -7 of *GALI*) and *PCK1* promoter (nucleotides -1425 to -1 of *PCK1*) sequences to generate pURA3-PGAL1-GFP and pURA3-PPCK1-GFP, respectively.

We hypothesized that expression induced from each of these promoters would be quantitatively different, providing expression level options to the researcher. P_{MET3} is regulated by the concentration of methionine in the medium, with 10 mM methionine causing near-complete repression of expression (Care *et al.*, 1999). In addition, variable levels of P_{MET3} repression can be achieved by adding between 0 and 10 mM methionine (Umeyama *et al.*, 2002). Expression from P_{GAL1} is induced by galactose (Gorman *et al.*, 1991; Mao *et al.*, 1999), but also retains significant activity in glucose-containing media (Srikantha *et al.*, 1996). In one study, expression of a P_{GAL1} -*ADE2* reporter construct was increased approximately 10-fold when induced with galactose as compared to glucose (Srikantha *et al.*, 1996). Expression from P_{PCK1} is induced by gluconeogenic carbon sources such as succinate and casamino acids (Leuker *et al.*, 1997). Notably, the addition of casamino acids resulted in two-fold greater expression levels of a *LAC4* reporter gene as compared to levels of expression seen with the addition of succinate (Leuker *et al.*, 1997). Furthermore, maximal induction of P_{PCK1} -*LAC4* expression was reached approximately 8 h after shift of the culture from repressing (glucose) to inducing (succinate) conditions, indicating that a time of adaptation to glucose-free conditions is required to achieve full expression from P_{PCK1} (Leuker *et al.*, 1997). As with P_{GAL1} , a low level of expression from P_{PCK1} may be present even in glucose-containing media (Leuker *et al.*, 1997).

To validate the usefulness of the promoter cassette plasmids, they were used as templates, along with gene-specific primers designed to facilitate integration immediately upstream of the *INT1* gene, to generate promoter-GFP modules by

PCR (Figure 1). *INT1* was chosen as the test gene because fluorescent protein fusions to the carboxy-terminus of Int1p are readily visualized at the mother-bud neck of yeast-form *C. albicans* cells (Gale *et al.*, 2001). Thus, expression of the new promoter-driven amino-terminal fusions could be screened initially by fluorescence microscopy. Primers FINT1 and RINT1 (Table 2) were used to amplify the promoter modules, as depicted in Figure 1.

BWP17 was transformed with each of the resulting PCR products to generate strains containing P_{MET3} -GFP, P_{GAL1} -GFP and P_{PCK1} -GFP integrated immediately upstream of, and in-frame with, the *INT1* ORF. PCR analysis of eight of the resulting transformants for each transformation showed that at least two (and as many as five transformants) out of each set of eight transformants contained the promoter module inserted at the *INT1* locus. Southern blotting showed that the FP cassette integrated only at the *INT1* locus and not in additional (ectopic) locations (data not shown). Fluorescence microscopy of these transformants indicated that amino-terminal GFP-tagged Int1p localized to the mother-bud neck of yeast-form cells (Figure 2), similar to the localization observed for the carboxy-terminal Int1-GFP fusion protein (Gale *et al.*, 2001). We saw the same specific localization for three individual transformants from each promoter-cassette transformation, further supporting the conclusion that this localization phenotype arises from the specific (*INT1*) fusion and not from a coincidental secondary integration. In general, GFP-Int1p rings were observed at the necks of small-, medium- and large-budded cells for all of the promoter constructs. However, in the case of expression from P_{MET3} , in 14 of 38 (37%) budded cells examined, an additional ring of GFP-Int1p remained, usually placed adjacent to the mother-bud neck (Figure 2). In comparison, lower numbers of budded cells were observed to have an additional ring of GFP-Int1p when *GFP-INT1* was expressed from P_{INT1} (8%), P_{GAL1} (12%) and P_{PCK1} (8%). The predominance of a second GFP-Int1p ring when expressed from P_{MET3} may indicate a prolonged persistence of Int1p (when overexpressed at these levels) at the bud-site of the previous cell cycle, especially since it is usually located adjacent to the current bud-site, in accordance with *C. albicans* bud-site selection patterns during yeast-form growth.

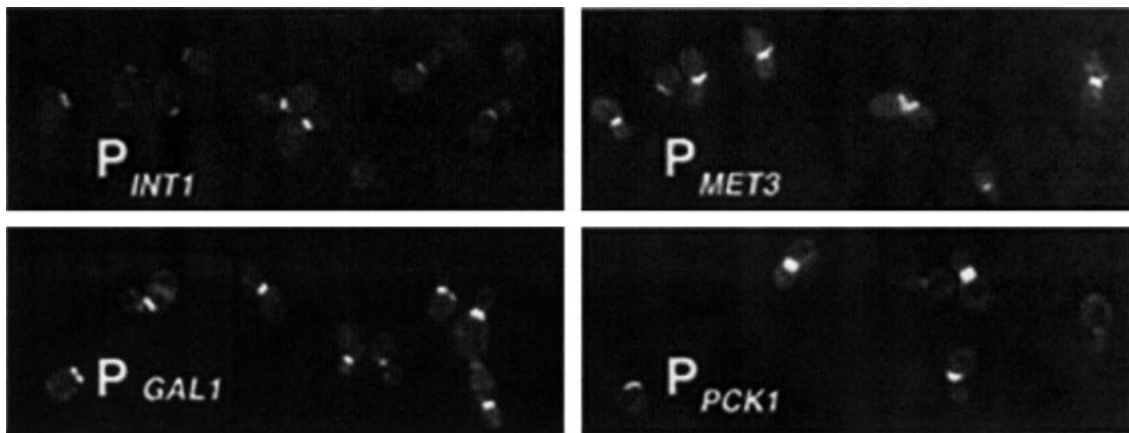


Figure 2. Immunofluorescence micrographs of GFP-Int1p expressed from four different promoters. Construction of strains expressing GFP-Int1p from the *PCK1* (P_{PCK1}), *MET3* (P_{MET3}) and *GAL1* (P_{GAL1}) promoters, and conditions for induction of expression of each, are described in Materials and methods. For comparison, a strain containing Int1p expressed from its native promoter (P_{INT1}) and tagged with GFP at its carboxy-, rather than its amino-terminus (Gale et al., 2001), is included in the analysis (top left). Strains were grown in inducing conditions for 6 h prior to analysis by fluorescence microscopy. Relative exposure times to give the intensity of the signals shown are 0.5 s (P_{MET3}), 1 s (P_{GAL1} , P_{PCK1}) and 2 s (P_{INT1})

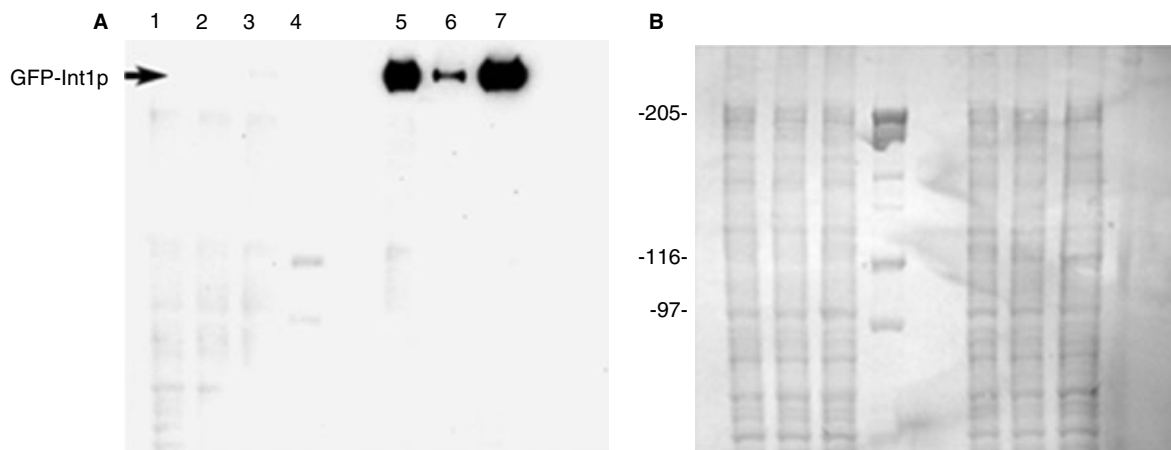


Figure 3. (A) Protein blot showing the relative levels of GFP-Int1p expressed from the three different promoters (P_{MET3} , lanes 1 and 5; P_{GAL1} , lanes 2 and 6; P_{PCK1} , lanes 3 and 7). Cell lysates from strains grown in non-inducing conditions (lanes 1–3) and in inducing conditions (lanes 5–7), as described in the text, are included as shown. Equal amounts of protein were loaded in each lane, as determined prior to electrophoresis by absorption of protein samples at 280 nm, and standardized after electrophoresis by quantitation of the India ink-stained protein blot (B). Sizes of molecular weight standards (A and B, lane 4) are indicated

To measure the levels of GFP-Int1p expression from each of the promoters relative to expression from the native promoter, yeast cell lysates were separated by polyacrylamide gel electrophoresis and then analysed on Western blots using anti-GFP antibodies. Expression of GFP-Int1p was undetectable when the strains containing P_{MET3} -GFP-INT1, P_{PCK1} -GFP-INT1

and P_{GAL1} -GFP-INT1 were grown in non-inducing conditions (Figure 3A, lanes 1–3). In contrast, expression of GFP-Int1p, by the same strains grown in inducing conditions, was readily detected with expression being highest from P_{MET3} (9 \times) and P_{PCK1} (8 \times) followed by P_{GAL1} (1 \times) (Figure 3A, lanes 5–7), after standardization for differences in protein loading (Figure 3B; see also Materials

and methods). Importantly, we did not observe GFP signals at sizes other than that predicted for GFP–Int1p, further supporting the conclusion that *GFP* integrated at only the *INT1* locus and not at ectopic locations. In four separate Western blot experiments, Int1p expression was always greatest from P_{MET3} and P_{PCK1} , as compared to expression from P_{GAL1} . In addition, in three of the four experiments, P_{MET3} gave higher Int1p expression levels than P_{PCK1} . Although we observed this hierarchy with regard to Int1p expression, expression levels for other proteins may differ somewhat (e.g. due to the specific induction conditions used or post-translational regulation) and should be determined empirically for each particular experiment.

Potential uses

The *URA3*–promoter–*GFP* cassettes facilitate the PCR-mediated construction of regulated genes in *C. albicans*, obviating the need to clone full-length genes into an expression plasmid. In addition, by using reverse primer R3 (Table 2, Figure 1), a *GFP* tag can be incorporated into the constructs, providing a means to introduce an amino-terminal fluorescent protein tag. Furthermore, this PCR technique generates chromosomally integrated genes whose expression should be more stable than plasmid-borne constructions, because plasmid loss occurs with significant frequency in *C. albicans*, even on selective media. The use of regulated promoters to construct conditional mutants has become an important technique that has allowed the study of many essential gene functions in *C. albicans* (Bockmuhl *et al.*, 2001; Munro *et al.*, 2001; Nishikawa *et al.*, 2002; Ushinsky *et al.*, 2002; Weber *et al.*, 2001). Furthermore, relative overexpression of a gene often results in phenotypes that provide insights into the functions and/or interactions of the gene product.

The ability to construct amino-terminal fluorescent protein fusions extends the repertoire of tools available to construct epitope-tagged proteins in *C. albicans* (Magee *et al.*, 2003), facilitating protein localization studies, as well as protein isolation, detection and interaction analyses by Western blot and immunoprecipitation techniques. In cases where a carboxy-terminal GFP fusion may interfere with the normal localization of a protein, amino-terminal fusions can be constructed and analysed in parallel with the carboxy-terminal GFP fusion.

In addition, the relative overexpression of GFP-tagged fusion proteins may permit the visualization of fusion proteins expressed at levels from their native promoters that are so low that their signals are difficult or impossible to detect microscopically. While information regarding protein localization can be gained in this way, overexpression constructs may cause proteins to localize to ectopic sites or to persist at sites, as was seen for the P_{MET3} -controlled GFP–Int1p (Figure 2). Furthermore, the vectors described here do not permit the construction of 5'–GFP–gene fusions expressed from their native promoters. Thus, as is true for all epitope-tagged proteins, results obtained from amino-terminal GFP fusions should not be analysed in isolation. Rather, they should be used in concert with other techniques, such as traditional immunofluorescence methods and/or placement of the tag at another location within the gene. Nonetheless, the relative ease and speed with which these promoter and promoter–*GFP* fusions can be generated make them an extremely useful tool for gaining initial information regarding protein function and localization.

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