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Transcriptional Regulators Cph1p and Efg1p Mediate Activation of the *Candida albicans* Virulence Gene *SAP5* during Infection

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The opportunistic fungal pathogen *Candida albicans* can cause superficial as well as systemic infections. Successful adaptation to the different host niches encountered during infection requires coordinated expression of various virulence traits, including the switch between yeast and hyphal growth forms and secretion of aspartic proteinases. Using an in vivo expression technology that is based on genetic recombination as a reporter of gene activation during experimental candidiasis in mice, we investigated whether two signal transduction pathways controlling hyphal growth, a mitogen-activated protein kinase cascade ending in the transcriptional activator Cph1p and a cyclic AMP-dependent regulatory pathway that involves the transcription factor Efg1p, also control expression of the *SAP5* gene, which encodes one of the secreted aspartic proteinases and is induced by host signals soon after infection. Our results show that both transcriptional regulators are important for *SAP5* activation in vivo. *SAP5* expression was reduced in a *cph1* mutant, although filamentous growth in infected tissue was not detectably impaired. *SAP5* expression was also reduced, but not eliminated, in an *efg1* null mutant, although this strain grew exclusively in the yeast form in infected tissue, demonstrating that in contrast to in vitro conditions, *SAP5* activation during infection does not depend on growth of *C. albicans* in the hyphal form. In a *cph1 efg1* double mutant, however, *SAP5* expression in infected mice was almost completely eliminated, suggesting that the two signal transduction pathways are important for *SAP5* expression in vivo. The avirulence of the *cph1 efg1* mutant seemed to be caused not only by the inability to form hyphae but also by a loss of expression of additional virulence genes in the host.

The yeast *Candida albicans* is a member of the microflora on mucosal surfaces of healthy people, but it can also cause superficial and life-threatening systemic infections, especially in immunocompromised patients. The success of *C. albicans* both as a colonizer and as an infectious microorganism probably depends on many different characteristics of the fungus, including its ability to adhere to a variety of host tissues, its ability to switch between yeast and hyphal growth forms, and its secretion of hydrolytic enzymes, such as proteinases and phospholipases (3, 5, 24). Since *C. albicans* encounters different body niches during an infection, a flexible reaction to different environmental conditions is a prerequisite for optimal adaptation to the host at different infection stages. For example, the switch from yeast growth to hyphal growth may facilitate tissue invasion and evasion of macrophages, and the generation of yeasts budding off from the hyphae may allow more efficient spread through the bloodstream during disseminated infection and multiplication in infected tissues. Accordingly, mutants restricted to either the yeast form or the hyphal form are attenuated in terms of virulence (2, 18). The secreted aspartic proteinases (Saps) are also necessary for full virulence of *C. albicans* (6, 10, 14, 28). Different roles have been suggested for the Saps based on in vitro experiments; these roles include nitrogen supply (30), adherence (33), degradation of host barriers (4, 23), and evasion of host defense mechanisms (11, 25). *C. albicans* has a large gene family encoding Saps (21), and it is likely that the individual Sap isoenzymes evolved for optimal adaptation to specific functions or host niches. In fact, by using an in vivo expression technology (IVET) that is based on genetic recombination as a reporter of gene expression and allows detection of gene activation in single cells, we could demonstrate that individual *SAP* genes were differentially activated during infection depending on the host niche and the infection stage (31).

It is likely that expression of different virulence traits has to be coordinated, and common signal transduction pathways may be used to ensure induction of an appropriate set of virulence genes in response to environmental signals. A mitogen-activated protein kinase cascade ending in the transcription factor Cph1p and a cyclic AMP-dependent signaling pathway ending in the transcriptional regulator Efg1p are both necessary for induction of filamentous growth in *C. albicans*, depending on the conditions used (17, 18). Expression of some of the *SAP* genes, *SAP4* to *SAP6*, in vitro has been linked to the hyphal form of *C. albicans* (9, 34). This linkage was confirmed by the finding that in contrast to expression in the wild-type parent strain, expression of *SAP4* to *SAP6* was not detectable in an *efg1* mutant in vitro under conditions in which the strain failed to form hyphae (29). However, recent studies have dem-

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TABLE 1. *C. albicans* strains used in this study

Strain(s)	Parent	Genotype[a]	Reference
CAI4		Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	8
CFI1	CAI4	*ACT1*/*act1*::*FRT-MPA*^{r}-*FRT*	32
S2FI5B	CFI1	*sap2-1*::P_{SAP2-1}-*ecaFLP-URA3*/*SAP2-2*	31
S5FI2A	CFI1	*sap5-1*::P_{SAP5}-*ecaFLP-URA3*/*SAP5-2*	31
S5FI2B	CFI1	*SAP5-1*/*sap5-2*::P_{SAP5}-*ecaFLP-URA3*	31
JKC18	CAI4	*cph1*::*hisG*/*cph1*::*hisG*	17
CFI2	JKC18	*ACT1*/*act1*::*FRT-MPA*^{r}-*FRT*	This study
C2S2F1A and C2S2F1B	CFI2	*sap2-1*::P_{SAP2-1}-*ecaFLP-URA3*/*SAP2-2*	This study
C2S5F1A and C2S5F1B	CFI2	*sap5-1*::P_{SAP5}-*ecaFLP-URA3*/*SAP5-1*/*SAP5-2*	This study
HLC67	CAI4	*efg1*::*hisG*/*efg1*::*hisG*	18
CFI3	HLC67	*ACT1*/*act1*::*FRT-MPA*^{r}-*FRT*	This study
C3S2F1D and C3S2F1E	CFI3	*sap2-1*::P_{SAP2-1}-*ecaFLP-URA3*/*SAP2-1*	This study
C3S5F1A	CFI3	*SAP5-1*/*sap5-2*::P_{SAP5}-*ecaFLP-URA3*	This study
C3S5F1B	CFI3	*sap5-1*::P_{SAP5}-*ecaFLP-URA3*/*SAP5-2*	This study
HLC69	CAI4	*cph1*::*hisG*/*cph1*::*hisG efg1*::*hisG*/*efg1*::*hisG*	18
CFI4	HLC69	*ACT1*/*act1*::*FRT-MPA*^{r}-*FRT*	This study
C4S2F1B and C4S2F1C	CFI4	*sap2-1*::P_{SAP2-1}-*ecaFLP-URA3*/*SAP2-2*	This study
C4S5F1A	CFI4	*SAP5-1*/*sap5-2*::P_{SAP5}-*ecaFLP-URA3*	This study
C4S5F1B	CFI4	*sap5-1*::P_{SAP5}-*ecaFLP-URA3*/*SAP5-2*	This study

[a] Apart from the features indicated all strains are identical to their parents.

onstrated that the pattern of expression of virulence genes in pathogenic microorganisms in an infected host can be totally different from the expression pattern observed in vitro, including the dependence on regulatory factors (15, 16). Indeed, when IVET was used to study expression of the *SAP* genes during infection, we observed that the *SAP5* gene, one of the putative hypha-specific genes, was induced very soon after contact with the host, at a time when no hyphae were detected in the infected animals (31). We suggested that expression of *SAP5* during infection might be activated by signals that also induce hyphal growth, possibly involving the same signal transduction pathways, but might be independent of the hyphal morphology itself. Therefore, in the present study we investigated whether the transcriptional regulators *CPH1* and *EFG1*, representing the two best-studied signaling pathways regulating hyphal formation in *C. albicans* (7, 35), were also necessary for *SAP5* activation under experimental infection conditions. The *SAP5* gene was chosen for this analysis because activation of this gene was observed in a large proportion of infecting cells and a negative influence of a regulatory mutation on *SAP5* expression in vivo would be detected most easily (31). In addition, an *efg1* mutation strongly attenuates virulence and prevents tissue invasion, so that certain host niches that induce activation of specific virulence genes are not accessible to an *efg1* mutant (18). Since the *SAP5* gene is activated very soon after infection, it was possible to study a possible effect of regulatory mutations on gene expression in a body location that is reached by both wild-type and mutant strains (i.e., under comparable in vivo conditions).

MATERIALS AND METHODS

Strains and growth media. The *C. albicans* strains used in this study are listed in Table 1. The strains were maintained on minimal agar (6.7 g of yeast nitrogen base without amino acids [Bio 101, Vista, Calif.] per liter, 20 g of glucose per liter, 0.77 g of complete supplement medium [Bio 101] per liter, 15 g of agar per liter). For routine growth of the strains, YPD liquid medium (10 g of yeast extract per liter, 20 g of peptone per liter, 20 g of glucose per liter) was used. For induction of the *SAP2* promoter, cells were grown overnight in YCB-BSA (23.4 g of yeast carbon base per liter, 4 g of bovine serum albumin per liter; pH 4.0). Screening for mycophenolic acid (MPA)-sensitive colonies was performed after 2 days of growth at 30°C on minimal agar containing 1 μg of MPA ml^{-1}, which resulted in generation of large MPA^r and small MPA^r colonies (32). Uridine (100 μg ml^{-1}) was added to the media to support growth of *ura3* mutant strains.

***C. albicans* transformation.** *C. albicans* strains were transformed by electroporation (13) with the following gel-purified linear DNA fragments: a *Sac*I-*Sac*I fragment from pAFI3 (32) containing the *FRT-MPA*^{r}-*FRT* cassette between flanking *ACT1* sequences (Fig. 1A), an *Xba*I-*Sac*I fragment from pSFL53 (31) containing a P_{SAP5}-*ecaFLP* reporter gene fusion (Fig. 2A), and an *Xba*I-*Sac*I fragment from pSFL213 (31) containing a P_{SAP2-1}-*ecaFLP* reporter gene fusion

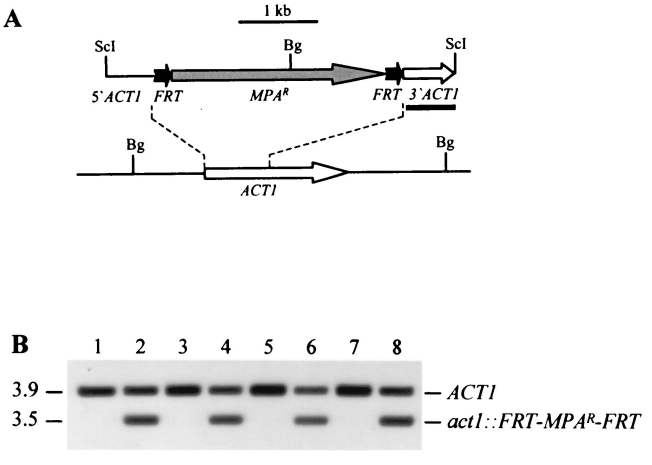

FIG. 1. Integration of the deletable *FRT-MPA*^{r}-*FRT* cassette into one of the *ACT1* alleles of strains JKC18 (*cph1*/*cph1*), HLC67 (*efg1*/*efg1*), and HLC69 (*cph1*/*cph1 efg1*/*efg1*). (A) Integration scheme. The *ACT1* coding region is represented by the open arrow, and the *MPA*^{r} marker is represented by the grey arrow. The 34-bp *FRT* site is not drawn to scale. The probe used for verification of correct integration by Southern hybridization is represented by the black bar, and the diagnostic *Bgl*II sites are shown. Bg, *Bgl*II; ScI, *Sac*I. (B) Southern hybridization of *Bgl*II-digested genomic DNA of parent strains and their transformants carrying the *FRT-MPA*^{r}-*FRT* cassette with an *ACT1*-specific probe. The positions of the fragments are indicated on the right, and molecular sizes (in kilobases) are indicated on the left. Lane 1, CAI4; lane 2, CFI1; lane 3, JKC18; lane 4, CFI2; lane 5, HLC67; lane 6, CFI3; lane 7, HLC69; lane 8, CFI4.

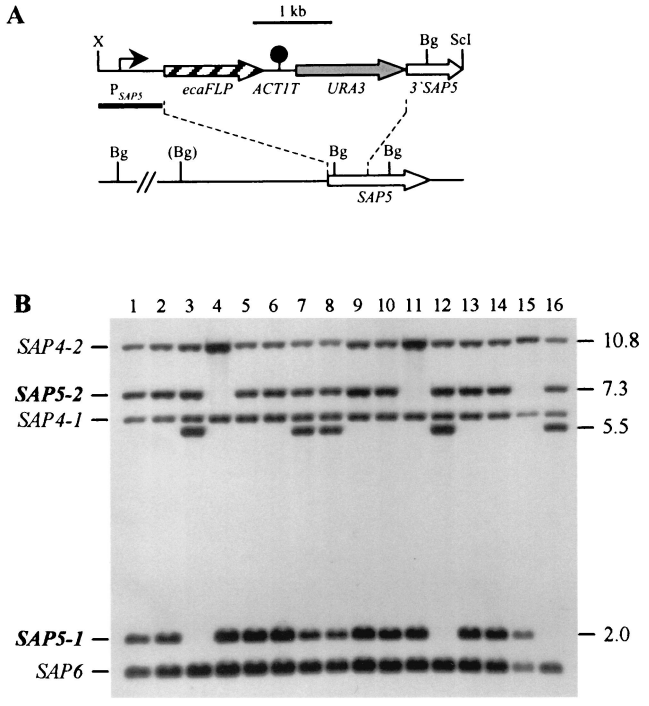

FIG. 2. Integration of the P_{SAP5}-*ecaFLP* fusion into one of the *SAP5* alleles of strains CFI2 (*cph1*/*cph1*), CFI3 (*efg1*/*efg1*), and CFI4 (*cph1*/*cph1 efg1*/*efg1*). (A) Integration scheme. The coding regions of the *SAP5* and *ecaFLP* genes are represented by the open and cross-hatched arrows, respectively. The *SAP5* promoter (P_{SAP5}) is indicated by the solid arrow, the *ACT1* transcription termination sequence (*ACT1T*) is indicated by the solid circle, and the *URA3* selection marker is indicated by the grey arrow. The probe used for verification of correct integration by Southern hybridization is represented by the black bar, and the diagnostic *Bgl*II sites are shown. The *Bgl*II site in parentheses is not present in the *SAP5-2* allele of strain CAI4 and its derivatives. Bg, *Bgl*II; ScI, *Sac*I; X, *Xba*I. (B) Southern hybridization of *Bgl*II-digested genomic DNA of parent strains and their transformants carrying the *ecaFLP* reporter gene with the *SAP5* promoter fragment as the probe. The positions of the wild-type *SAP5* alleles (boldface) and the cross-hybridizing *SAP4* and *SAP6* fragments are indicated on the left. The molecular sizes (in kilobases) of the wild-type *SAP5* fragments and the fragments corresponding to the reporter gene fusions are indicated on the right. Lane 1, CAI4; lane 2, CFI1; lane 3, S5FI2A; lane 4, S5FI2B; lane 5, JKC18; lane 6, CFI2; lane 7, C2S5F1A; lane 8, C2S5F1B; lane 9, HLC67; lane 10, CFI3; lane 11, C3S5F1A; lane 12, C3S5F1B; lane 13, HLC69; lane 14, CFI4; lane 15, C4S5F1A; lane 16, C4S5F1B.

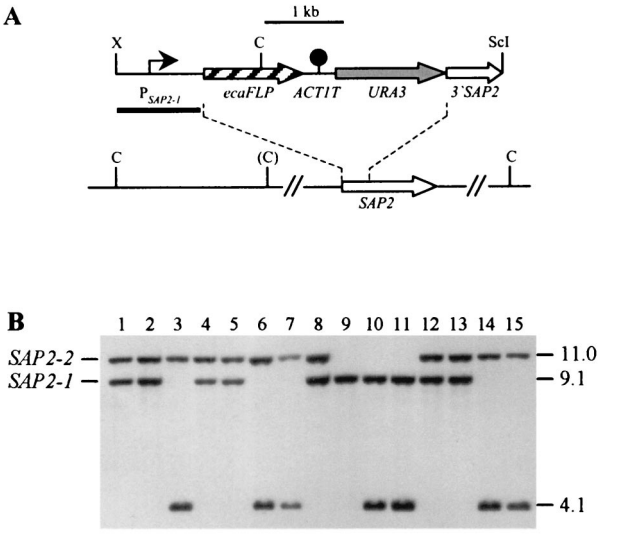

FIG. 3. Integration of the P_{SAP2}-*ecaFLP* fusion into one of the *SAP2* alleles of strains CFI2 (*cph1*/*cph1*), CFI3 (*efg1*/*efg1*), and CFI4 (*cph1*/*cph1 efg1*/*efg1*). (A) Integration scheme. The coding regions of the *SAP2* and *ecaFLP* genes are represented by the open and cross-hatched arrows, respectively. The *SAP2* promoter (P_{SAP2-1}) is indicated by the solid arrow, the *ACT1* transcription termination sequence (*ACT1T*) is indicated by the solid circle, and the *URA3* selection marker is indicated by the grey arrow. The probe used for verification of correct integration by Southern hybridization is represented by the black bar, and the diagnostic *Cla*I sites are shown. The *Cla*I site in parentheses is not present in the *SAP2-2* allele of strain CAI4 and its derivatives. C, *Cla*I; ScI, *Sac*I; X, *Xba*I. (B) Southern hybridization of *Cla*I-digested genomic DNA of parent strains and their transformants carrying the *ecaFLP* reporter gene with the *SAP2* promoter fragment as the probe. The positions of the wild-type *SAP2* alleles are indicated on the left, and the molecular sizes (in kilobases) of the wild-type *SAP2* fragments and the fragment corresponding to the reporter gene fusion are indicated on the right. Lane 1, CAI4; lane 2, CFI1; lane 3, S2FI5B; lane 4, JKC18; lane 5, CFI2; lane 6, C2S2F1A; lane 7, C2S2F1B; lane 8, HLC67; lane 9, CFI3; lane 10, C3S2F1D; lane 11, C3S2F1E; lane 12, HLC69; lane 13, CFI4; lane 14, C4S2F1B; lane 15, C4S2F1C.

(Fig. 3A). MPA-resistant transformants were selected on minimal agar plates containing 100 μg of uridine ml^{-1} and 10 μg of MPA ml^{-1}. Single colonies were picked after 5 to 7 days of growth at 30°C and restreaked on the same medium. Uridine-prototrophic transformants were selected on minimal agar plates without uridine.

Isolation of chromosomal DNA and Southern hybridization. Genomic DNA of *C. albicans* strains was isolated as described previously (20). DNA (10 μg) was digested with appropriate restriction enzymes, separated on a 1% (wt/vol) agarose gel, and, after ethidium bromide staining, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. Southern hybridization with enhanced chemiluminescence-labeled probes was performed with an ECL labeling and detection kit obtained from Amersham (Braunschweig, Germany) according to the instructions of the manufacturer.

In vivo experiments. For the in vivo experiments, 8- to 12-week-old female BALB/c mice (Harlan, Borchen, Germany) were used. To prepare the inoculum, *C. albicans* yeast cells grown overnight in YPD broth at 30°C were washed twice in phosphate-buffered saline (PBS) (Gibco, Karlsruhe, Germany) and resuspended in the same buffer. The phenotype of injected cells was controlled by spreading the inocula on MPA indicator plates. The percentage of MPA-sensitive cells was always less than 1%.

Mice were each infected intraperitoneally with 1×10^8 blastoconidia in 1.0 ml of PBS. At 30 min postinfection *C. albicans* cells were recovered by peritoneal lavage with 10 ml of PBS. Cells that adhered to the liver surface at 4 h but had not yet invaded (as determined by microscopic examination) were recovered after the peritoneal cavity was first washed to remove nonadherent cells. The organ was then cut out and homogenized with Tenbroeck tissue grinders (Wheaton Scientific) in 10 ml of sterile distilled water. After 24 and 48 h cells that adhered to or had invaded the liver were recovered in the same way. Aliquots of the lavages and the homogenates were spread on indicator plates to determine the percentage of MPA-sensitive cells.

Histology and alanine aminotransferase (ALT) activity determination. For histological examination, organs were dissected, and blocks of tissue were fixed in a 10% formaldehyde solution in PBS. The tissue samples were further processed by using standard methods for paraffin embedding and cutting. Five-micrometer sections of the organs were cut. To stain the *C. albicans* cells, the periodic acid-Schiff reaction was used. The tissue sections were incubated in a 1% solution of periodic acid in distilled water for 5 min, and this was followed by washing with distilled water and incubation with Schiff reagent (Sigma), which consisted of 1% (wt/vol) pararosaniline HCl and 4% (wt/vol) sodium bisulfite in hydrochloric acid (0.25 mol/liter). The periodic acid-Schiff reagent reaction mixture was developed in tap water for 10 min. The tissue sections were counter-

stained with Mayer's hemalum solution (Merck) for 10 s, dehydrated, and embedded in DePeX (Serva, Heidelberg, Germany).

ALT activity, an indicator of liver damage in infected mice, was determined as described previously (14).

RESULTS

Construction of *C. albicans* reporter strains in *cph1* and *efg1* mutant backgrounds. So that we could use IVET to analyze the influence of *CPH1* and *EFG1* on virulence gene expression during infection, we integrated the deletable MPA resistance marker, flanked by direct repeats of the minimal FLP recombination target (*FRT*), into the genome of the *cph1* mutant JKC18, the *efg1* mutant HLC67, and the *cph1 efg1* double mutant HLC69 (Fig. 1A). Southern hybridization analysis demonstrated that the resulting MPA-resistant transformants, CFI2 (Fig. 1B, lane 4), CFI3 (lane 6), and CFI4 (lane 8), had the deletable marker integrated into one of the *ACT1* alleles in the same way as in previously described strain CFI1 (lane 2), as shown by the appearance of an expected 3.5-kb fragment in addition to the 3.9-kb wild-type fragment (lanes 1 to 8) hybridizing with an *ACT1*-specific probe.

A fusion of the *ecaFLP* reporter gene to the *SAP5* promoter (P_{SAP5}) was then integrated into the various signal transduction mutants carrying the deletable *MPA*^{r} marker (Fig. 2A). The two *SAP5* alleles in strain CAI4 and its derivatives can be distinguished by a *Bgl*II restriction site polymorphism. As previously shown for strains S5FI2A and S5FI2B (Fig. 2B, lanes 3 and 4) carrying the P_{SAP5}-*ecaFLP* fusion in a wild-type background, derivatives of the *efg1* mutant CFI3 and the *cph1 efg1* double mutant CFI4 were isolated in which the reporter gene fusion was integrated into one of the two possible *SAP5* alleles (strains C3S5F1A and C3S5F1B [lanes 11 and 12] and strains C4S5F1A and C4S5F1B [lanes 15 and 16]). All of the transformants of strain CFI2 tested had the reporter gene fusion integrated into the *SAP5-1* allele; however, the original wild-type fragment did not disappear in these transformants, as was expected after allelic replacement (lanes 7 and 8). Close examination of the Southern blot revealed that the relative signal intensity of the band corresponding to the *SAP5-1* allele was stronger in strain JKC18 (lane 5) and its derivative, CFI2 (lane 6), than in the other parent strains, CAI4 (lane 1), HLC67 (lane 9), and HLC69 (lane 13), and their derivatives, CFI1 (lane 2), CFI3 (lane 10), and CFI4 (lane 14). This suggests that duplication of the *SAP5-1* allele had occurred in our copy of strain JKC18 and was maintained in all of its derivatives. Two independent transformants carrying the P_{SAP5}-*ecaFLP* fusion integrated into one of the duplicated *SAP5-1* alleles in the *cph1* mutant background, strains C2S5F1A and C2S5F1B (lanes 7 and 8), were used for further analysis.

To control for a possible effect of the *cph1* and *efg1* mutations on FLP activity, a $P_{SAP2\text{-}1}$-*ecaFLP* fusion was also integrated into strains CFI2, CFI3, and CFI4 (Fig. 3A), since the *SAP2* promoter could be induced by growth of the cells in YCB-BSA. The two *SAP2* alleles in strain CAI4 and its derivatives could be distinguished by *Cla*I restriction site polymorphism. Wild-type control strain S2FI5B contained the $P_{SAP2\text{-}1}$-*ecaFLP* fusion integrated into the *SAP2-1* allele (Fig. 3B, lane 3). To ensure optimal comparability, the reporter fusion was integrated into the same *SAP2* allele in two independent transformants of strains CFI2 (strains C2S2F1A and C2S2F1B [lanes 6 and 7]), CFI3 (strains C3S2F1D and C352F1E [lanes 10 and 11]), and CFI4 (strains C4S2F1B and C4S2F1C [lanes 14 and 15]). We found in this analysis that heterozygosity was lost in strain CFI3, presumably due to a recombination event that may have been induced by the electroporation, so that this strain carried two copies of only the *SAP2-1* allele based on the *Cla*I hybridization pattern (Fig. 3B, lane 9).

Induction of the *SAP2* promoter resulted in excision of the *MPA*^{r} marker from the genomes of all reporter strains with the same efficiency, irrespective of the genetic background (all of the cells were MPA^s after overnight growth in YCB-BSA). However, in contrast to the wild-type strains and the *cph1* mutants, the *efg1* and *cph1 efg1* mutants lost the *MPA*^{r} marker after growth in *SAP2*-repressing minimal medium (more than 10% of the cells in each strain), suggesting that the *SAP2* promoter is somewhat derepressed in the absence of a functional *EFG1* gene. Therefore, *SAP2* expression was not investigated further. However, these results demonstrated that the FLP recombinase works normally in all of the mutant backgrounds tested.

***CPH1* and *EFG1* contribute to *SAP5* activation during infection.** The various reporter strains carrying the P_{SAP5}-*ecaFLP* fusion were then tested for *SAP5* activation in a mouse model of *Candida* peritoneal infection. The wild-type reporter strains and the *cph1* mutants formed germ tubes that had adhered to the surfaces of parenchymatous organs at 4 h and had invaded deeply 24 h after inoculation into the peritoneal cavity (Fig. 4A and B). In contrast, no germ tubes were observed with the *efg1* single and *cph1 efg1* double mutants (Fig. 4C and D), in agreement with previous studies performed by other researchers (18). The invasiveness of the strains correlated with organ damage, as measured by ALT activity 24 h after infection (14). The ALT activities of mice infected with the wild-type control strains (124 and 177 U in the two mice tested) and with the *cph1* mutants (between 102 and 150 U in four mice) were similar. In contrast, the ALT activities were strongly reduced in both the *efg1* single mutants (between 8 and 11 U in four mice) and the *cph1 efg1* double mutants (between 7 and 10 U in four mice).

In agreement with our previous results, the *SAP5* gene was detectably activated in a significant proportion of wild-type cells soon after infection, before invasion of internal organs had occurred (at 4 h), and a minor subpopulation of the cells was induced for *SAP5* expression as soon as 30 min after inoculation into the peritoneal cavity (Fig. 5). Compared with the wild-type reporter strains, the proportion of cells in which the *SAP5* gene was detectably activated was reduced in the *cph1* and *efg1* single mutants at all infection stages. The early activation of *SAP5* was strongly dependent on the presence of a functional *EFG1* gene, since FLP-mediated marker excision was detected only in a negligible percentage of the cells 4 h after infection (2.3% ± 1.9% MPA^s cells [mean ± standard deviation], compared with 36.7% ± 12.1% MPA^s cells for the wild type). Later, significant *SAP5* activation was also observed in the *efg1* mutants (9.6% ± 2.8% and 8.5% ± 4.3% MPA^s cells at 24 and 48 h after infection, respectively, compared with 27% ± 4.9% and 37.4% ± 6.5% MPA^s cells for the wild type), although the cells were not able to form hyphae and invade the liver.

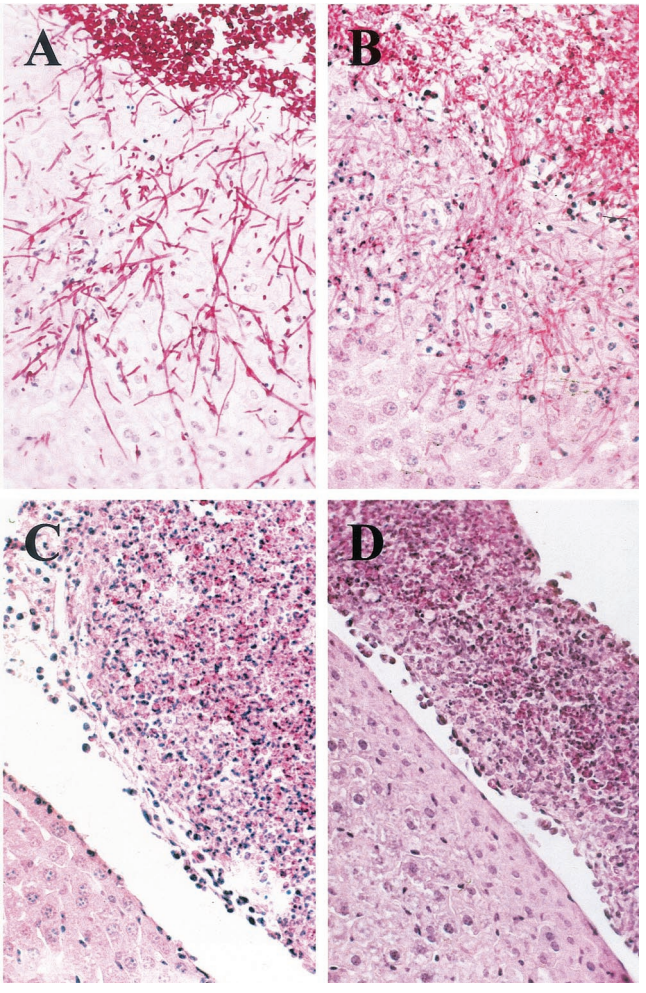

FIG. 4. Microscopic appearance of reporter strains S5FI1A (wild type) (A), C2S5F1A (*cph1*) (B), C3S5F1A (*efg1*) (C), and C4S5F1A (*cph1 efg1*) (D) 24 h after infection. Peritoneal infection by the *efg1* single mutant and the *cph1 efg1* double mutant was characterized by inflammatory infiltrates composed of yeast cells surrounded by inflammatory cells which were attached to the liver surface (C and D), whereas the wild-type strain and the *cph1* mutant invaded the liver (A and B).

In contrast, the negative effect of *cph1* inactivation on *SAP5* expression was less pronounced than the effect of the *efg1* mutation but was nevertheless clearly detectable at all infection stages (17.3% ± 3.3%, 17.5% ± 2.6%, and 18.9% ± 6.9% MPA^s cells after 4, 24, and 48 h, respectively), although the invasiveness of the *cph1* mutant was comparable to that of the wild type. In the absence of both *CPH1* and *EFG1*, *SAP5* expression was almost completely eliminated, since very few MPA-sensitive cells were recovered at all times investigated (1.1% ± 0.6%, 4.5% ± 2.1%, and 1.9% ± 0.8% MPA^s cells after 4, 24, and 48 h, respectively). In each genetic background, the two independently constructed reporter strains gave comparable results. Together, these results demonstrated that *SAP5* activation can occur in the absence of *CPH1* or *EFG1* and independent of hyphal growth, but both transcriptional regulators are necessary for full *SAP5* activation in the host.

DISCUSSION

The complex environments encountered by pathogens within their host during an infection can influence gene expression patterns in a way that is very different from the organism's response to the simpler parameters that regulate gene activity in vitro (19). In vivo, the induction of virulence genes can depend on regulatory factors which are not required for expression in vitro, and conversely, the dependence of virulence gene expression on specific regulators in vitro may be bypassed in the host by the use of alternative signaling pathways, illustrating the importance of studying regulation of virulence genes in the context of bona fide host-pathogen interactions (15, 16).

The recombination-based IVET detects expression of a target gene whenever the promoter is sufficiently activated in a cell to result in recombinase-mediated marker excision. As in a previous study (31), expression of the *SAP5* gene was observed only in a subpopulation of cells recovered from infected tissue. The heterogeneity in expression status can be explained in several ways. Both yeasts and hyphae were present in the infected host, and the different morphological forms probably differed with respect to gene expression. It is also likely that fungal cells reisolated from whole organs in fact inhabited many different microniches, only some of which may have induced *SAP5* expression. Apart from these factors, it can be assumed that individual cells in a given population differ with respect to the activation status of genes even in the same environment, so that in some cells the promoter activity is below the threshold that is necessary to detect gene expression by IVET. As long as no differences are observed in the infection progress of two strains that are compared, a reduction in the number of cells in which a target gene is detectably activated can be assumed to be caused by reduced promoter activity in the population. This should have been the case for the *cph1* mutants at all infection stages which we investigated, since we did not observe major differences in the capacities of these strains to infect internal organs in our experimental model system compared with the wild-type controls, in agreement with observations made by other workers (18). In contrast, the *efg1* and *cph1 efg1* mutants were unable to form hyphae and invade the internal organs. Especially at the late infection stages, when wild-type cells had invaded deep tissue, the different host niches in which wild-type and mutant cells were located, and not the defective signal transduction pathways alone, contributed to differences in gene expression. Nevertheless, at the early infection stages the mutants had access to the same host niches as the wild-type strains (i.e., the peritoneal cavity and the surfaces of the parenchymatous organs). Since in the wild-type strains *SAP5* activation was clearly detectable in these host niches, the failure of the mutants to induce *SAP5* at a significant level indicates the importance of *EFG1* for *SAP5* expression at this infection stage. At later times, even in the absence of hyphal formation and tissue invasion, reduced but significant *SAP5* activation was observed also with the *efg1* single mutants, which may have been induced by changes in the host environment caused by the infecting *C. albicans* cells. It seems that this activation was mediated by the Cph1p transcription factor, because almost no *SAP5* induction was detected in the *cph1 efg1* double mutants, indicating the

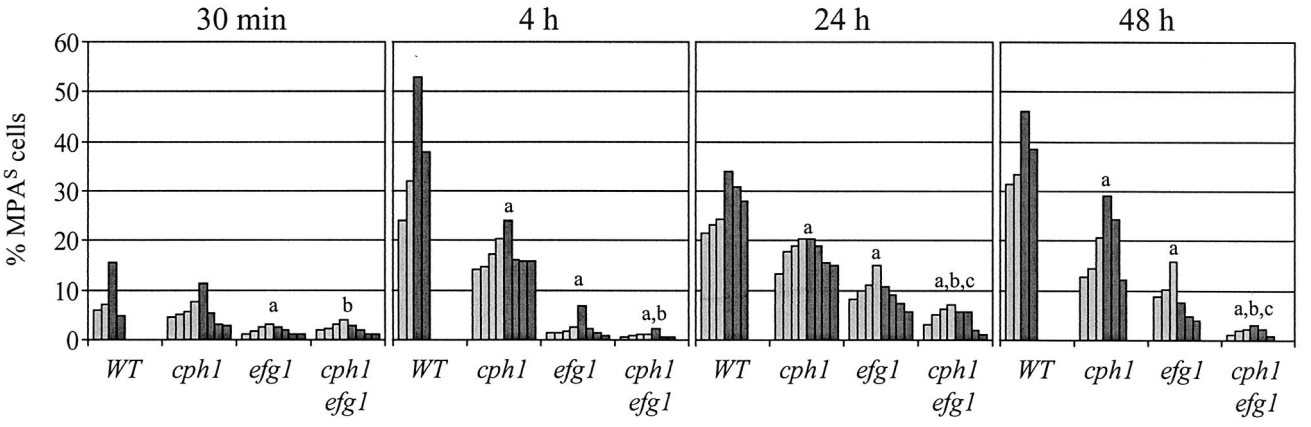

FIG. 5. Expression of the *SAP5* gene in wild-type (*WT*) and *cph1*, *efg1*, and *cph1 efg1* mutant strains at various stages of an intraperitoneal infection. In vivo *SAP5* induction was monitored by determining the percentage of MPA-sensitive cells recovered by peritoneal lavage after 30 min and recovered from the liver after 4, 24, and 48 h. The results are results from two independent experiments in which one or two mice were infected per strain and time point; each bar represents one animal. The light grey bars show the results obtained with strains S5FI2A, C2S5F1A, C3S5F1A, and C4S5F1A, and the dark grey bars show the results obtained with strains S5FI2B, C2S5F1B, C3S5F1B, and C4S5F1B. a, mutant strains were significantly different from the wild type ($P < 0.005$, as determined by Student's *t* test); b, *cph1 efg1* mutants were significantly different from the *cph1* single mutant; c, *cph1 efg1* mutants were significantly different from the *efg1* single mutant.

importance of the two signal transduction pathways for *SAP5* activation in an infected host. Interestingly, Efg1p also seems to negatively regulate expression of some genes directly or indirectly, since expression of the *SAP2* gene was derepressed in all strains lacking a functional *EFG1* gene.

Transcripts hybridizing with a probe specific for the *SAP4* to *SAP6* genes have also been detected during hyphal growth of *C. albicans* in vitro (9, 29). Because of the high levels of homology of the three genes, Northern hybridization did not reveal whether all three genes or only one or two of them were expressed under these conditions. In contrast, using antisera that recognize this subgroup of Saps, Borg-von Zepelin et al. observed expression of *SAP4* to *SAP6* only after phagocytosis by murine peritoneal macrophages and not in hyphae alone (1). We tried to detect *SAP5* expression in vitro by incubating the strains containing the P_{SAP5}-*ecaFLP* reporter fusion in a wild-type background in RPMI medium containing serum at 37°C, conditions that favor hyphal growth of *C. albicans*. Although germ tube formation and formation of hyphae were induced very efficiently, *SAP5* expression was hardly detected, since very few cells became MPA sensitive (data not shown). This result was confirmed with strains carrying a different reporter gene, *GFP*, under the control of the *SAP5* promoter. The fluorescence of the cells was hardly above the background level (unpublished observations), although like *FLP*, *GFP* has been demonstrated to be a useful reporter of *SAP2* expression under *SAP2*-inducing conditions (22). Therefore, the transcripts detected by other workers during hyphal growth either may correspond to *SAP4* and/or *SAP6* but not *SAP5* or *SAP5* expression under hypha formation conditions in vitro is below the detection limit of our reporter systems. These observations, which are in agreement with those of Borg-von Zepelin et al. (1), suggest that *SAP5* activation during infection is much stronger than the possible low-level expression under hypha-inducing conditions in vitro.

An issue to be considered when our IVET is used is that some of the colonies obtained after cells recovered from infected tissue are plated may be derived from more than one cell. If only one of the cells contains the *MPA*^{r} marker, this gives rise to an MPA^r colony, so the actual percentage of MPA^s cells is underestimated. Microscopic analysis demonstrated that there was no clumping of cells that might be responsible for the reduced percentage of MPA^s colonies of the *efg1* and *cph1 efg1* mutants. However, since the wild-type strains and the *cph1* single mutants form hyphae in infected tissue, many colonies of these strains are derived from mycelial fragments, which can consist of several unseparated cells. Therefore, the percentages of MPA^s cells of these strains may in fact have been somewhat higher than the observed percentages of MPA^s colonies, and the difference between the wild type and the *efg1* and *cph1 efg1* mutants may have been even more pronounced. However, this would only emphasize our conclusion that full activation of the *SAP5* gene during infection depends on *CPH1* and *EFG1*.

We observed that our copy of *cph1* mutant JKC18 contained a duplication of one of the *SAP5* alleles. In addition, a change from heterozygosity to homozygosity for the *SAP2* gene occurred during the construction of strain CFI3, which was derived from *efg1* mutant HLC67. Such changes may be induced by genetic manipulation of strains (transformation, fluoroorotic acid selection) but may also occur due to natural genomic alterations (26, 27). These changes were detected only later when the reporter fusions were introduced into the loci and only because we made efforts to distinguish between the two alleles of the target locus. Nonspecific genomic alterations in derivatives of a parent strain may therefore be more common than suggested by the usually limited analyses of genetically engineered strains. However, we do not think that the additional copy of *SAP5* or the homozygosity for *SAP2* influenced our results for *SAP5* regulation by *CPH1* and *EFG1*, since no such alterations were observed in the reporter strains derived from the *cph1 efg1* double mutant. The contribution of both

CPH1 and *EFG1* to *SAP5* induction was clearly observable when the *SAP5* expression in the double mutant strains was compared with the *SAP5* expression in each of the single mutants.

Several important conclusions can be drawn from our results. *SAP5* expression and hyphal morphology seem to be coincidentally linked in vitro, because both phenotypes are induced by the same or similar signals. Our results obtained with the *efg1* mutants demonstrate that *SAP5* can be expressed in the absence of hyphal formation in vivo, although *SAP5* activation occurred at a reduced level in these strains due to the involvement of *EFG1* in *SAP5* induction. In the absence of both transcription factors, *SAP5* expression was almost completely eliminated in vivo, at least in the infection model used in this study. Therefore, the avirulence of the *cph1 efg1* mutant seems to be caused not only by the nonfilamentous phenotype but also by the loss of expression of additional virulence genes. Although the ability to grow in the hyphal form is certainly important for the virulence of *C. albicans*, it will be difficult to assess the contribution of morphology per se since many other cellular characteristics change upon the yeast-hypha transition (12). These observations illustrate the complex regulatory networks that control and coordinate virulence gene expression in *C. albicans* within its host.

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