Yeast-enhanced green fluorescent protein (yEGFP): a reporter of gene expression in *Candida albicans*

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The green fluorescent protein (GFP) of *Aequorea victoria* has been developed here as a reporter for gene expression and protein localization in *Candida albicans*. When wild-type (wt) GFP was expressed in *C. albicans*, it was not possible to detect fluorescence or a translation product for the wt protein. Since this was probably due in part to the presence of the non-canonical CTG serine codon in the *Aequorea* sequence, this codon was changed to the leucine codon TTG. *C. albicans* cells expressing this construct contained GFP mRNA but were non-fluorescent and contained no detectable translation product. Hence a codon-optimized GFP gene was constructed in which all of the 239 amino acids are encoded by optimal codons for *C. albicans*. In this gene were also incorporated two previously identified mutations in the chromophore that increase GFP fluorescence. *C. albicans* cells expressing this yeast-enhanced GFP gene (yEGFP3) are fluorescent and contain GFP protein. yEGFP3 can be used as a versatile reporter of gene expression in *C. albicans* and *Saccharomyces cerevisiae* and the optimized GFP described here should have broad applications in these and other fungal species.

INTRODUCTION

Candida albicans, an important human pathogen (Cutler, 1991; Odds, 1988, 1994), has no known sexual cycle. In addition, because it is diploid (Scherer & Magee, 1990), classical genetic analyses have been very difficult. Most genetic analyses have relied on 'reverse genetics', involving gene cloning and targeted gene disruption followed by phenotypic analysis. Furthermore, it has proven difficult to functionally express proteins from other species in *C. albicans*, and as a result, until recently there has been no reporter system for *C. albicans*. This difficulty in heterologous protein expression has been generally ascribed to the presence in *C. albicans* (and some other related species) of a non-canonical genetic code, in which CTG codons are decoded as serine instead of leucine (Ohama *et al.*, 1993; Santos *et al.*, 1994; Santos & Tuite, 1995; White *et al.*, 1995). Recently, the *C. albicans URA3* gene (Brown *et al.*, 1996; Myers *et al.*, 1995), the *LAC4* gene of *Kluyveromyces lactis* (Leuker *et al.*, 1992) and the *Renilla reniformis* luciferase which contains no CTG codons (Srikantha *et al.*, 1996) have been developed as reporters of gene transcription.

We were interested in using the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* as a marker for gene expression and protein localization in *C. albicans*. GFP absorbs light with an excitation maximum of 395 nm, and fluoresces with an emission maximum of 510 nm (Morise *et al.*, 1974; Ward *et al.*, 1980). Since this can occur in the absence of any cofactors, GFP has been extremely useful as a marker for gene expression and as a tag in studying protein localization in a variety of organisms (Chalfie *et al.*, 1994; Cubitt *et al.*, 1995; Sidorova *et al.*, 1995; Wang & Hazelrigg, 1994). However, the *Aequorea* GFP sequence contains a single CTG codon, potentially precluding its use in *Candida*.

It is shown here that neither the wild-type (wt) GFP gene, nor a mutated GFP gene in which the CTG codon has been changed to TTG, is expressed in *C. albicans*. However, a synthetic GFP gene that carries optimal codons for translation in *C. albicans*, as well as two chromophore mutations that increase GFP fluorescence (Cormack *et al.*, 1996), is functional in *C. albicans*. This yeast-enhanced GFP (yEGFP3) is evaluated here as an efficient reporter of gene expression in *Saccharomyces cerevisiae* as well as *C. albicans*, and as a reporter in the context of infected host tissue.

METHODS

Strain maintenance. The *C. albicans* strain CAI4 (*ura3*Δ::*imm434*/*ura3*Δ::*imm434*) (Fonzi & Irwin, 1993) is a ura3⁻ derivative of Ca 5314 and was a gift from Bill Fonzi (Georgetown University Medical Center, Washington, DC, USA). CAI4 was grown in YPD (1%, w/v, yeast extract; 2%, w/v, peptone; 2%, w/v, dextrose) supplemented with 25 mg uridine l⁻¹. The *S. cerevisiae* strain W303-1B (*MAT*a *ade2 his3 leu2 trp1 ura3*) (Thomas & Rothstein, 1989) was propagated on YPD agar. *C. albicans* and *S. cerevisiae* were grown in synthetic media (2%, w/v, glucose, galactose or maltose; 0·65% Yeast Nitrogen Base without amino acids; and the appropriate supplements at 50 mg l⁻¹; Guthrie & Fink, 1991) at 30 °C with shaking at 200 r.p.m., and harvested during exponential growth (OD$_{600}$ 0·8) for RNA and protein analyses.

Yeast transformation. Transformation for *S. cerevisiae* was as previously described by Gietz *et al.* (1992) and Ito *et al.* (1983). *C. albicans* was transformed by the protoplasting method of Kurtz *et al.* (1986), or using a modification of the *S. cerevisiae* protocol. Cells were grown in YPD to early-exponential phase, and collected by centrifugation (1000 *g*, 5 min). They were washed twice with water, and resuspended in 0·1 M LiAc, 1 mM EDTA and 10 mM Tris/HCl (pH 7·5). To the cells were added 10 μg transforming DNA with 100 μg denatured salmon sperm DNA and 0·5 ml 0·1 M LiAc, 40% (w/v) PEG (3350), 1 mM EDTA and 10 mM Tris/HCl (pH 7·5). The mix was incubated at 30 °C for 30 min, and heat-shocked at 45 °C for 15 min. The cells were collected by centrifugation (1000 *g*, 5 min) and plated on synthetic medium lacking uridine.

Construction of the codon-optimized yEGFP genes. The nomenclature for the codon-optimized GFP molecules follows that previously published (Cormack *et al.*, 1996). Thus, the chromophore mutations in GFPmut1, GFPmut2 and GFPmut3 (Cormack *et al.*, 1996) are present in the yeast-optimized genes yEGFP1, yEGFP2 and yEGFP3, respectively. The yEGFP3 gene was constructed in three parts using nine oligonucleotides. The sequences of the oligonucleotides are given in Table 1.

Oligonucleotides A1–3, B1–3 and C1–3 were used in three PCR reactions to generate three fragments (Fig. 1). Fragment A was cloned into pUC19 after digestion with *Hin*dIII and *Eco*RI. Fragment B was blunt cloned into the vector pCRII (Invitrogen). Fragment C was cloned into pUC19 after digestion with *Eco*RI and *Pst*I. All three fragments were sequenced to verify that there were no mutations introduced by the PCR. Subsequently, fragment A was digested with *Hin*dIII and *Nde*I, fragment B with *Nde*I and *Hpa*I and fragment C with *Hpa*I and *Pst*I, and the three products were cloned in a quadruple ligation into pUC19 digested with *Hin*dIII and *Pst*I. The resulting vector, pYGFP3, was designed such that the GFP was in-frame with the *lacZ* of pUC19, and therefore the final clone was highly fluorescent in *E. coli*.

For the construction of two additional GFP mutant genes, yEGFP1 and yEGFP2, we replaced the *Hin*dIII–*Nde*I fragment of pYGFP3 with *Hin*dIII–*Nde*I fragments obtained by PCR of pYGFP3 with the following oligonucleotides:

(1) 5′ GGAATTCAAAGCTTTATTAAAATGTCTAAAG-GTGAA

Table 1. Oligonucleotides used for constructing yEGFP3

Restriction sites used for cloning are in bold.

Oligo	Sequence
A1	GGGATCCAAAGCTTTATTAAAATGTCTAAAGGTGAAGAATTATTCACTGGTGTTGTCCCAATTTTGGTTGAATTAGATGGTGATGTTAATGGTCACAAATTTTCTGTCTCC
A2	AATGGTCACAAATTTTCTGTCTCCGGTGAAGGTGAAGGTGATGCTACTTACGGTAAATTGACCTTAAAATTTATTTGTACTACTGGTAAATTG
A3	GGGAATTCCATATGATCTGGGTATCTAGCAAAACATTGAACACCATAACCGAAAGTAGTGACTAAGGTTGGCCATGGAACTGGCAATTTACCAGTAGTACAAATAAA
B1	GGGAATTCCCATATGAAACAACATGACTTTTTCAAGTCTGCCATGCCAGAAGGTTATGTTCAAGAAAGAACTATTTTTTTCAAAGATGACGGTAACTACAAGACCAGAGCTGAA
B2	AACTACAAGACCAGAGCTGAAGTCAAGTTTGAAGGTGATACCTTAGTTAATAGAATCGAATTAAAAGGTATTGATTTTAAAGAAGATGGTAACATTTTAGGT
B3	TCTAATTTTGAAGTTAACTTTGATACCATTCTTTTGTTTGTCAGCCATGATGTAAACATTGTGAGAGTTATAGTTGTATTCCAATTTGTGACCTAAAATGTTACCATCTTC
C1	GACAGAATTCGTTAACTTCAAAATTAGACACAACATTGAAGATGGTTCTGTTCAATTAGCTGACCATTATCAACAAAATACTCCAATTGGTGATGGTCCAGTCTTGTTACCA
C2	GATGGTCCAGTCTTGTTACCAGACAACCATTACTTATCCACTCAATCTGCCTTATCCAAAGATCCAAACGAAAAG
C3	AAAAAACTGCAGTTATTTGTACAATTCATCCATACCATGGGTAATACCAGCAGCAGTAACAAATTCTAACAAGACCATGTGGTCTCTCTTTTCGTTTGGATCTTTGGA
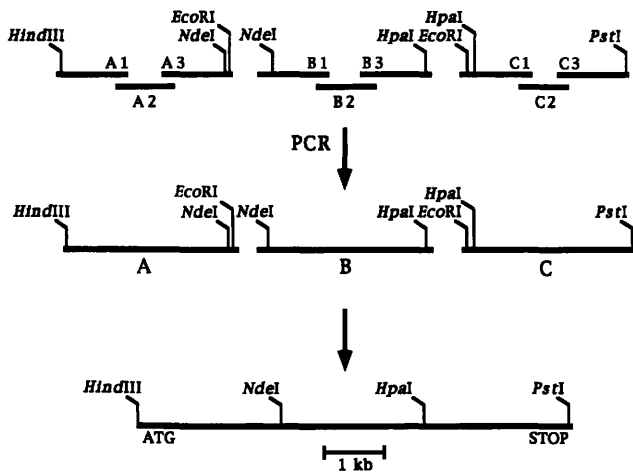
Fig. 1. Scheme for synthesis of the yEGFP3 gene, using *C. albicans* optimal codons.

(2) yEGFP1: GGGAATTCCATATGATCTGGGTATCTA-GAAAAACATTGAACACCATAAGTTAAAGTAGTGGAC

(3) yEGFP2: GGGAATTCCATATGATCTGGGTATCTA-GCAAAACATTGTAAACCATAAGCGAAAGTAGTGAC

The restriction sites used for cloning are shown in bold. The plasmids corresponding to these mutant GFPs are pYGFP1 and pYGFP2.

A fourth GFP gene (GFP$_{TTG}$) was made in which the single CTG codon was replaced with the optimal leucine codon, TTG. Oligonucleotides corresponding to the top and bottom strands of the wt GFP gene (GFP$_{wt}$) between the *Mun*I and *Bst*BI sites were made with this CTG to TTG mutation. These oligonucleotides were annealed and cloned into gel-purified pGFP (Clontech) digested with *Mun*I and *Bst*BI. The GFP$_{TTG}$ mutation was confirmed by DNA sequencing (Sanger *et al.*, 1977).

Gene expression. Constitutive expression of the GFP genes in *C. albicans* and *S. cerevisiae* was achieved using pYPB-ADHpt. This expression vector contains a *C. albicans* autonomously replicating sequence, the *C. albicans* URA3 selectable marker, *S. cerevisiae* 2μ sequences, the *E. coli* ampicillin-resistance gene and the *C. albicans* ADH1 promoter and terminator regions (Bertram *et al.*, 1996) separated by unique *Eco*RV, *Bgl*II, *Xho*I and *Stu*I sites (G. Bertram, I. D. Broadbent, P. J. F. Feldmann & A. J. P. Brown, unpublished). The GFP$_{wt}$ and GFP$_{TTG}$ genes were PCR-amplified using primers that introduced 5′-*Bgl*II and 3′-*Xho*I sites, and cloned between the *Bgl*II and 3′-*Xho*I sites in pYPB-ADHpt. yEGFP3 (the *Hind*III–*Pst*I fragment from pYGFP3) was blunted using the Klenow fragment of DNA polymerase I and inserted into the *Eco*RV site of pYPB-ADHpt.

yEGFP3 was expressed inducibly in *C. albicans* using pRC2312MAL. This vector was constructed as follows. The *C. albicans* MAL2 (Geber *et al.*, 1992) promoter (−540 to the ATG) was amplified by PCR using the primers:

M1: GAACGCATGCGTCGACGATATTTTTGTCTAGT-ACCATC

M2: GAACTCTAGATGTAGTTGATTATTAGTTAAACC

This promoter was cloned as a *Sph*I–*Xba*I fragment; the restriction sites are in bold. The 3′ untranslated region (including 256 bp 3′ to the stop codon) of the MAL2 gene was amplified using the oligonucleotides:

M3: GAAAGGATCCATACGCTTTGCAGGTGGTGTTG-ATCCAAATTCC

M4: CTTCTCTAGAGAGCTCAGAGGAATTCGATTAA-ATATACACTAGATGCTATC

This was cloned as a *Bam*HI–*Xba*I fragment; restriction sites are shown in bold. The 5′ and 3′ regions of the MAL2 gene were combined into the high-copy-number vector pRC2312 (Cannon *et al.*, 1992), digested with *Hind*III and *Bam*HI. This vector, p2312MAL, now contained the 5′ and 3′ untranslated regions of the MAL2 promoter flanking a polylinker containing *Xba*I, *Sac*I and *Eco*RI sites. yEGFP3 (the *Hind*III–*Pst*I fragment from pYGFP3) was blunted into the *Xba*I site of p2312MAL.

yEGFP1–3 was expressed inducibly in *S. cerevisiae* using the GAL1,10 vector pTS210. This vector, a gift from Tim Stearns (Department of Biological Sciences, Stanford University), is a YCp50-based vector with the GAL1,10 promoter and transcriptional terminator of the ACT1 gene. Between these two is a polylinker containing *Bam*HI, *Hind*III and *Xba*I sites. The polylinker sequence is GGATCCGCAAGCTTGCTCTAGA. The three codon-optimized GFPs were first cloned as *Hind*III–*Pst*I fragments into pUC19. From there, they were cloned as *Hind*III–*Xba*I fragments into PTS210, digested with *Hind*III and *Xba*I. The GFP$_{wt}$ construct in pTS210 was made by Tim Stearns and is called pTS395. This construct has GFP$_{wt}$ cloned into the *Xba*I site of pTS210.

Northern analysis. RNA was prepared from *C. albicans and S. cerevisiae* as described previously (Brown, 1994; Lindquist, 1981). Approximately equal amounts of total RNA were subjected to Northern analysis (Moore *et al.*, 1991) using radiolabelled GFP probes prepared by random-priming purified restriction fragments (Feinberg & Vogelstein, 1983). The insert from pGFP (Clontech) was used to detect the GFP$_{wt}$ and GFP$_{TTG}$ mRNAs, and the insert from pYGFP3 was used to detect the yEGFP3 mRNA. However, both probes cross-reacted with the heterologous GFP genes.

Western analysis. Protein extracts (Hostetter *et al.*, 1990) were analysed by 10% (w/v) SDS-PAGE (Laemmli, 1970), electroblotted onto nitrocellulose membranes, and GFP polypeptides were detected using an anti-GFP polyclonal antibody (Clontech). The secondary antibody was alkaline-phosphatase-conjugated mouse anti-rabbit IgG (Sigma).

Microscopy and fluorescence-activated cell sorting (FACS) analysis. GFP expression was visualized in yeast cells by epifluorescence microscopy, with fluorescein isothiocyanate (485/510 nm, excitation/emission) and tetramethylrhodamine isothiocyanate (540/605 nm, excitation/emission) filters. For growth in serum, *C. albicans* transformed with the ADH–yEGFP3 construct was grown in YPD plus 10% (v/v) foetal calf serum overnight to saturation. For tissue preparation from infected mice, mice were killed at 24 h post-infection, and the kidneys were recovered. These were embedded in OCT (Tissue-Tek, Milles), frozen on dry ice, and cryosectioned in 5–10 μm sections. The sections were fixed in 4% (v/v) paraformaldehyde and mounted in Vectashield (Vector Laboratories) with 0·5 mg propidium iodide ml^{-1}.

A FACStar (Becton Dickinson) instrument equipped with an argon laser emitting at 488 nm was used for FACS analysis. Fluorescence emission was read with a 515/40 band pass filter.

Fluorescence and forward scatter data were collected with logarithmic amplifiers. The mean fluorescence intensities were calculated using the CellQuest software.

RESULTS

The CTG codon does not explain the lack of GFP expression in C. albicans

Our initial objective was to use the wt GFP gene as a reporter in C. albicans. However, when this gene was
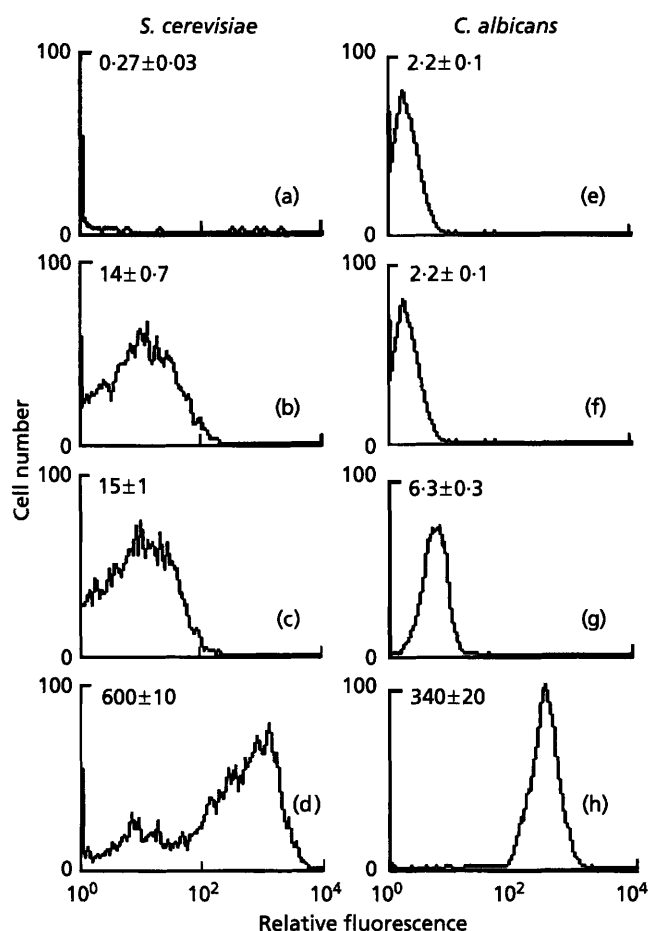
Fig. 2. FACS analysis of S. cerevisiae or C. albicans strains carrying various GFP constructs. Experiments were carried out on three independent transformants. The histograms shown are representative; the mean and standard deviations shown are calculated from the mean fluorescence for the three cultures. The vectors carried in each of the strains are as follows: (a, e) pYPB-ADHpt; (b, f) pYPB-ADHpt-GFP$_{wt}$; (c, g) pYPB-ADHpt-GFP$_{TTG}$; (d, h) pYPB-ADHpt-yEGFP. Each histogram represents a total of 2×10^4 events. The mean fluorescence of each culture is shown in arbitrary units. The S. cerevisiae baseline (a) was set lower than that for C. albicans to permit comparison of fluorescence in (a–c) with the extremely high fluorescence in (d). Thus direct comparisons of fluorescence should be made only within the S. cerevisiae and C. albicans groups. Since the histogram in (a) is offscale, a second set of measurements were taken for the cultures in (a) and (b), with the baseline set to allow accurate analysis of both histograms. The fluorescence value of 0·27 was calculated from the ratio of the values for cultures (a) and (b) in this set of measurements.

cloned into the expression vector pYPB-ADHpt and transformed into C. albicans, no fluorescence was observed, although the same construct yielded high levels of fluorescence in S. cerevisiae (Fig. 2b, f). The lack of expression in C. albicans might have been due to the translation of the single CTG codon in the GFP$_{wt}$ gene (Prasher et al., 1992) as serine rather than leucine (Santos & Tuite, 1995). Hence, we changed codon 201 to the preferred leucine codon, TTG (Brown et al., 1991; Lloyd & Sharp, 1992), and compared the expression of this GFP$_{TTG}$ gene in C. albicans and S. cerevisiae (Fig. 2c, g). High levels of fluorescence were observed in S. cerevisiae, but no fluorescence was seen in C. albicans. Therefore, the presence of a CTG codon in GFP$_{wt}$ is not sufficient to account for the lack of expression of this gene in C. albicans.

Construction of codon-optimized GFP

A codon-optimized GFP (see Methods and Fig. 1) was constructed in which all the codons are optimal for C. albicans. The codon usage for the starting and final genes is shown in Fig. 3. For the codon-optimized genes (yEGFP3), we were guided by a codon usage table derived from three highly expressed Candida genes: TEF3, ENO1 and HSP70 [assembled by S. Scherer (University of Minnesota School of Medicine, MN, USA) and available at http://alces.med.umn.edu/candida/codons.html]. In addition to the codon usage changes in the optimized GFP gene, two amino acid changes relative to the wt gene (S65G; S72A) were included. GFP with this double amino acid change fluoresces 75 times more brightly than wt GFP when expressed in E. coli and excited at 488 nm (Cormack et al., 1996). The mutations shift the absorbance maxima from 395 nm to 490 nm, and increase the solubility of the protein.

TTT Phe 8	8	TCT Ser 3	6	TAT Tyr 4	4	TGT Cys -	2
TTC Phe 5	5	TCC Ser 1	3	TAC Tyr 7	7	TGC Cys 2	-
TTA Leu 3	12	TCA Ser 2	-	TAA Stop 1	1	TGA Stop -	-
TTG Leu 1	7	TCG Ser 1	-	TAG Stop -	-	TGG Trp 1	1
CTT Leu 11	-	CCT Pro 2	-	CAT His 5	5	CGT Arg 1	-
CTC Leu -	-	CCC Pro 2	-	CAC His 5	5	CGC Arg -	-
CTA Leu 3	-	CCA Pro 6	10	CAA Gln 6	8	CGA Arg -	-
CTG Leu 1	-	CCG Pro -	-	CAG Gln 2	-	CGG Arg -	-
ATT Ile 8	9	ACT Thr 7	10	AAT Asn 5	5	AGT Ser 3	-
ATC Ile 3	3	ACC Thr 2	5	AAC Asn 8	8	AGC Ser 1	-
ATA Ile 1	-	ACA Thr 6	-	AAA Lys 15	15	AGA Arg 5	6
ATG Met 6	6	ACG Thr -	-	AAG Lys 5	5	AGG Arg -	-
GTT Val 8	11	GCT Ala 3	7	GAT Asp 12	12	GGT Gly 7	23
GTC Val 6	6	GCC Ala 2	2	GAC Asp 6	6	GGC Gly 3	-
GTA Val 3	-	GCA Ala 3	-	GAA Glu 13	16	GGA Gly 9	-
GTG Val -	-	GCG Ala -	-	GAG Glu 3	-	GGG Gly 3	-

Fig. 3. Codon usage tables for GFP. The first column in each set of two shows codon usage for the wt GFP gene from A. victoria. Codon usage for the optimized yEGFP is given in the second column. Note that in yEGFP there are two fewer serines and additional alanine and glycine residues relative to the original GFP sequence. This reflects the mutations in the chromophore that result in increased fluorescence.
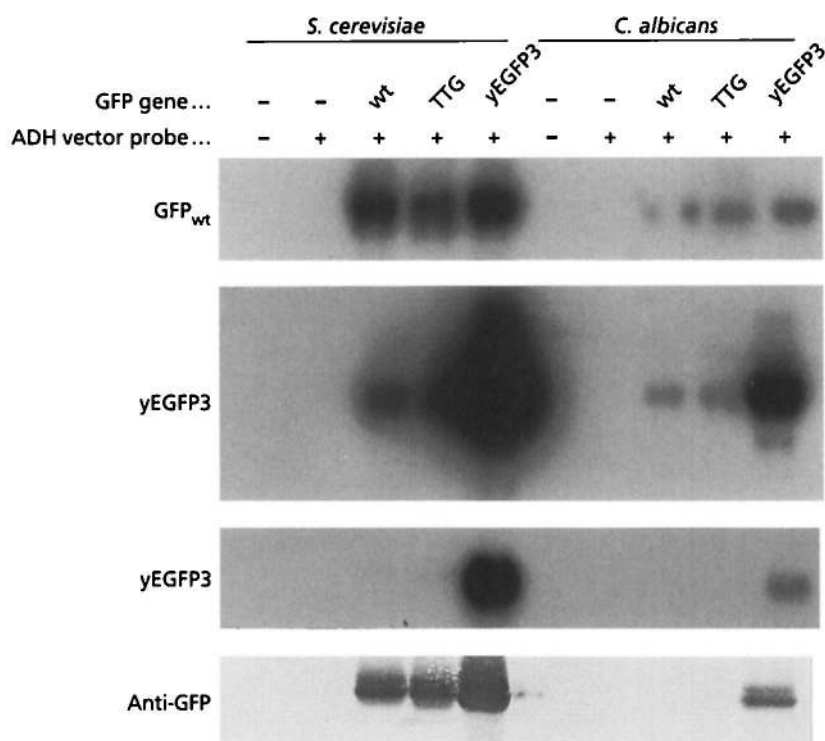
Fig. 4. GFP mRNA and protein levels in C. albicans and S. cerevisiae. RNA and protein extracts were prepared from exponential cultures of C. albicans and S. cerevisiae carrying the GFP$_{wt}$, GFP$_{TTG}$ or yEGFP3 genes (shown at top of figure). Control cultures of untransformed cells, or cells transformed with the parent ADH1 expression vector were also analysed. Northern analysis was performed on approximately equal amounts of RNA and the filters were probed with the GFP$_{wt}$ and yEGFP3 genes (shown at the side of figure). Two exposures of the same yEGFP3-probed filter are shown because of the high abundance of the yEGFP3 mRNA in S. cerevisiae. Western analysis was performed on approximately equal amounts of protein using an anti-GFP polyclonal antibody. The GFP band was calculated to be 24 kDa, in agreement with the prediction.

The synthetic GFP gene generates fluorescence in S. cerevisiae and C. albicans

The expression of the various GFP genes in S. cerevisiae and C. albicans was analysed by Northern and Western blotting (Fig. 4). In S. cerevisiae, no GFP transcript or protein was observed in untransformed cells or in cells transformed with the parent expression vector, whereas GFP transcript and protein was observed in cells transformed with vector containing each of the GFP genes. Not surprisingly, all S. cerevisiae transformants expressing GFP were fluorescent when illuminated at 488 nm (Fig. 2b–d). In C. albicans, Northern analysis revealed the presence of GFP mRNA in cells transformed with all ADH–GFP fusions (Fig. 4). However, Western analysis revealed GFP protein only in C. albicans strains containing the codon-optimized GFP gene; no protein could be detected in the negative controls, in cells transformed with the GFP$_{wt}$, or even in cells transformed with the GFP$_{TTG}$ gene (Fig. 4). These cells were also non-fluorescent (Fig. 2e–g). In contrast, C. albicans cells expressing yEGFP3 were highly fluorescent (Figs 2h and 6a, b).

Comparing expression of GFP in S. cerevisiae and C. albicans, it was noticed that the levels of GFP mRNA were lower in C. albicans than in S. cerevisiae, consistent with a high copy number in S. cerevisiae due to the presence of 2μ sequences on the vector. Similar observations were made when pYPB-ADHpt was used to express the C. albicans sequences HST7 and HYR1 in S. cerevisiae and C. albicans (Bailey et al., 1996; Leberer et al., 1996). Interestingly, both in S. cerevisiae and C. albicans, the levels of the codon-optimized GFP mRNA were significantly higher than that for the GFP$_{wt}$ and GFP$_{TTG}$ mRNAs. In both organisms, this correlated with elevated protein levels, and with elevated fluorescence.

Inducible expression of yEGFP3

To test if yEGFP3 could be used to measure promoter strength, it was placed under the control of the maltase promoter on the high-copy-number vector pRC2312MAL (see Methods). Two transformants were grown in synthetic medium containing glucose or synthetic medium containing maltose overnight to saturation. The fluorescence of the cultures was assessed by FACS analysis (Fig. 5a). While the cultures grown in glucose were virtually non-fluorescent, the cultures grown in maltose were 20 times more fluorescent. Different transformants of the pRC2312MAL–GFP did not behave identically. All transformants were more fluorescent in maltose than in glucose, but in some transformants, a substantial percentage of the cells remained non-fluorescent after induction (data not shown). It is not known if this was a result of plasmid loss, failure to induce the MAL2 promoter, or a failure of GFP to fluoresce in all cells. The latter explanation is less likely, since all cells fluoresce with virtually identical intensity when GFP is expressed from the constitutive ADH1 promoter (Fig. 2h).

yEGFP is highly fluorescent in S. cerevisiae

The mean fluorescence for S. cerevisiae cells transformed with the ADH1–yEGFP3 construct was approximately 2×10^3 times greater than for the untransformed parental strain (Fig. 2d), and 40 times greater than for
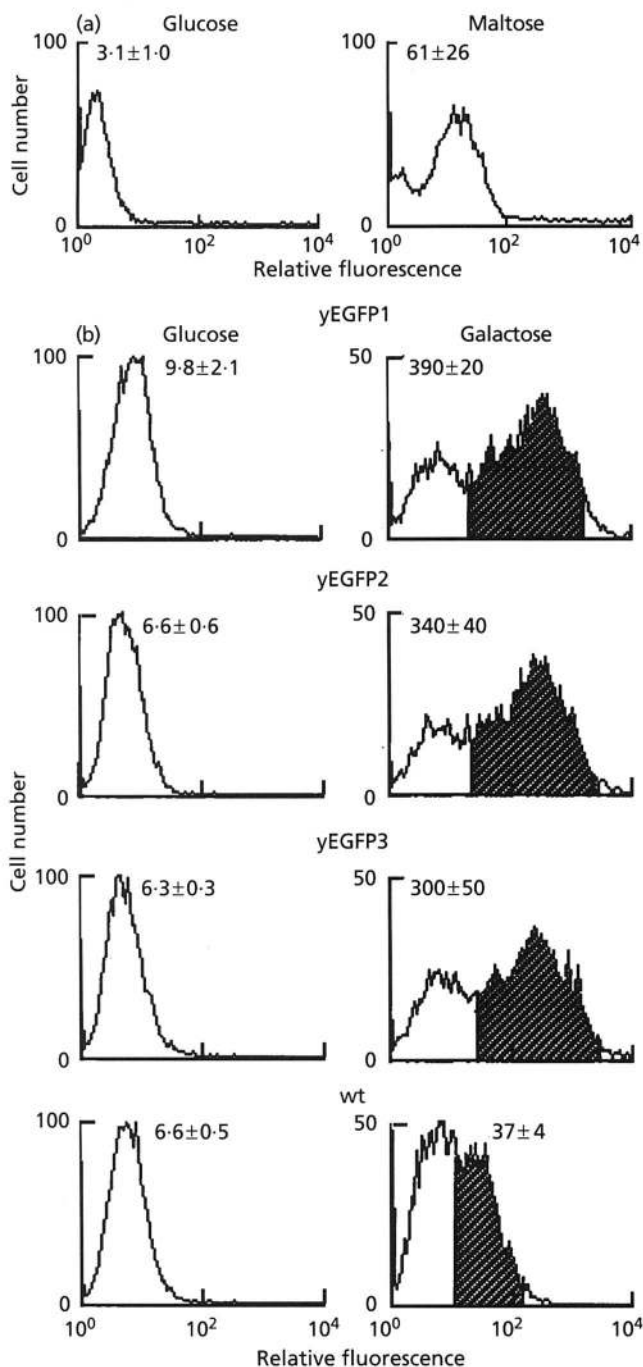
Fig. 5. (a) FACS analysis of *C. albicans* strains carrying the *MAL2*–GFP construct. The histograms shown are representative; the means and standard deviations shown are calculated from the mean fluorescence for three independent cultures. Transformants were grown overnight in synthetic media without uridine, and supplemented with either glucose or maltose (2%, w/v). Each histogram represents a total of 2×10^4 events. The geometric mean fluorescence of each culture is shown in arbitrary units. (b) FACS analysis of *S. cerevisiae* strains transformed with pTS210 carrying either GFP$_{wt}$, yEGFP1, yEGFP2 or yEGFP3. Transformants were grown overnight in synthetic media without uracil, and supplemented with either glucose or galactose (2%, w/v). After growth in galactose, the populations of yeast cells were largely bimodal for fluorescence. For these populations, the mean fluorescence indicated in the figure was

strains transformed with a plasmid carrying GFP$_{wt}$ expressed from the *ADH1* promoter. This demonstrates that the optimized yEGFP3 gene gives higher signals than GFP$_{wt}$ in *S. cerevisiae*. Codon-optimized versions of two other highly fluorescent mutants of GFP, mut1 and mut2 (Cormack *et al.*, 1996), were also tested. When expressed in *S. cerevisiae* from a *GAL1,10* promoter, all three of the GFP mutants gave comparable results, paralleling their comparable fluorescence in *E. coli*. With this promoter, strains expressing the codon-optimized, fluorescence-enhanced GFPs were approximately 10 times more fluorescent than GFP$_{wt}$ expressed from the same promoter (Fig. 5).

In vivo expression of yEGFP3

Lastly, because GFP fluoresces in the absence of any added cofactors, theoretically it can be used as a marker inside live infected animals. We therefore tested whether *C. albicans* expressing yEGFP3 could be detected in tissue from infected animals. BALB/c mice were injected intravenously with 1×10^6 CAI4 cells transformed with the ADH1–yEGFP3 construct. After 24 h, the mice were killed, and sections of the infected kidneys were examined. Numerous fluorescing *C. albicans* cells were visible in all sections examined. Fig. 6(c, d) shows a strongly fluorescent hyphal mass in the kidney of an infected mouse. Thus, *C. albicans* cells expressing yEGFP3 can be detected easily in infected tissue.

DISCUSSION

It is shown here that a codon-optimized and fluorescence-enhanced version of GFP can be used as an efficient reporter in *S. cerevisiae* and *C. albicans*. This optimized GFP is one of only a few reporters available for work in *C. albicans*. Development of such reporters has been complicated by the difficulty in expressing foreign genes in *C. albicans*, a failure that is generally assumed to result from the aberrant translation of CTG codons in this organism. However, when *C. albicans* was transformed with a GFP construct in which the CTG codon was changed to the preferred leucine codon TTG, GFP protein or fluorescence could not be detected, although GFP mRNA was present in these cells. This could be explained by the presence of a second problematic codon in *C. albicans*. Almost certainly, such a codon would be infrequently used, since the codon-optimized GFP is functional. Theoretically, this problematic codon could either be mistranslated, as in the case of the CTG codon, or alternatively it could be translated correctly but at a very low efficiency.

yEGFP3 is much more fluorescent than GFP$_{wt}$ in both *S. cerevisiae* and *C. albicans*. Part of this increase in

calculated using only the fluorescent population, indicated by the hatching. The mean fluorescence values calculated using the entire population of cells were 23 ± 7, 280 ± 4, 270 ± 55 and 212 ± 43 for wt and yEGFP1, 2 and 3, respectively.
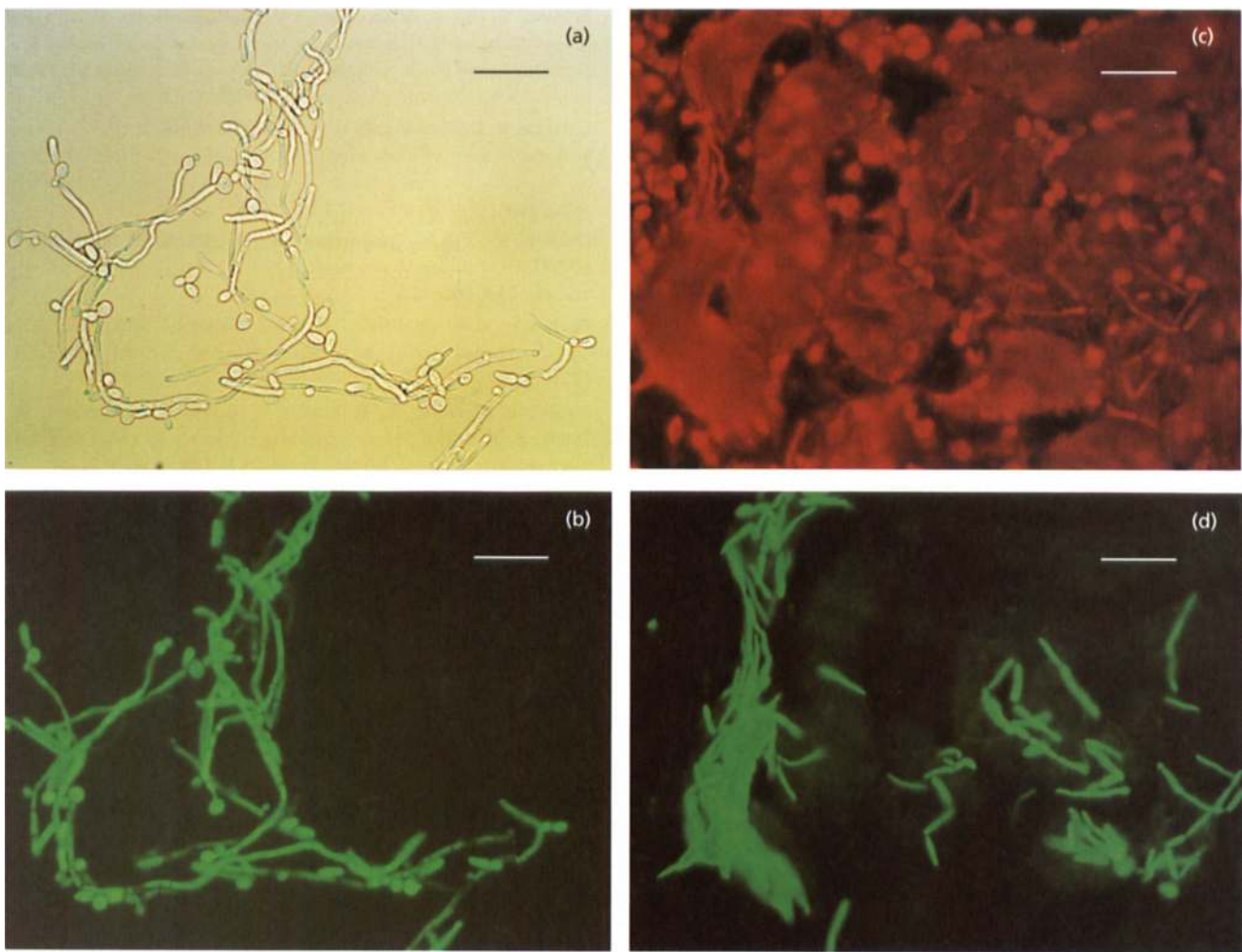
Fig. 6. (a) *C. albicans* expressing yEGFP3 visualized after growth overnight in YPD plus 10% foetal calf serum (Gibco-BRL). (b) The same field viewed by epifluorescence using 485/510 nm excitation/emission filters. (c) *C. albicans* expressing yEGFP3 visualized in murine kidney 24 h post-infection. A 10 μm section of frozen kidney tissue stained with propidium iodide and viewed by epifluorescence using 540/605 nm excitation/emission filters is shown. (d) The same field viewed by epifluorescence using 485/510 nm excitation/emission filters. Bars, 10 μm.

fluorescence is almost certainly due to the increased translational efficiency of the codon-optimized gene. It seems likely, however, that the relatively high yEGFP3 mRNA levels also contribute to the elevated fluorescence. These elevated mRNA levels in *S. cerevisiae* and *C. albicans* may have been due to possible increases in the copy number of the ADH1–yEGFP3 plasmid. Alternatively, the codon optimization might have influenced the stability of the GFP mRNA either through direct effects upon mRNA stability determinants, or indirectly via an interplay between mRNA translation and degradation (Beelman & Parker, 1995; Ross, 1995).

Apart from the GFP molecules described here, recently developed reporter systems exploit the β-galactosidase of *K. lactis*, and the luciferase of *R. reniformis*. GFP, β-galactosidase and luciferase all have certain advantages as reporters of transcription. The β-galactosidase and luciferase seem to be sensitive over a larger range than yEGFP3, and they are possibly more easy to quantify

than GFP. Even expressing yEGFP3 from the fairly strong *ADH1* promoter, the fluorescence in *C. albicans* was not overwhelming, being 150-fold above background. Hence, it seems likely that in general, β-galactosidase and luciferase will be more useful than yEGFP3 as reporters of transcription *in vitro*.

On the other hand, yEGFP3 has other important advantages. First, unlike β-galactosidase or luciferase, yEGFP3 can be monitored at the level of a single cell. Secondly, since GFP requires no cofactors, yEGFP3 can be used as a marker in applications where it is advantageous to maintain cell viability. Hence FACS sorting could be used to separate yeast cells on the basis of differential GFP expression levels, thereby permitting new types of genetic screens in *C. albicans*. In addition, as shown here, yEGFP3 can be used as a reporter in animal tissue, potentially allowing the dissection of transcriptional programmes that respond to the complex microenvironments of the host. Lastly, as a marker for

protein localization, yEGFP3 is a uniquely powerful tool. As has been demonstrated in many other systems, fusions of GFP to endogenous proteins can be used to visualize the normal subcellular localization of those proteins. Therefore, yEGFP3 should be useful in the analysis of protein localization during growth and development in *C. albicans*, both *in vivo* and *in vitro*.

There remain some unresolved issues with GFP expression in *C. albicans*. It is possible that yEGFP1, which is approximately 30 % more fluorescent than yEGFP3 in *S. cerevisiae* (Fig. 5), will also be more fluorescent in *C. albicans*. We have no estimate for the time lag between yEGFP3 protein production and fluorescence in *C. albicans*, a parameter that will be important for some studies of gene regulation. Furthermore, it is well known that the formation of the GFP chromophore requires oxygen and that the redox state of the cell can influence fluorescence (Heim *et al.*, 1994). Hence it will be interesting to see whether yEGFP3 shows differential fluorescence in *C. albicans* under different growth conditions, or in different growth phases. These concerns notwithstanding, yEGFP3 should prove a powerful tool in the study of *C. albicans* cell biology and pathogenicity.

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