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The Sea Pansy *Renilla reniformis* Luciferase Serves as a Sensitive Bioluminescent Reporter for Differential Gene Expression in *Candida albicans*

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The infectious yeast *Candida albicans* progresses through two developmental programs which involve differential gene expression, the bud-hypha transition and high-frequency phenotypic switching. To understand how differentially expressed genes are regulated in this organism, the promoters of phase-specific genes must be functionally characterized, and a bioluminescent reporter system would facilitate such characterization. However, *C. albicans* has adopted a nontraditional codon strategy that involves a tRNA with a CAG anticodon to decode the codon CUG as serine rather than leucine. Since the luciferase gene of the sea pansy *Renilla reniformis* contains no CUGs, we have used it to develop a highly sensitive bioluminescent reporter system for *C. albicans*. When fused to the galactose-inducible promoter of *GAL1*, luciferase activity is inducible; when fused to the constitutive *EF1α2* promoter, luciferase activity is constitutive; and when fused to the promoter of the white-phase-specific gene *WH11* or the opaque-phase-specific gene *OP4*, luciferase activity is phase specific. The *Renilla* luciferase system can, therefore, be used as a bioluminescent reporter to analyze the strength and developmental regulation of *C. albicans* promoters.

Reporter genes which code for bioluminescent gene products, like the luciferases, have provided a very rapid method for analyzing the regulation of gene expression (4) and a highly sensitive method for single-cell analysis (38). Recently, we used the firefly luciferase gene (*FLUC*) fused in frame with the phase-regulated *WH11* gene of *Candida albicans* as a reporter to functionally characterize the 5′ upstream regulatory region of *WH11* (29), but the analyses were restricted to Northern (RNA) blots because we were unable to identify a translation product of the firefly luciferase, either through enzyme activity or as a FLUC-related peptide in Western blots (immunoblots; unpublished observations). The lack of a detectable translation product was most likely due to a nontraditional codon strategy adopted by *C. albicans* and related species (19, 22, 23). These organisms use a tRNA with a CAG anticodon to decode the codon CUG as serine, while most organisms use CAG to decode the codon CUG as leucine. Recently, it was demonstrated that the traditional leucine isoacceptor tRNA for CUG from *Saccharomyces cerevisiae* is toxic to *C. albicans* (13). Furthermore, direct determination of the amino acid sequences of peptides derived from three aspartyl proteinases of *C. albicans* confirmed the presence of serine instead of leucine at nucleotide positions containing the CUG codon (38). *FLUC* contains nine in-frame CUG codons, making it highly unlikely that a functional luciferase could be expressed in *C. albicans*. In order to circumvent this codon problem, we have developed a reporter system for *C. albicans* using the luciferase gene *RLUC* of the sea pansy *Renilla reniformis*, which contains no CUG codons in its open reading frame (ORF). We have fused the *Renilla* luciferase gene to a number of promoters of *C. albicans* and demonstrate here that it is expressed as a functional protein in *C. albicans* and can be used to analyze the strength and developmental regulation of *C. albicans* promoters. We also demonstrate that the *Renilla* luciferase assay can be further modified to measure gene expression in intact cells. The development of this reporter system will facilitate the analysis of gene regulation in the two developmental programs of *C. albicans*, the bud-hypha transition (26) and high-frequency phenotypic switching (27), as well as in pathogenesis.

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MATERIALS AND METHODS

Strain maintenance and transformation. Strain Red 3/6, an *ade2* derivative of strain WO-1, was used in all transformations. Red 3/6 switches between the white and the opaque phases at frequencies comparable to those observed in the parental strain (30). Red 3/6 was maintained on supplemented Lee's medium (1) containing 3 mM adenine sulfate. To obtain highly enriched (>99%) populations of white or opaque cells, Red 3/6 cells from a stock culture were plated on agar and cells from a homogeneous white and a homogeneous opaque colony were collected. Spheroplasts of white or opaque cells were generated according to methods previously described (29, 30).

Construction of the *Renilla* luciferase reporter plasmid cPRW3 and promoter derivatives. A 950-bp *Pst*I-*Sma*I fragment of the *Renilla* luciferase gene (referred to subsequently as *RLUC*) ORF from pRLUC4.1 (14) was subcloned in pCaAHI (30) between the *Pst*I and *Pvu*II sites to generate pCHRL.2. The fragment contained an ATG codon immediately downstream from the *Pst*I site. pCRW3 was derived by inserting a 200-bp PCR product of the *WH11* transcription termination sequence at the 3′ end of the *RLUC* ORF. The PCR product was generated with two oligonucleotide primers, XSA (Fig. 1), which is homologous to bp +189 to +223 of *WH11* and contains a *Xho*I, *Stu*I, and *Apa*I site, and XN (Fig. 1), which is homologous to bp +481 to +505 of *WH11* and contains a *Nco*I site, and the plasmid pWhg.4, containing the *WH11* gene (29, 30), as the template. The PCR product was gel purified and digested with restriction enzymes *Xho*I and *Nco*I. The resulting 200-bp fragment was ligated between the *Xho*I and *Nco*I sites of pCHRL.2. The subcloning of this PCR fragment was accomplished by deleting a 0.65-kb fragment of pCHRL.2 spanning the T7 promoter and the 3′ half of the *CaARS*, which contained the distal autonomously replicating sequence core element (5). The resulting plasmid pCRW3 (Fig. 2) survived autonomously in *C. albicans* even though it contained only one of two autonomously replicating sequence core elements.

pCRW3 was used to derive test plasmids which contained the 5′ upstream regions of the *C. albicans* gene *WH11* (29), *EF1α2* (33), *GAL1* (9), and *OP4* (18) inserted between the *Sma*I and *Pst*I sites of pCRW3 (Fig. 2). To derive pCRW5,

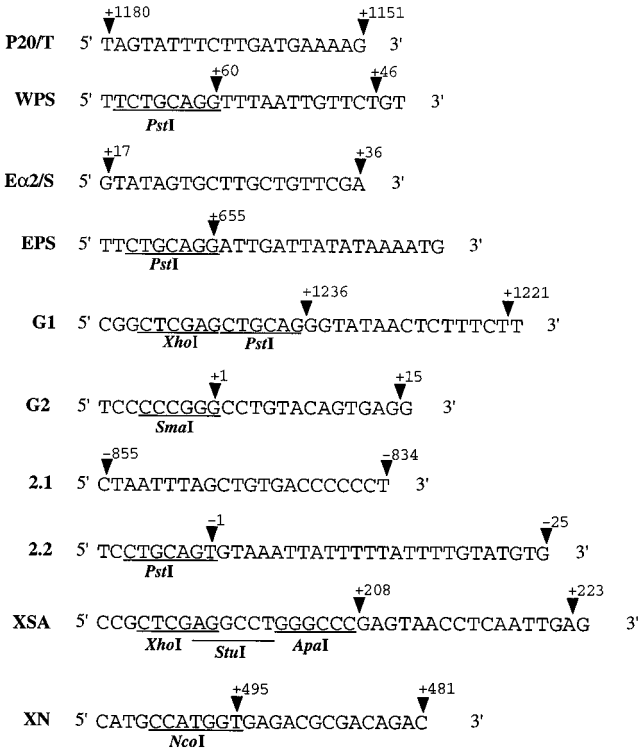

FIG. 1. Oligonucleotides used to generate various constructs in this study. Appropriate restriction enzymes sites (underlined) were included at the 5′ end of most of these oligonucleotides for the purpose of directional cloning of PCR products. Oligonucleotides P20/T, WPS, XSA, and XN are from reference 30; oligonucleotides Eα2/S and EPS are from reference 33; oligonucleotides G1 and G2 are from reference 8a; and oligonucleotides 2.1 and 2.2 are from reference 26.

which contains the *WH11* promoter (Fig. 2), a PCR product of the 1,200-bp 5′ upstream region of *WH11* was generated with the oligonucleotide primers P20/T and WPS (Fig. 1) and pWHg.4 was used as the template. The PCR product was gel purified, cut with *Ssp*I and *Pst*I, and fractionated in Tris-borate-EDTA–agarose, and a 0.80-kb *Ssp*I-*Pst*I fragment was ligated between the *Sma*I and *Pst*I sites of pCRW3.

To derive pCRE3, which contains the *EF1α2* promoter (Fig. 2), a PCR product was generated with the oligonucleotide primers Eα2/S and EPS (Fig. 1), with mp *EF1α2* as the template (33). The PCR product was gel purified, cut with *Ssp*I, end repaired with T4 DNA polymerase, and fractionated in Tris-borate-EDTA–agarose, and a 0.55-kb *Ssp*I-*Pst*I fragment was ligated at the *Sma*I site of pCRW3.

To derive pCRG1, which contains the *GAL1* promoter (Fig. 2), a PCR product was generated with the oligonucleotide primers G1 and G2 (Fig. 1), designed from the *GAL1* sequence (8a), with total DNA of *C. albicans* WO-1 as the template. The PCR product was gel purified, cut with *Sma*I and *Pst*I, and ligated between the *Sma*I and *Pst*I sites of pCRW3.

To derive pCROP31, which contains the *OP4* promoter (Fig. 2), a PCR product of the 0.85-kb 5′ upstream region of OP4 was generated with the oligonucleotide primers 2.1 and 2.2 (Fig. 1) and with λ *OP4* as the template. The PCR product was gel purified, end repaired with T4 DNA polymerase, and ligated at the *Pst*I site of pCRW3 following end repair with T4 DNA polymerase.

For integrative transformation, 20 μg of a particular plasmid was linearized at the *ADE2* gene with *Nsi*I or at the *OP4* gene with *Sac*I. The details for integrative transformation have been described in earlier publications (29, 30).

Southern blot analysis. Total cellular DNA was extracted from putative transformant clones grown for 4 days on agar plates containing supplemented Lee's medium. Approximately 4 μg of DNA from each clone was digested with *Bam*HI and resolved by electrophoresis in a 0.8% agarose gel containing 1× Tris-borate-EDTA. DNA was transferred to a Hybond N^+ nylon membrane (Amersham, Arlington Heights, Ill.), hybridized with a 0.95-kb *Renilla* luciferase cDNA probe or the *ADE2* probe, and washed according to methods described previously (6). Blots were then autoradiographed following exposure at −70°C with an intensifying screen.

To demonstrate that integration was site specific, total cellular DNA of untransformed cells and cells transformed with plasmid linearized at the *ADE2* gene was digested with *Bam*HI and the Southern blots were probed either with the *RLUC* cDNA or the *ADE2* gene. A diagram of the expected configuration of site-specific integration at the *ade2* locus is shown in Fig. 3. The expected *Bam*HI fragments are labeled A through F. The transformed clones analyzed in this manner included pCRW5 (1-1 and 1-2), pCRE3 (2-1 and 2-3), pCRG1 (3-1 and 3-2), pCRW3 (5-1), and pCROP31 (2-5 and 2-7). Untransformed Red 3/6 DNA contained no sequences homologous to *RLUC* cDNA (Fig. 4a). However, the DNA from transformants contained one common band of 4.6 kb which represented the *Bam*HI E fragment and bands of various sizes which represented the predicted *Bam*HI B, C, or D fragment (Fig. 3). Fragment B in pCRW5 was smaller than fragment B in pCRG1 because of differences in the sizes of the promoters and are distinguished as B2 and B1, respectively.

The Southern blot was stripped and rehybridized with the *ADE2* probe (Fig. 4b). While the untransformed Red 3/6 DNA contained only the expected A and F bands with molecular sizes of 20 and 11 kb, respectively, the digested DNA of all transformants contained in addition to the A and F bands the expected 4.6-kb E band and the variable B, H, and G bands (Fig. 4b), consistent with the fragment sizes predicted from the model for site-specific integration (Fig. 3). Again, the B bands differed between pCRW5 and pCRG1 and are distinguished as B2 and B1, respectively. We also analyzed two transformant clones, 3-3 and 3-7, in which pCROP31 integration was targeted to the *Op4* locus by linearizing the plasmid at the *OP4* promoter sequence prior to transformation. In both cases, only the 4.6-kb E fragment hybridized with both the *RLUC* and *ADE2* probes, the expected result for integration at a locus other than *ADE2* (data not shown). These results support previous observations that the integration of linearized plasmids is site specific (8, 9, 12, 30).

In vitro assay of *RLUC* activity. Transformants were plated on Lee's modified agar medium (1), and white and opaque colonies were isolated prior to an analysis of luciferase activity. Cells were then grown to mid-log phase in supplemented Lee's medium (1). Cells (2×10^8) were washed two times with sterile water and once with *RLUC* buffer (0.5 M NaCl, 0.1 M K_2HPO_4 [pH 6.7], 1 mM Na_2 EDTA, 0.6 mM sodium azide, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.02% bovine serum albumin) (16). The final cell pellet was resuspended in 200 μl of buffer, 2 volumes of glass beads (0.45-mm diameter) were added, and the cells were disrupted with a Bead Beater (Biospec Products, Bartlesville, Okla.) through four cycles of 20-s duration at 4°C. Extracts were clarified by centrifugation for 15 min at 13,000 × *g* in a microcentrifuge at 4°C. To 100 μl of *RLUC* buffer containing 0.5 μM coelentrazine (Molecular Probes, Inc., Eugene, Oreg.) 1 to 2 μl of diluted cell extract was mixed in 4-ml tubes for light measurements. The *RLUC* buffer used in the assay mixture was flushed with nitrogen gas to prevent auto-oxidation of the coelentrazine. The stock solution of coelentrazine was stored in acid-methanol at −20°C. Immediately after mixing, light emission was measured at 480 nm in the integration mode for 10 or 30 s with a Monolight 2001 luminometer (Analytical Luminescence, San Diego, Calif.). The transformant cell-free protein extracts were usually diluted between 1/1,000 and 1/10,000 to obtain luminescence measurements in the assayable range. Activity is presented as relative luminescence per 10 s per μg of protein. This represents arbitrary units not normalized to a standard for light emission. Protein was measured by the Bradford assay (Bio-Rad Laboratories, Hercules, Calif.).

In vivo assay of *RLUC* activity. Cells of transformant clones were grown in 25 ml of supplemented Lee's medium to a density of 4×10^6 to 10^7 cells per ml. Cells were washed twice in distilled water and resuspended in *RLUC* buffer at a concentration of 10^8 cells per ml. For each assay, 50 μl of cell suspension, containing 10^7 cells, was mixed with 50 μl of a 10 μM solution of coelentrazine. Luminescence was immediately recorded for 30 s in an integration mode. Again, activity is presented as relative luminescence per 10 s per 10^7 cells. In mixing experiments, 25 μl of control cells in *RLUC* buffer derived from transformant clones harboring pCRW3 at the *ADE2* locus were mixed with 25 μl of cells in *RLUC* buffer containing the appropriate *Renilla* promoter construct. To this cell mixture 50 μl of a 10 μM solution of coelentrazine was added, and luminescence was measured.

For microtiter plate assays, cells were diluted in *RLUC* buffer to a final density of 2×10^8 to 2×10^2 cells per ml. Fifty microliters of each diluted cell suspension was added to individual wells of a Dynatech Microlite 2 microtiter plate (Dynatech Laboratories, Chantilly, Va.). With a multipipetter, 50 μl of a 1 μM solution of coelentrazine was mixed with each cell suspension. Ortho-G X-ray film (Eastman Kodak Co., Rochester, N.Y.) was then firmly placed at the bottom of the microtiter plate, and the plate was covered with aluminum foil. The film was exposed for 6 h at 25°C.

RESULTS

***Renilla* luciferase (*RLUC*) activity measured by an in vitro assay: constitutive expression when under *EF1α2* promoter regulation.** To test whether the *RLUC* gene product can be measured in an in vitro bioluminescence assay, we first compared the in vitro activities of extracts from cells transformed with the pCRW3 plasmid, which contains a promoterless *RLUC* gene, and cells transformed with pCRE3, which contains *RLUC* fused to the constitutive *EF1α2* promoter (Fig. 2).

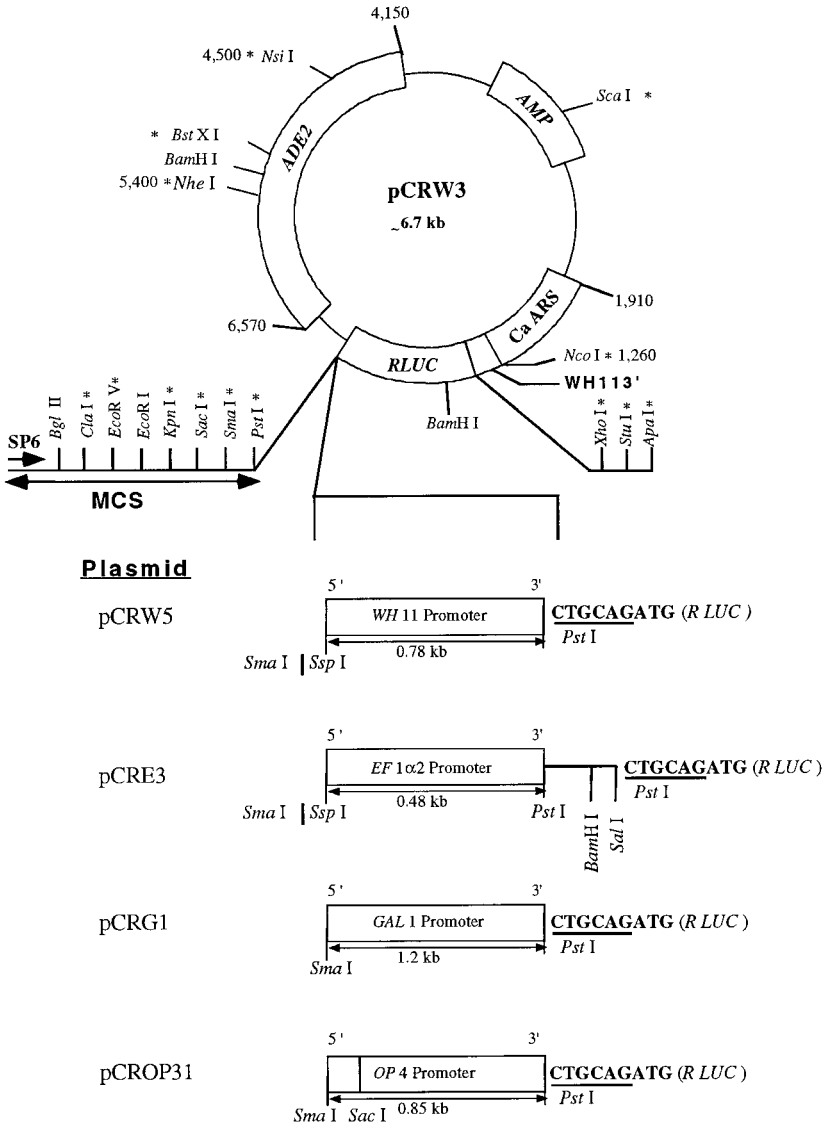

FIG. 2. Construction of the pCRW3 plasmid, which contains the *RLUC* gene preceded by a multiple cloning site (MCS), and derivative plasmids containing the *WH11* promoter, the *EF1α2* promoter, the *GAL1* promoter or the *OP4* promoter upstream of *RLUC*. Unique restriction sites are marked with an asterisk. *ADE2*, the *C. albicans ADE2* gene; *AMP*, the ampicillin-resistant gene; *RLUC*, the *Renilla* luciferase gene ORF; WH113′, the 3′ downstream transcription termination sequence of gene *WH11*; Ca ARS, *C. albicans* autonomous replication site; SP6, transcription start site; MCS, multiple cloning site.

In both cases, the plasmids were linearized by cutting at an *Nsi*I site in the *ADE2* gene in order to direct integration to an *ade2* locus of strain Red 3/6 (30). The activity of the pCRW3 transformants was the same as that of the background (Table 1), suggesting that *C. albicans* does not contain any endogenous luciferase-like activity. In contrast, the level of luciferase activity of the pCRE3 transformants in both opaque and white cell-free protein extracts was 10^5-fold greater than that of the background (Table 1). These results demonstrate that the level of luciferase activity of cells containing the *RLUC* gene is roughly 5 orders of magnitude higher than that of the background and that *RLUC* expression is regulated constitutively in both white and opaque cells by the *EF1α2* promoter.

Luciferase activity is inducible when under *GAL1* promoter regulation. Inducible promoters serve as powerful tools to study the feasibility of reporter genes for the analysis of induction and repression kinetics as well as the mechanisms regulating gene activity. In order to test whether *RLUC* can be used as a reporter for an inducible promoter, the promoter of the *C. albicans GAL1* gene was inserted upstream of the *RLUC* ORF in pCRG1 (Fig. 2). Three independent clones transformed with pCRG1 were then grown in defined medium containing either 2% glucose or 2% galactose for three to four generations to mid-log phase and analyzed for *RLUC* activity by the in vitro assay. The level of luciferase activity in galactose was between 9- and 10-fold greater than that in glucose (Table 2). The level of luciferase activity was approximately 1.5×10^5-fold higher than that of the background (Table 2). These results demonstrate that *RLUC* can be used as a reporter for the analysis of induction of *GAL1* by galactose in particular and suggest that it can be used as a reporter for other inducible genes.

Luciferase activity is white phase specific when under the regulation of the promoter of *WH11*. Strain Red 3/6, used in this study, switched spontaneously, reversibly, and at high fre-

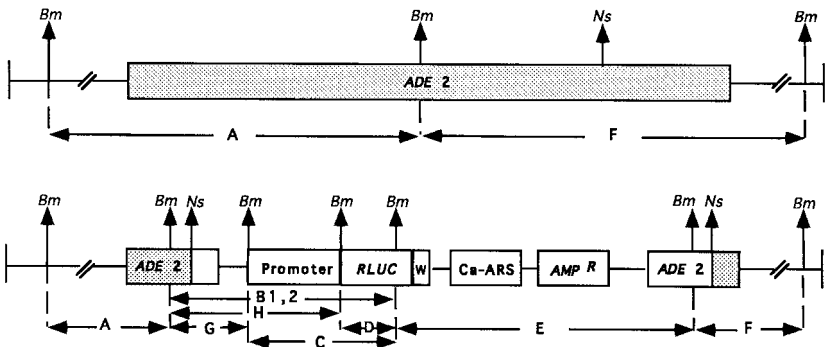

FIG. 3. A model of site-specific integration of pCRW3 derivatives at an *ADE2* locus of *C. albicans*. The upper diagram represents the native *ADE2* locus and the lower diagram the integrated locus. The plasmid was linearized with *Nsi*I at the *ADE2* gene prior to integration. A through F represent expected *Bam*HI fragments. Bm, *Bam*HI sites; Ns, *Nsi*I sites. Details of the integrated plasmid are described in the legend to Fig. 2.

quencies between a white and an opaque CFU in the white-opaque transition (25, 29, 30). In the white budding phase, cells differentially express the white-phase-specific gene *WH11* (30), which encodes a protein with homology to the glucose-lipid-regulated protein 1 (GLP1) of *S. cerevisiae* (32). To test whether *RLUC* is expressed in a white-phase-specific manner, the *WH11* promoter (29) was inserted upstream of the *RLUC* ORF in pCRW5 (Fig. 2) and the plasmid was linearized by cutting with *Nsi*I for targeted integration into the *ADE2* locus. Four independent clones transformed with pCRW5 were each independently grown in liquid medium and plated at low densities on agar, and cells from a white and an opaque colony of each clone were grown to mid-log phase in liquid growth medium. The cell-free protein extract of each clone was then analyzed for luciferase activity in the white and opaque phases. The luciferase activity in the white phase ranged between 124 and 180 times that in the opaque phase (Table 3), demonstrating that luciferase activity was under the stringent developmental regulation of the white-phase-specific *WH11* promoter. Although the level of luciferase activity in opaque cells was on average 150-fold less than that in white cells (Table 3), it was at least 45 times higher than that of the background (Table 1). The low but significant level of *RLUC* activity in pCRW5 transformants was proportional to the level of white cells present in the opaque cell populations as a result of spontaneous switching (2, 21, 25). As an internal control for differential expression, white and opaque cells of four independent clones transformed with pCRE3, which contains the constitutive *EF1α2* promoter upstream of *RLUC*, were analyzed for *RLUC* activity. There was no significant difference in the levels of luciferase activity between the two cell types (Tables 1 and 2), demonstrating that the differential expression in pCRW5-transformed cells is not due to the differential stability of the *Renilla* luciferase in white- and opaque-phase cells.

Luciferase activity is opaque phase specific when under the regulation of the promoter of *OP4*. In the opaque phase of the white-opaque transition of strain WO-1, cells differentially transcribe the opaque-phase-specific gene *OP4* (18). To test whether *RLUC* is expressed in an opaque-phase-specific manner when fused to an opaque-phase-specific gene promoter, the *OP4* promoter (28) was inserted upstream of the *RLUC* ORF in pCROP31 (Fig. 2) and the plasmid was linearized by cutting with *Nsi*I for targeted integration into the *ADE2* locus. Five independent clones transformed with pCROP31 were each grown in liquid medium and plated at low densities on agar, and cells from a white and an opaque colony were grown to mid-log phase in liquid growth medium. The cell-free protein extract of each clone was then analyzed for luciferase

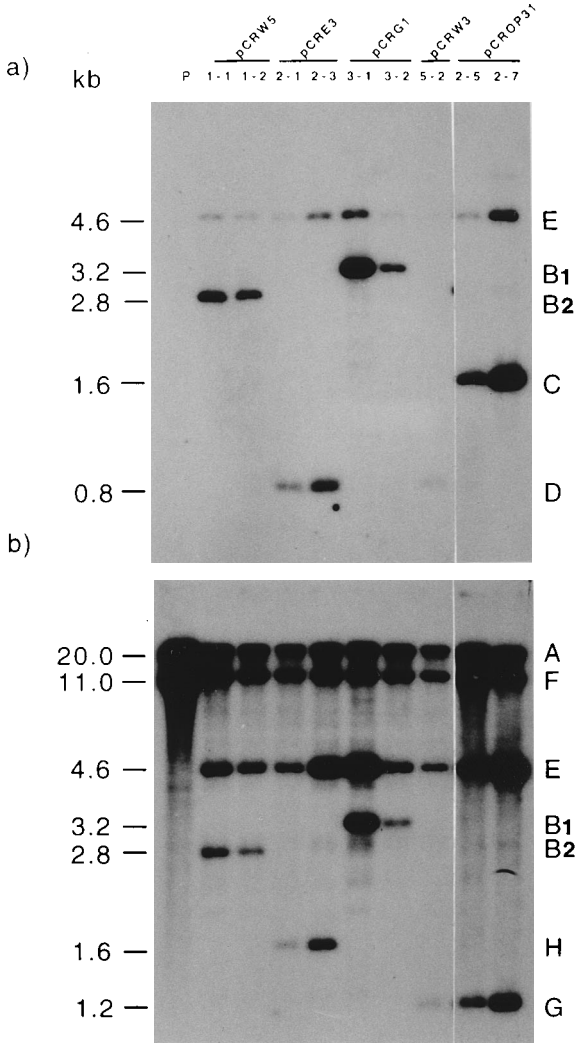

FIG. 4. Southern blot hybridization analysis of *C. albicans* transformants with plasmids pCRW5, pCRE3, pCRG1, pCRW3, and pCROP31. The DNA of the parental strain Red 3/6 (P) and one or two independent transformants with each noted plasmid was digested with *Bam*HI. (a) Hybridization with *RLUC*. (b) hybridization with *ADE2*. Molecular weights of the *Bam*HI fragments are shown to the left of each blot, and the predicted fragments from the model shown in Fig. 3 are noted to the right of each blot. In all cases, the predicted fragments matched the hybridization bands, demonstrating site-specific integration. Note that fragment B varied between pCRW5 and pCRG1 as a result of promoter sizes and are designated B1 and B2, respectively.

TABLE 1. In vitro luciferase activities of *C. albicans* WO-1 derivative Red 3/6[a]

Promoter	Promoter construct (*ADE2* integrant)	Clone	Phase	Sp. act. of *RLUC* (U/μg of protein) (10^6)[b]	Fold difference (white/opaque)
No promoter[c]	pCRW3	5-1	White	<0.001	
			Opaque	<0.001	
		5-4	White	<0.001	
			Opaque	<0.001	
EF1α2 promoter	pCRE3	2-1	White	95.1 ± 1.6	
			Opaque	87.0 ± 3.0	1.1
		2-3	White	79.4 ± 0.8	
			Opaque	81.2 ± 4.6	1.0
		2-4	White	102.1 ± 4.1	
			Opaque	94.3 ± 4.7	1.1
		2-5	White	100.5 ± 3.7	
			Opaque	91.6 ± 2.2	1.1
Mean ± standard deviation			White	94.3 ± 10.3	1.1 ± 0.1
			Opaque	88.5 ± 5.7	

[a] *C. albicans* WO-1 derivative Red 3/6 was transformed by the *RLUC* reporter system in which *RLUC* is under the regulation of the constitutive *EF1α2* promoter. Specific activities represent the means ± standard deviations of four independent measurements.
[b] Although the accuracy of the assay was less than 0.001, values for the promoter constructs are presented with only one decimal place.
[c] The measured activities were below 0.001 for the promoterless luciferase construct. Activities were measured in undiluted extracts, and the values, presented with three decimal places, accurately reflect the sensitivity of the assay.

activity in the white and opaque phases. The level of luciferase activity in the opaque phase ranged between 1,448 and 2,226 times that in the white phase (Table 3), thus demonstrating that *RLUC* activity was under the stringent developmental regulation of the *OP4* promoter. *RLUC* expression under the regulation of the *OP4* promoter was roughly 10-fold higher than *RLUC* expression under the regulation of the *WH11* promoter, suggesting that the *OP4* promoter is transcriptionally stronger than the *WH11* promoter. This correlates with the earlier observation that transcripts of *OP4* and *PEP1* (17) are abundant, while the transcript of *WH11* is of significantly low abundance (31).

In order to test whether the integration of the *OP4* promoter at the ectopic locus *ADE2* has any effect on the level or phase specificity of expression, the plasmid pCROP31 was linearized with *Sac*I for targeted integration at the *OP4* locus. Five independent clones transformed with pCROP31 were analyzed in the white and opaque phases. The levels of activity in the white and opaque phases (Table 3) were similar to those of clones containing pCROP31 at the *ADE2* locus (Table 3). These results demonstrate that the strength of phase-specific promoters and the levels of gene expression are independent of the site of integration.

Phase-specific regulation of *RLUC* activity is at the level of transcription. To demonstrate that phase-specific regulation of *RLUC* activity is at the level of transcription, Northern analysis was performed on total cellular RNA from white- and opaque-phase cells of two clones (1-1 and 1-2) transformed with pCRW5, in which *RLUC* is under the regulation of the *WH11* promoter (Fig. 2), and two clones (2-5 and 2-7) transformed with pCROP31, in which *RLUC* is under the regulation of the *OP4* promoter (Fig. 2). Northern blots of pCRW5 transformants were probed with the *RLUC* cDNA, the *WH11* cDNA, or the *ADE2* gene, while Northern blots of pCROP31 transformants were probed with the *RLUC* cDNA, the *OP4* cDNA, or the *ADE2* gene (Fig. 5). In both sets of transformants, the *RLUC* transcript was demonstrated to be phase specific. In the clones transformed with pCRW5, both the *RLUC* transcript and the *WH11* transcript were present only in the white phase, while in the clones transformed with pCROP31, both the *RLUC* transcript and the *OP4* transcript were present only in the opaque phase. In all of the transformants the constitutively expressed *ADE2* transcript was present at similar levels in both white- and opaque-phase cells (Fig. 5). These results demonstrate that differential expression of *RLUC* luciferase activity in the two phases of the white-

TABLE 2. In vitro luciferase activities of *C. albicans* WO-1 derivative Red 3/6[a]

Promoter	Promoter construct (*ADE2* integrant)	Clone	Sugar	Sp. act. of *RLUC* (U/μg of protein) (10^6)[b]	Fold increase (Gal/Glu)
GAL1 promoter	pCRG1	3-1	Glu	12.1 ± 1.0	
			Gal	120.5 ± 3.4	10.0
		3-2	Glu	18.0 ± 3.0	
			Gal	160.1 ± 5.5	8.9
		3-3	Glu	24.1 ± 3.4	
			Gal	220.0 ± 8.8	9.1
Mean ± standard deviation			Glu	18.0 ± 6.0	9.3 ± 0.6
			Gal	166.9 ± 50.1	

[a] *C. albicans* WO-1 derivative Red 3/6 was transformed by the *RLUC* reporter system in which *RLUC* is under the regulation of the galactose-inducible *GAL1* promoter. Specific activities represent the means ± standard deviations of four independent measurements. Glu, glucose; Gal, galactose.
[b] Although the accuracy of the assay was less than 0.001, values for the promoter constructs are presented with only one decimal place.

TABLE 3. In vitro luciferase activities of *C. albicans* WO-1 derivative Red 3/6[a]

Promoter	Promoter construct	Clone	Phase	Sp. act. of *RLUC* (U/μg of protein) (10^6)	Fold difference (white/opaque or opaque/white)
WH11 promoter	pCRW5 (*ADE2* integrant)	1-1	White	6.51 ± 0.63	
			Opaque	0.05 ± 0.01	130[b]
		1-2	White	5.40 ± 0.18	
			Opaque	0.03 ± 0.01	180[b]
		1-3	White	7.11 ± 0.95	
			Opaque	0.04 ± 0.01	165[b]
		1-6	White	6.20 ± 0.99	
			Opaque	0.05 ± 0.01	124[b]
Mean ± standard deviation[c]			White	6.30 ± 0.71	150 ± 27[b]
			Opaque	0.04 ± 0.01	
OP4 promoter	pCROP31(*ADE2* integrant)	2-5	Opaque	54.01 ± 4.39	
			White	0.03 ± 0.01	1,865[d]
		2-6	Opaque	66.13 ± 3.31	
			White	0.04 ± 0.01	1,574[d]
		2-7	Opaque	69.20 ± 2.91	
			White	0.03 ± 0.01	2,035[d]
		2-9	Opaque	57.91 ± 2.82	
			White	0.04 ± 0.01	1,448[d]
		2-10	Opaque	60.11 ± 3.35	
			White	0.03 ± 0.01	2,226[d]
Mean ± standard deviation[c]			Opaque	61.47 ± 6.16	1,830 ± 321[d]
			White	0.03 ± 0.01	
OP4 promoter	pCROP31(*OP4* integrant)	3-3	Opaque	72.91 ± 2.58	
			White	0.04 ± 0.01	1,823[d]
		3-6	Opaque	65.61 ± 2.08	
			White	0.03 ± 0.01	2,050[d]
		3-7	Opaque	55.71 ± 2.12	
			White	0.04 ± 0.01	1,466[d]
		3-8	Opaque	67.15 ± 1.88	
			White	0.04 ± 0.00	1,638[d]
		3-9	Opaque	52.23 ± 1.94	
			White	0.03 ± 0.01	1,934[d]
Mean ± standard deviation[c]			Opaque	62.72 ± 8.50	1,782 ± 233[d]
			White	0.04 ± 0.01	

[a] *C. albicans* WO-1 derivative Red 3/6 was transformed by the *RLUC* reporter system in which *RLUC* is under the regulation of the white-phase-specific *WH11* promoter or the opaque-phase-specific *OP4* promoter. Specific activities represent the means of four independent measurements.
[b] White/opaque.
[c] The means and standard deviations for the white and opaque phases for all tested clones were computed from the average values of four independent measurements of each clone.
[d] Opaque/white.

opaque transition (Table 3) corresponds to the levels of *RLUC* mRNA (Fig. 5).

***RLUC* activity measured by an in vivo assay.** The in vitro reporter assay affords a sensitive measure of promoter strength and regulation. However, in order to exploit the potential of the reporter system as a positive marker to screen for transformants, we should be able to measure the luciferase activity in intact cells. We, therefore, tested whether the luciferase activities of intact cells transformed with pCRW5 containing *RLUC* under *WH11* promoter regulation or with pCRE3 containing *RLUC* under *EF1α2* promoter regulation could be assayed in vivo, using coelentrazine as a substrate. As a control, we used cells harboring pCRW3, which contains no promoter upstream of *RLUC*. In addition, white- and opaque-phase cells of each transformant clone were analyzed in order to test for phase-specific expression in vivo. The in vivo luciferase activities of white-phase cells of two clones (1-1 and 1-2) harboring pCRW5 were approximately 1,000 times greater than that of clone 5-2, harboring pCRW3 (Table 4). The in vivo activities of white-phase cells of the two clones 1-1 and 1-2 were 61- and 94-fold greater, respectively, than that of opaque-phase cells, demonstrating that the in vivo luciferase activity reflects the phase-specific regulation of the *WH11* promoter. The in vivo luciferase activity of cells harboring pCRE3 was approximately 1,500 times greater than that of cells of clone 5-2 harboring pCRW3 (Table 4). In vivo activity with the *EF1α2* promoter was similar in both white- and opaque-phase cells, demonstrating the constitutive regulation of this promoter. However, the in vivo activity (Table 4) was significantly lower than in vitro activity (Tables 1, 2, and 3). To test whether the reduced in vivo activity was due to the loss of emitted light through absorption by cells in suspension, different concentrations of untrans-

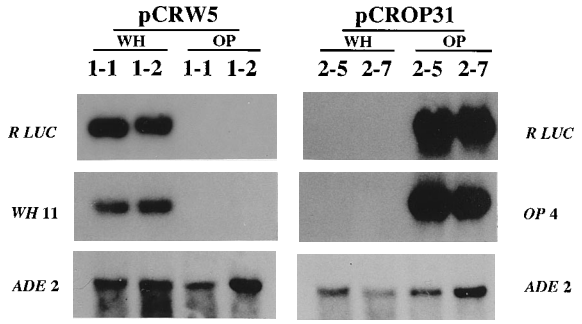

FIG. 5. Northern blot hybridization of cells transformed with pCRW5, which contains the white phase-specific *WH11* promoter upstream of *RLUC*, and pCROP31, which contains the opaque phase-specific *OP4* promoter upstream of *RLUC*. 1-1 and 1-2 are independent clones transformed with pCRW5, and 2-5 and 2-7 are independent clones transformed with pCROP31. Northern blots were serially hybridized with *RLUC*, *WH11*, and *ADE2* in the case of the pCRW5 transformants and *RLUC*, *OP4*, and *ADE2* in the case of pCROP31. RNA was analyzed from cells in the white (WH) and opaque (OP) phases for each clone.

formed white or opaque cells were mixed with a constant concentration of pCRW5-transformed cells expressing luciferase. No significant effect was observed on in vivo-measured activity (Table 4).

If one could visualize the expression of *RLUC* in intact cells by autoradiography, it would facilitate the use of this reporter system as a dominant marker for screening transformants. We tested this possibility by incubating transformant clones with the substrate using a microtiter plate assay and exposing the samples to X-ray film. The autoradiographic results shown in Fig. 6 demonstrate that activity was assayable only in the white-phase cell cultures of pCRW5 transformants (clones 1-1 and 1-2), which contain luciferase under the control of the *WH11* promoter, in opaque-phase cell cultures of pCROP31 transformants (2-5 and 2-7), which contain luciferase under the control of the *OP4* promoter, and in both white and opaque cell cultures of the pCRE3 transformant (2-1), which contains luciferase under the control of the constitutive *EF1α2* promoter. Neither white nor opaque cell cultures of the pCRW3 transformant (5-2), which contains a promoterless luciferase, exhibited luciferase activity (Fig. 6). In each case in which luciferase activity was expressed, the intensity of the exposed photographic emulsion was proportional to cell concentration and reflected the strength of the promoter. To determine whether the in vivo activity measured either in suspension with a luminometer or by autoradiography was associated with intact cells and not a result of leakage of luciferase or cell lysis, cell suspensions of two independent clones (1-1 and 1-2) harboring pCRW5 were used to measure luminescence in the supernatant and cell pellet fractions. The cell suspension was first mixed with coelentrazine, and luciferase activity was measured. Cells were then pelleted and resuspended in fresh buffer containing coelentrazine. The cell pellet fractions of clone 1-1 and 1-2 contained 673 and 976 times, respectively, the level of luciferase activity measured in supernatant fractions (data not shown). However, the level of activity of opaque cells was roughly 100 times lower than that of white cells but the residual activity was still present in the cell pellet fraction (data not shown). A similar experiment with a transformant clone harboring pCROP31 again demonstrated the association of luciferase activity in the opaque phase with the cell pellet fraction (data not shown).

TABLE 4. In vivo luciferase activities of *C. albicans* WO-1 derivative Red 3/6[a]

Promoter	Promoter construct (*ADE2* integrant)	Clone	Phase	No. of *RLUC*-free cells in assay mixture[b]	*RLUC* activity (10^4)	Fold difference (white/opaque)	Fold difference (in vitro/in vivo)
No promoter	pCRW3	5-2	White	NA[c]	0.03 ± 0.01		
			Opaque	NA	0.03 ± 0.01	NA	NA
WH11 promoter[d]	pCRW5	1-1	White	NA	32.29 ± 1.03		
			Opaque	NA	0.53 ± 0.11	61	60
			White	2×10^7 (W)	30.60 ± 1.31		
			White	10^7 (W)	31.60 ± 1.91		
			White	10^6 (W)	32.00 ± 2.05		
			White	2×10^7 (Op)	29.60 ± 2.31		
			White	10^7 (Op)	30.20 ± 1.06		
			White	10^6 (Op)	31.20 ± 1.43		
		1-2	White	NA	29.21 ± 2.29		
			Opaque	NA	0.31 ± 0.11	94	54
			White	2×10^7 (W)	27.60 ± 2.97		
			White	10^7 (W)	28.40 ± 1.85		
			White	10^6 (W)	28.90 ± 2.41		
			White	2×10^7 (Op)	26.90 ± 1.84		
			White	10^7 (Op)	27.80 ± 2.59		
			White	10^6 (Op)	28.20 ± 3.89		
EF1α2 promoter	pCRE3	2-1	White	NA	47.60 ± 2.42		
			Opaque	NA	44.50 ± 2.82	1.1	300
		2-4	White	NA	56.25 ± 2.80		
			Opaque	NA	49.42 ± 1.49	1.1	333

[a] *C. albicans* WO-1 derivative Red 3/6 was transformed by the *RLUC* reporter system in which *RLUC* is under the regulation of the *WH11* or *EF1α2* promoter. Specific activities represent the means of four independent measurements.
[b] W, white; Op, opaque.
[c] NA, not applicable.
[d] Assays in which RLUC-free cells were added contained 10^7 cells expressing luciferase.

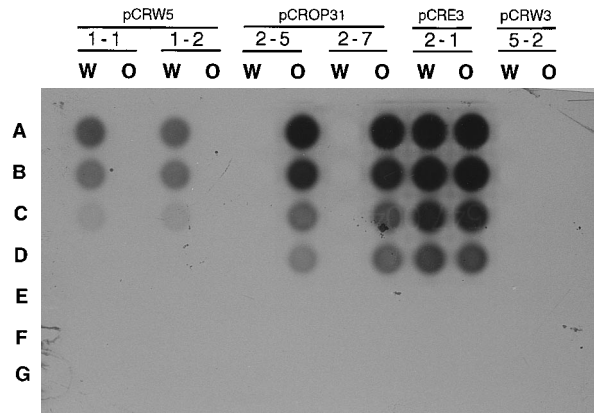

FIG. 6. Autoradiographic visualization of *RLUC* activity in intact cells by a microplate titer assay. 1-1 and 1-2 are individual transformants of pCRW5, which contains the *WH11* promoter; 2-5 and 2-7 are individual transformants of pCROP31, which contains the *OP4* promoter; 2-1 is a transformant of pCRE3, which contains the *EF1α2* promoter; and 5-2 is a transformant of pCRW3, a promoterless construct. Each transformant was analyzed in the white (W) and opaque (O) phases. A through G reflect the following cell densities (cells per 100 μl) in microtiter wells: A, 2×10^7; B, 2×10^6; C, 2×10^5; D, 2×10^4; E, 2×10^3; F, 2×10^2; G, 0.

DISCUSSION

The pathogenic yeast *C. albicans* undergoes phenotypic change through the bud-hypha transition (26) and high-frequency phenotypic switching (27). It has also been assumed that different environmental conditions induce the expression of traits which facilitate pathogenesis. In all of these processes, differential gene expression will, without doubt, play a crucial role in phenotypic expression, and phase-specific genes have already been cloned which are regulated by the bud-hypha transition (3, 24, 31) and by high-frequency phenotypic switching (10, 17, 18, 31, 37). To understand how these differentially expressed genes are regulated, their promoters will have to be functionally analyzed and *trans*-acting factors will have to be identified and characterized (e.g., see reference 29). To facilitate promoter analysis, we initially developed a reporter system in which the firefly luciferase gene (*FLUC*) was fused to several promoters of interest (29, 30), but we soon discovered that a functional *FLUC* gene product was not expressed. In the past few years, the reason for the lack of a functional reporter gene product has become evident. *C. albicans* has an altered codon strategy in which CUG encodes serine rather than leucine residues (19, 22, 23). By sequence analysis, it has been demonstrated that the serine tRNA contains the anticodon CAG for decoding the CUG codon to serine (34). It has also been demonstrated that the serine tRNA aminoacylates serine instead of leucine (23). This can lead to functionless gene products for heterologous genes which contain functionally important leucines encoded by CUGs. *FLUC* contains nine in-frame CUGs (7) in its ORF, and we have concluded that this must be the basis for a functionless or unstable *FLUC* gene product in *C. albicans*. When we examined the CUG contents of six additional potential bioluminescent reporter genes, it became apparent that the luciferase of *R. reniformis* had the greatest potential for expression in *C. albicans*. While the luciferases of *Vargula hilgendorfii* (37), *Luciola cruciata* (15), and *Luciola lateralis* (35) have 13, 5, and 2 CUGs, respectively, and while the green-fluorescent protein (20) and the aequorin (11) of *Aequorea victoria* have one CUG each, the luciferase of *R. reniformis* (14) has no CUGs. We have, therefore, constructed a vector in which the *Renilla* luciferase gene has been placed downstream of a multiple cloning site in which promoter sequences can be inserted for functional analysis. The expression vector contains an *ADE2* gene, which can be used as a selectable marker in the Red 3/6 strain, an *ade2* derivative of strain WO-1 (30). A *WH11* transcription termination sequence has been fused to the 3′ terminus of the *RLUC* ORF to facilitate proper transcription termination of the *RLUC* transcript (31). We have found that under the regulation of *C. albicans* promoters, *RLUC* can be expressed at levels several thousand times higher than that of the background. We have fused constitutive, galactose-inducible, and phase-specific promoters with the *RLUC* ORF, and in every case we have observed that the promoter functions in the predictable manner. The *EF1α2* promoter directs similar levels of expression in both white- and opaque-phase cells, and expression under the regulation of the *GAL1* promoter is induced 10-fold by shifting cells from glucose to galactose. Finally, we have demonstrated that *RLUC* expression is phase specific in the white-opaque transition when expression is under the regulation of either the white-phase-specific *WH11* promoter or the opaque-phase-specific *OP4* promoter and that the comparative levels of phase-specific expression reflect the differences in the abundance of the *WH11* and *OP4* transcripts, as predicted from the representations of the phase-specific mRNAs in cDNA libraries (18, 31). Phase-specific expression of *RLUC* under the regulation of the *OP4* promoter was also similar when integrated at different sites, an ectopic *ADE2* locus and the homologous *OP4* locus, demonstrating that both the levels of expression and phase specificity are regulated by the promoter itself. Together, these results demonstrate that *RLUC* represents a highly effective bioluminescent reporter of promoter function in *C. albicans*.

We have, in addition, demonstrated that *RLUC* activity can be assayed in intact cells, and experiments comparing the culture supernatant and the cell pellet suggest that the luciferase activity measured in the in vivo assay is intracellular. We have also demonstrated that luciferase activity in intact cell cultures can be assayed autoradiographically, which may eventually be modified to provide a single-cell assay. However, the activity of luciferase measured in intact cell cultures is at least 60-fold less than the activity of cell lysates, suggesting that in vivo measurements are less efficient than in vitro measurements. This could be due to a number of variables related to cell geometry, but it could also be due to the lower level of accessibility of the substrate coelentrazine to the cell cytoplasm. This latter problem may be circumvented either through the use of modified derivatives of coelentrazine with higher hydrophobicity or through the development of a secreted version of *Renilla* luciferase by fusing a secretory signal peptide similar to the secretory form of luciferase from *V. hilgendorfii* (37).

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