Use of Green Fluorescent Protein and Reverse Transcription-PCR To Monitor *Candida albicans* Agglutinin-Like Sequence Gene Expression in a Murine Model of Disseminated Candidiasis

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***Candida albicans* PALS-green fluorescent protein (GFP) reporter strains were inoculated into mice in a disseminated candidiasis model, and GFP production was monitored by immunohistochemistry and reverse transcription-PCR (RT-PCR). GFP production from the *ALS1* and *ALS3* promoters was detected immunohistochemically. *ALS1*, *ALS2*, *ALS3*, *ALS4*, and *ALS9* transcription was detected by RT-PCR, further identifying ALS genes expressed in this model.**

Disseminated candidiasis is a life-threatening disease manifestation with mortality rates as high as 40% (1, 14). *Candida albicans* is the fourth most commonly isolated organism from bloodstream infections in hospitalized patients (13). As a resident of normal intestinal flora, *C. albicans* can enter the bloodstream across the intestinal wall. Alternatively, contaminated central venous catheters can serve as a nidus for disseminated infections (5). One of the most commonly used animal models of disseminated candidiasis is the murine tail vein model (12). After injection of yeast into the lateral tail vein of a mouse, *C. albicans* leaves the circulatory system and is capable of invading essentially every parenchymal organ. Death generally occurs from renal failure, but fungal elements have been observed in the spleen, liver, heart, lungs, and central nervous system (12). Therefore, in the progression of disseminated candidiasis, the fungus is exposed to multiple microenvironments that can influence expression of genes involved in host-pathogen interactions.

ALS genes are large open reading frames that encode proteins with features of cell-surface glycoproteins (8). The cell surface localization of some of these proteins is known (6, 9, 10) and assumed to be the same for the remaining proteins in the family. In an initial study, an antiserum raised against four 10-mer peptide sequences from Als1p was used for immunohistochemical analysis of tissue sections from *C. albicans*-infected mice (10). This antiserum recognized several different Als proteins on Western blots (10). Als proteins were distributed uniformly on the cell wall of fungal elements within parenchymal organs of mice inoculated via lateral tail vein injection. In this study, all fungal cells in each tissue section were detected using the antiserum, suggesting the widespread presence of Als proteins within the host. This staining pattern was found for all *C. albicans* strains tested and over the time course of infection. Although this work demonstrated the production of Als proteins in vivo, it was limited by the lack of availability of specific antisera for each of the Als proteins. The goal of this study was to detect expression of individual ALS genes to learn

which Als proteins may be produced during disseminated infection in the mouse model.

Key to the approach taken here is a set of *C. albicans* strains in which the green fluorescent protein (GFP) reporter gene is under control of different ALS promoters. Construction and validation of these strains was described previously (7a). In each strain, GFP is integrated downstream of the ALS promoter at the native ALS locus. Strains 2225 (*PALS1*-GFP), 2185 (*PALS3*-GFP), 2227 (*PALS5*-GFP), 2223 (*PALS6*-GFP), 2224 (*PALS7*-GFP), and 2337 (*PALS9*-GFP) were used in this study. A *C. albicans* strain that produces GFP under the control of the strong, constitutive *TPII* promoter was constructed as a positive control for these analyses. For the control strain, GFP was cloned into a modified version of CIP10 (11), called plasmid 1105. To create plasmid 1105, the BglII site of CIP10 was destroyed by digestion, filling in with Klenow fragment, and religation of the blunt ends. The resulting construct was digested with KpnI-MluI to remove the polylinker region. The vector was modified to include a *C. albicans* *TPII* promoter and terminator with a polylinker between the sequences for high-level constitutive expression of *C. albicans* genes. The DNA sequence of *TPII* is deposited in the GenBank database under accession number AF124845.

The *TPII* promoter-terminator construct was originally made for plasmid pRC2312 (2) by amplification of the *TPII* promoter from strain 1161 genomic DNA by the use of primers Exp2 (5' CCC GCG GCC GCA ACC CGG GAA CTC GAG TGT TTC TAA AAT TGT ATA AAT GTA TTA ATT G 3') and Exp5 (5' CCA AGC TTG AAG TGG TTC AAG TGG AGT TAC GAA G 3'). The *TPII* terminator was amplified from strain 1161 genomic DNA by the use of primers Exp3 (5' CCC GCG GCC GCA AAG ATC TTA GCT AAG TGA ACA GTA TAT TAA AAA CTA TAT GCC TAT AG 3') and Exp6 (5' CCC CTG CAG CTG CTT GTA GAG TTG ATA TTA ATC ATC 3'). The promoter-containing fragment was digested with HindIII-NotI, and the terminator was digested with NotI-PstI and ligated into HindIII-PstI-cut pRC2312 in a three-part ligation.

Amplification with these primers created the promoter-terminator fragment with a polylinker between them that included the following sites (5' to 3'): XhoI-SmaI-NotI-BglII.

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DNA sequencing of the construct ensured accurate amplification and the presence of all expected restriction sites. This entire fragment was amplified from the pRC2312-based construct by the use of primers Exp7 (5' CCC GGT ACC GAA GTG GTT CAA GTG GAG TTA CGA AGA G 3') and Exp8 (5' CCC ACG CGT CTG CTT GTA GAG TTG ATA TTA ATC ATC 3'), digested with KpnI-MluI, and cloned into KpnI-MluI-digested BglII-less Clp10 to form plasmid 1105. GFP was amplified from plasmid pYGF3, which was a generous gift from Brendan Cormack (Johns Hopkins University) (4), by the use of primers GFPXhoI (5' CCCTCGAGTAT TAAATGTCTAAAGGTGAAGAATTATCACT 3') and GFPBglII (5' CCCACGCGTCTGCTTGTAGAGTTGATAG GAATCATC 3'). The GFP PCR product was digested with XhoI and BglII and cloned into plasmid 1105, which had been digested with the same enzymes. The resulting plasmid (called 1110) was linearized with StuI to direct integration to the *RP10* locus and transformed into *C. albicans* CAI4 by use of the methods described above. The resulting strain, 1143, was verified by BglII restriction mapping and Southern blotting with probes for *TP11*, the β -lactamase-encoding gene, and GFP as described elsewhere (7a). Strain 1143 exhibited the expected high-level, constitutive production of GFP as visualized by fluorescence microscopy, further validating the construct.

Female BALB/cByJ mice (7 weeks old) were purchased from Jackson Laboratories. *C. albicans* strains were grown in YPD liquid medium (10 g of yeast extract/liter, 20 g of peptone/liter, 20 g of dextrose/liter) as described previously for growth rate analysis (15). Cells were washed three times in phosphate-buffered saline, diluted, and counted in triplicate using a hemacytometer. A total of 2 million yeast cells in a volume of 0.1 ml were injected into the lateral tail vein of each mouse. Each *C. albicans* strain was injected into six mice, two of which were euthanized at each time point (12, 24, and 48 h) after infection. All animal protocols were conducted under approval by the University of Illinois Institutional Animal Care and Use Committee. Mice were dissected to remove the kidneys, heart, liver, spleen, and lungs. One kidney was diced with a fresh razor blade, flash frozen in liquid nitrogen, and stored at -80°C for subsequent RNA isolation and reverse transcription-PCR (RT-PCR) analysis (see below). The remainder of the tissues were fixed in 10% neutral buffered formalin for approximately 48 h, trimmed, embedded in paraffin, and sectioned. Sections were stained with hematoxylin-eosin and Gomori methenamine silver (GMS) for histopathology analysis. Unstained sections were used for immunohistochemical analysis that followed the method of Hoyer et al. (10) with two exceptions. First, formalin fixation masked GFP epitopes, requiring an antigen retrieval process. Slides were immersed in pH 6.0 citrate buffer (18 ml of 0.1 M monobasic citric acid/liter, 82 ml of 0.1 M sodium citrate/liter), brought to boiling for 4 min in a microwave, and maintained at an intermittent boil for 10 min. Slides were cooled at room temperature for approximately 30 min and then washed three times (5 min each) with phosphate-buffered saline (pH 7.2) before quenching of endogenous peroxidase activity with 0.5% hydrogen peroxide in methanol. Second, a polyclonal anti-GFP preparation was used in this study (catalog number NB 600-308; Novus) at a 1:100 dilution and incubated on slides overnight at 4°C .

Total RNA was recovered from mouse kidneys by homoge-

nizing tissue in Tris-EDTA-sodium dodecyl sulfate and following an acid-phenol extraction protocol (3). RNA yield was determined spectrophotometrically, and approximately 100 μg of total RNA was further purified using an RNeasy kit (QIAGEN) according to instructions. Samples were then DNase treated (Ambion) at 37°C overnight, and 200 ng of RNA was screened by PCR with the *ALS9* RT-PCR primers to check for genomic DNA as previously described (7). A parallel reaction that included *C. albicans* genomic DNA was run as a positive control for the PCR. DNA-free RNA was precipitated for 30 min at -80°C with ethanol, 5 M ammonium acetate, and linear acrylamide (Ambion) and then pelleted. RNA pellets were resuspended in 20 μl of RNase-free water, and the yield was determined spectrophotometrically. An additional control reaction for the absence of genomic DNA was run after RNA precipitation by the use of a quantity of RNA equal to that used in the RT-PCRs. RT-PCR followed the protocol of Green et al. (7); 2 μg of RNA were added to each cDNA synthesis, and 1/10 of the final cDNA product was added to PCRs specific for each ALS gene. GFP RT-PCRs used the primers GFPRTF (5' TCTGTCTCCGGTGAAGGTGAAG 3') and GFPRTTR (5' GGCATGGCAGACTTGAAAAAG 3').

Tissue sections recovered from each mouse were GMS stained to visualize fungal elements. The extent of fungal burden in each organ was determined by microscopic examination. The time course of infection for these mice matched that described by Hoyer et al. (10), which showed a hierarchy of organ infection (Table 1). In both cases, the kidneys had the most extensive fungal burden, followed by the heart. The liver and spleen in each animal were lightly infected with fungal elements mainly restricted to the phagocytic cells. Fungal elements were not observed in lung tissue in any of the mice. Tissue sections were also immunostained with an anti-GFP polyclonal antiserum. Examination of sections containing the *PTP11*-GFP positive control strain and strain CAI12 (non-GFP-producing negative control strain) validated the assay (Fig. 1; Table 1). For each mouse in which fungal elements were detected in the kidneys and heart by GMS staining, GFP production from the *ALS1* and *ALS3* promoters was also observed by immunostaining (Table 1). Representative images from mice inoculated with the *PALS1*-GFP and *PALS3*-GFP strains are shown in Fig. 1. Although GMS-stained fungal cells were present in mice inoculated with the other reporter strains, fungi did not stain immunohistochemically with the anti-GFP serum. These results could be due to lack of transcriptional activity from the other ALS promoters in this model or to transcriptional activity that was below the detection limit for this assay.

To distinguish between these possibilities, total RNA extracted from *C. albicans*-infected kidneys was analyzed by RT-PCR. In previous work, RT-PCR analysis of cultured *C. albicans* cells was sensitive enough to detect transcription from all ALS promoters in the presence of sufficient quantities of fungal RNA (7, 7a). RT-PCR using GFP-specific primers showed that under the control of the strong, constitutive *TP11* promoter, GFP-specific transcript was detected in the total kidney RNA from each mouse tested (Table 2). Signals from the *ALS1* and *ALS3* promoters were also observed, with detection increasing later in the time course of infection. This result presumably is due to the increased number of fungal cells in the kidney at later time points and the consequent increased

TABLE 1. Observations from GMS- and immunohistochemically stained *C. albicans*-inoculated mouse tissue sections^a

Inoculation strain	Time point (h)	Result ^b					
		Kidney		Heart		Liver	Spleen
		GMS	IHC	GMS	IHC	GMS	GMS
Uninfected CAI12	48	—	—	—	—	—	—
	12	+/+	-/-	+/+	-/-	-/+	-/-
	24	+/+	-/-	+/+	-/-	+/+	-/-
PTPII-GFP	48	+/+	-/-	+/+	-/-	+/+	-/-
	12	+/+	+/+	-/-	-/-	-/+	-/+
	24	+/+	+/+	-/-	-/-	+/+	+/+
PALS1-GFP	48	+/+	+/+	-/+	-/+	-/-	-/+
	12	+/+	+/+	-/+	-/+	-/-	-/-
	24	+/+	+/+	+/+	+/+	+/+	-/+
PALS3-GFP	48	+/+	+/+	+/+	+/+	-/-	-/-
	12	+/+	+/+	+/+	+/+	-/-	-/-
	24	+/+	+/+	-/+	-/+	-/-	-/-
PALS5-GFP	48	+/+	+/+	-/-	-/-	-/-	+/+
	12	+/+	-/-	+/+	-/-	+/+	+/+
	24	+/+	-/-	+/+	-/-	+/+	-/-
PALS6-GFP	48	+/+	-/-	-/+	-/-	-/+	-/-
	12	-/-	-/-	-/-	-/-	-/-	-/-
	24	+/+	-/-	-/+	-/-	-/-	-/-
PALS7-GFP	48	+/+	-/-	-/-	-/-	-/-	-/+
	12	+/+	-/-	+/+	-/-	-/+	-/-
	24	+/+	-/-	+/+	-/-	+/+	-/-
PALS9-GFP	48	+/+	-/-	+/+	-/-	+/+	-/-
	12	+/+	-/-	-/-	-/-	-/-	-/-
	24	+/+	-/-	-/-	-/-	-/-	-/-
	48	+/+	-/-	-/-	-/-	+/+	+/+

^a Tissues from PALS-GFP-infected mice were analyzed using Gomori methanamine silver (GMS) staining and anti-GFP immunohistochemical (IHC) detection. Each entry in the table shows results from a pair of mice at each time point.

^b +, presence of fungal element(s) stained black for GMS or brown for IHC visible at $\times 400$ magnification. —, absence of fungal element(s) stained by GMS or lack of staining by IHC in the presence of GMS-positive fungal element(s). All lung sections were negative for GMS and IHC staining at all time points in all animals. All liver and spleen sections were negative by IHC staining, so only GMS results are reported in the table.

TABLE 2. Results of GFP-specific RT-PCRs from analysis of total kidney RNA from mice inoculated with *C. albicans* PALS-GFP reporter strains

Duration of infection (h)	Inoculation strain ^a			
	PTPII-GFP	PALS1-GFP	PALS3-GFP	PALS9-GFP
12	+/+	+/-	-/-	-/-
24	+/+	+/+	-/-	+/+
48	+/+	+/+	+/+	-/-

^a RNA was isolated from kidneys of animals inoculated with the specified strain, and RT-PCR was performed using GFP-specific primers. Each entry in the table shows results from a pair of mice at each time point. +, presence of RT-PCR product. —, lack of detectable RT-PCR product. Strains not listed produced all negative reactions. PALS2-GFP and PALS4-GFP reporter strains were not included in the analysis.

ratio of fungal RNA to host RNA in the assayed samples. RT-PCR with GFP-specific primers detected transcription from the *ALS9* promoter at the 24 h time point (Table 2). RT-PCR analysis was also done using a set of primers specific for each *ALS* gene (7). Results were similar to those from the GFP-specific RT-PCR analysis in that transcription from the *ALS1*, *ALS3*, and *ALS9* promoters was detected (Table 3). Transcription from the *ALS2* promoter was observed with a frequency similar to that from *ALS1*, mirroring results from a previous study that suggested that the levels of *ALS1* and *ALS2* transcript were similar in cultured cells (7). Transcription from the *ALS4* promoter was observed in a minority of the samples assayed. Transcription from the *ALS5*, *ALS6*, and *ALS7* promoters was not detected with any method used.

In the experiments described here, various methods of detecting *ALS* gene expression were used as a means to infer which *Als* proteins are produced in the murine model of dis-

FIG. 1. GMS-stained (upper panels) and anti-GFP immunohistochemically stained (lower panels) tissue sections from *C. albicans*-infected mice. The strains used for inoculation are listed above each pair of panels. The left three panels (strains PTPII-GFP, CAI12, and PALS1-GFP) show kidney tissue 48 h after infection. The right two panels (PALS1-GFP and PALS3-GFP) show heart tissue 24 h after infection. Inoculation of mice with the PTPII-GFP strain produced strong, constitutive GFP production and obvious immunohistochemical staining. Staining of tissues infected with strain CAI12, which does not produce GFP, was performed as a negative control. Anti-GFP staining of strains producing GFP under the control of the *ALS1* or *ALS3* promoter was obvious in all kidney and heart sections in which GMS-stained fungal cells were present. Images were taken at $\times 400$ magnification.

TABLE 3. Percent positive ALS-specific RT-PCR from analysis of total kidney RNA from mice inoculated with *C. albicans*^a

Duration of infection (h)	% Of positive ALS-specific RT-PCR reactions							
	ALS1	ALS2	ALS3	ALS4	ALS5	ALS6	ALS7	ALS9
12	14	0	0	0	0	0	0	0
24	71	86	93	7	0	0	0	7
48	86	93	86	0	0	0	0	14

^a RT-PCR was performed with ALS-specific primers (7) on total kidney RNA from the experimental mice. Each data point represents analysis of 14 mice (2 mice each inoculated with CA112 or one of the PALS-GFP reporter strains). Percentages represent the frequency with which individual ALS-specific transcripts were detected.

seminated candidiasis. Immunostaining of reporter protein and RT-PCR to detect reporter gene and ALS transcription detected expression of *ALS1*, *ALS2*, *ALS3*, *ALS4*, and *ALS9* under the conditions tested. Expression of more ALS genes was detected using RT-PCR than was detected using immunostaining, presumably because of the amplification-based nature of the RT-PCR technique. The relative ease of detection of transcription of some genes over that of others suggests that these genes are characterized by a stronger transcriptional response. The hierarchy observed here is similar to that found in cultured cell models and other disease models (7, 7a) and further supports the conclusion that the ALS family can be divided into two groups: one that is regulated by strong transcriptional responses and another that is characterized by lesser activity. Indication of signals from *ALS5*, *ALS6*, and *ALS7* was absent from the present analysis. These ALS genes may be expressed during parenchymal organ infection, but the level of expression may simply be below the limit of detection for the assays utilized here. In this work and previous studies (7), the ratio of *C. albicans* RNA to host RNA appeared to strongly influence the ability to detect ALS transcripts. For example, it was easier to detect GFP in 12-h lesions by immunohistochemical staining than it was to detect GFP-specific transcript from the kidneys of the same animals. In this situation, the reporter protein was localized in an obvious region of a tissue section. RT-PCR at the same time point requires amplification of a specific transcript that is relatively rare compared to the host RNA present. It is therefore important to consider multiple approaches to gene expression analysis, as techniques differ in their limits of detection. Techniques that allow enrichment for fungal RNA among the total RNA extracted from *C. albicans*-infected kidneys would likely reveal the presence of *ALS5*-, *ALS6*-, and *ALS7*-specific transcripts. Alternatively, in situ PCR, with its combination of amplification and localization, might detect these transcripts, although designing probes that do not cross-react with other ALS sequences would be challenging. Ultimately, carefully validated monoclonal antibodies specific for each Als protein should be produced.

Use of these reagents would provide data regarding cellular localization of Als protein production with an improved detection limit, since the protein products from both ALS alleles could be assayed, rather than transcription and translation

from the single allele inherent in the GFP approach used here. Regardless of detection limit, the comparison of the original staining for Als protein with the results presented here provides a hierarchy of transcriptional activity, from each ALS locus, in the murine tail vein disease model, supporting the hypothesis that certain Als proteins are abundantly produced in vivo and that others are less so. The identity of these proteins and their hierarchical arrangement in quantity parallel results gathered from other experimental approaches and present a consistent picture of ALS gene expression both in vitro and in vivo.

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