Differential activation of a *Candida albicans* virulence gene family during infection

Peter Staib*, Marianne Kretschmar†, Thomas Nichterlein†, Herbert Hof†, and Joachim Morschhäuser*‡

*Zentrum für Infektionsforschung, Universität Würzburg, Röntgenring 11, D-97070 Würzburg, Germany; and †Institut für Medizinische Mikrobiologie und Hygiene, Klinikum der Stadt Mannheim, D-68135 Mannheim, Germany

Edited by John J. Mekalanos, Harvard Medical School, Boston, MA, and approved March 30, 2000 (received for review January 24, 2000)

The yeast *Candida albicans* is a harmless commensal in most healthy people, but it causes superficial as well as life-threatening systemic infections in immunocompromised patients. *C. albicans* can colonize or infect virtually all body sites because of its high adaptability to different host niches, which involves the activation of appropriate sets of genes in response to complex environmental signals. We have used an *in vivo* expression technology that is based on genetic recombination as a reporter of gene expression to monitor the differential activation of individual members of a gene family encoding secreted aspartic proteinases (Saps), which have been implicated in *C. albicans* virulence, at various stages of the infection process. Our results demonstrate that *SAP* expression depends on the type of infection, with different *SAP* isogenes being activated during systemic disease as compared with mucosal infection. In addition, the activation of individual *SAP* genes depends on the progress of the infection, some members of the gene family being induced immediately after contact with the host, whereas others are expressed only after dissemination into deep organs. In the latter case, the number of invading organisms determines whether induction of a virulence gene is necessary for successful infection. The *in vivo* expression technology allows the elucidation of gene expression patterns at different stages of the fungus–host interaction, thereby revealing regulatory adaptation mechanisms that make *C. albicans* the most successful fungal pathogen of humans and, at the same time, identifying the stage of an infection at which certain virulence genes may play a role.

The yeast *Candida albicans* is a member of the microflora in most healthy people, where it predominantly colonizes the mucosal surfaces of the gastrointestinal tract. However, especially in immunocompromised patients, *C. albicans* develops from a harmless commensal to an opportunistic pathogen that can cause superficial as well as life-threatening disseminated infections (1). Although the immune status of the host is the major factor that determines whether *C. albicans* can become a pathogen and cause infection, the fact that *C. albicans* is by far the most frequent cause of fungal infections in such debilitated patients indicates that it must possess traits that make it a more successful colonizer and pathogen than other medically important *Candida* species or the usually apathogenic yeast *Saccharomyces cerevisiae*. It is a general view that the pathogenicity of *C. albicans* is not caused by single dominant virulence factors (2). Rather, it seems to be the high adaptability of *C. albicans* to many different host niches, as illustrated by the possession of many different adhesins that mediate binding to a variety of tissues, which allows the fungus the colonization and infection of virtually all body locations (3–6). A prerequisite for this adaptability is the capacity to respond to complex environmental signals representing the different host niches by the expression of an appropriate set of virulence-related and other genes.

C. albicans possesses a gene family encoding secreted aspartic proteinases (7, 8), and these enzymes have been linked with the virulence of the fungus since their discovery (9–11). Proposed functions of the proteinases during infection include the digestion of host proteins for nutrient supply, the evasion of host defenses by degrading immunoglobulins and complement proteins, adherence, and degradation of host barriers during invasion (12). Individual members of the gene family might have their own special role in infection, and this might be reflected by a differential expression pattern at various stages of the infection process. In concordance with this assumption is the finding that the secreted aspartic proteinase (*SAP*) genes, which are not expressed in standard laboratory media, are differentially expressed *in vitro* under special induction conditions (13–16). However, although proteinase production has been observed during experimental infection (17), the *in vivo* regulation and role in pathogenicity of the individual *SAP* genes remains unclear.

For bacterial pathogens, different *in vivo* expression technologies (IVETs) have been developed to detect expression of a gene during infection (18). One of these technologies, which uses site-specific recombination as a reporter of gene activation, is especially suitable to detect the *in vivo* induction of genes that are only transiently expressed at a certain stage of infection (19). In *C. albicans*, the development of similar reporter techniques has been hampered by the unusual codon usage of this organism (20), which is responsible for the failure to express most heterologous genes (21–23). We recently adapted the recombination-based IVET described by Camilli *et al.* (19) for use in *C. albicans*. The *FLP* gene encoding a site-specific recombinase from *S. cerevisiae* was genetically modified for expression in *C. albicans*, and FLP-mediated excision of a marker that confers resistance against mycophenolic acid (MPA) served to monitor the induction of a promoter by using *FLP* as a reporter gene (24). In this report, we describe the use of an optimized version of this IVET with enhanced sensitivity to analyze the expression of six different members of the *C. albicans SAP* gene family during superficial and disseminated infection in mice.

Materials and Methods

Strains and Growth Conditions. *C. albicans* strains used in this study are listed in Table 1. Strains were maintained on minimal agar plates containing 6.7 g yeast nitrogen base without amino acids (Bio 101), 2 g glucose, and 0.77 g of complete supplement medium without uracil (CSM-URA; Bio 101) per liter. To screen for MPA-resistant and sensitive clones, 1 μg/ml of MPA was added to minimal agar plates. Other media used were YPD (20 g peptone, 10 g yeast extract, 20 g glucose) and YCB-BSA (23.4 g yeast carbon base, 4 g BSA, pH 4.0). Strains were routinely grown at 30°C. To support growth of strain CFI1, 100 μg/ml uridine was added to the media.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: MPA, mycophenolic acid; SAP, secreted aspartic proteinase; IVET, *in vivo* expression technology.

‡To whom reprint requests should be addressed. E-mail: joachim.morschhaeuser@mail.uni-wuerzburg.de.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.110031497. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.110031497

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

Strain	Description	Reference
CFI1	*Δura3*::*imm434*/*Δura3*::*imm434, ACT1*/*act1*::*FRT-MPAR-FRT*	24
S2FI1	as CFI1, but *SAP2-2*/*sap2-1*::*SAP2P-caFLP-URA3*	24
S1FI2A	as CFI1, but *SAP1-1*/*sap1-2*::*SAP1P-ecaFLP-URA3*	This study
S1FI2B	as CFI1, but *SAP1-2*/*sap1-1*::*SAP1P-ecaFLP-URA3*	This study
S2FI5B	as CFI1, but *SAP2-2*/*sap2-1*::*SAP2P-ecaFLP-URA3*	This study
S2FI5G	as CFI1, but *SAP2-1*/*sap2-2*::*SAP2P-ecaFLP-URA3*	This study
S3FI2B	as CFI1, but *SAP3*/*sap3*::*SAP3P-ecaFLP-URA3*	This study
S3FI2C	as CFI1, but *SAP3*/*sap3*::*SAP3P-ecaFLP-URA3*	This study
S4FI2A	as CFI1, but *SAP4-1*/*sap4-2*::*SAP4P-ecaFLP-URA3*	This study
S4FI2B	as CFI1, but *SAP4-2*/*sap4-1*::*SAP4P-ecaFLP-URA3*	This study
S5FI2A	as CFI1, but *SAP5-2*/*sap5-1*::*SAP5P-ecaFLP-URA3*	This study
S5FI2B	as CFI1, but *SAP5-1*/*sap5-2*::*SAP5P-ecaFLP-URA3*	This study
S6FI2A	as CFI1, but *SAP6*/*sap6*::*SAP6P-ecaFLP-URA3*	This study
S6FI2B	as CFI1, but *SAP6*/*sap6*::*SAP6P-ecaFLP-URA3*	This study

Site-Specific Mutagenesis. The four amino acid exchanges, P2S, L33S, Y108N, and S294P, (25) were introduced into the *C. albicans*-adapted *FLP* gene (*caFLP*) in several successive steps by using a PCR-based mutagenesis procedure. A fragment comprising positions 1–109 of the *caFLP* coding sequence was amplified from plasmid pFLP6123 (24) with the primers FLP12 (5′-CAGCAGCACATGATGCTATTTTC-3′) and FLP14 (5′-TCGAG**GTCGACAAAATGT**CACAATTTGATATATTATGTA-3′), thereby introducing the P2S and L33S exchanges (nucleotide substitutions are underlined) and a translation consensus sequence (AAA, ref. 26) between the *Sal*I site and the *FLP* start codon (shown in bold). The PCR product was phosphorylated and digested with *Sal*I. A second fragment comprising *caFLP* sequences from position 110 to the *Eco*RI site at position 750 was amplified from pFLP6123 with primer FLP13 (5′-AACTAACCTATTTATGTTGG-3′) and the universal primer. After digestion of the phosphorylated PCR product at a vector-derived *Sac*I site, the two *caFLP* fragments were fused at their blunt ends and ligated between the *Sal*I and *Sac*I sites of pBluescript. The generated plasmid, pFLPT12, served as template for the introduction of the Y108N mutation by amplification of a *caFLP* fragment with the primers FLP14 and FLP15 (5′-ATCAGATTGATGTTTTTGTCCATTGTAAGGAATAATTG-3′), which subsequently was used to replace the corresponding original fragment in the *Sal*I/*Eco*RV-digested pFLPT12, creating pFLPT123. The S294P mutation was first introduced into the wild-type *FLP* gene in pFLP1 (24) by amplification of *FLP* coding sequences downstream of the *Hin*dIII site (bold letters) with primer FLP16 (5′-CAATA**AAGCTT**TGAAGAAAAATGCTCCTTATCCAATCTTTGC-3′) and the universal primer and substituting the *Hin*dIII/*Xba*I-digested PCR product for the corresponding original fragment in pFLP1, thereby generating pFLPT4. Finally, the four amino acid exchanges were combined by cloning the *Sal*I–*Cla*I fragment from pFLPT123 into the *Sal*I/*Cla*I-digested pFLPT4. The resulting plasmid pFLPT1234 contains the previously described three CTG-TTG codon exchanges in the *caFLP* gene (24) as well as the four amino acid substitutions that confer enhanced activity on the FLP recombinase (25).

Construction of *C. albicans* Reporter Strains with *SAP-ecaFLP* Fusions. To facilitate subsequent cloning steps, part of the polylinker in plasmid pSFL26, which contains *caFLP* under the control of the *SAP2* promoter (*SAP2P*) (24), was deleted by digestion with *Xho*I/*Bam*HI, filling in the recessive ends and religation, yielding pSFL27. The *SAP2* promoter from the *SAP2P-FLP* fusion in the reporter strain S2FI1 (24) was amplified with the primers SAP22 (5′-ACTTTCACT**TCTAGA**TTTTTGCAAGC-3′) and FLP9 (5′-CGAACAAGCACCTTAGGTGG-3′). The PCR product was digested with *Xba*I/*Sal*I and ligated together with a *Sal*I–*Cla*I fragment from pFLPT123 into the *Xba*I/*Cla*I-digested pSFL27, generating pSFL212, which contains the P2S, L33S, and Y108N exchanges in the *caFLP* gene. The S294P mutation then was introduced by substitution of the *Cla*I–*Spe*I fragment from pFLPT4 for the corresponding fragment in pSFL212, generating pSFL213, which carries a *SAP2P-ecaFLP* fusion (Fig. 1).

Parts of the coding regions of the *SAP1*, *SAP3*, *SAP4*, *SAP5*, and *SAP6* genes were obtained by PCR with the following primer pairs derived from the published sequences (X56867, L22358, L25388, Z30191, Z30192): SAP1C (5′-GTTATG**CTGCAG**ACATCACTATTGG-3′) and SAP1D (5′-GACCGTTAGCG-**GAGCTC**AACGGAGC-3′), SAP3C (5′-TGATT**CTGCAG**TTTCATGTCAAGCTGG-3′) and SAP3D (5′-TATAGGCT-**GAGCTC**AAGAAATTATCACC-3′), SAP4C (5′-CGGTTCCTATT**CTGCAG**CTGCTTCTAGC-3′) and SAP4D (5′-GAGCCATG**GAGCTC**TTTCTATCATCC-3′), SAP5C (5′-ACCTG**CTGCAG**TTACTTTGCACAATGAAGC-3′) and SAP5D (5′-GGAACG**GAGCTC**TTGAGGTTATTACCA-3′), SAP6C (5′-CCCGTCGATGATA**CTGCAG**AAATGTAGG-3′) and SAP6D (5′-AAGCAGGAACG**GAGCTC**TTGAGGTTTT-3′). The PCR products were digested at the introduced *Pst*I and *Sac*I sites (bold letters; nucleotide substitutions are underlined) and used to replace the 3′*SAP2* fragment in pSFL27, yielding pSFL11, pSFL31, pSFL41, pSFL51, and pSFL61.

The *SAP* promoter regions immediately upstream of the start codons were obtained by PCR amplification from strain CFI1. For *SAP1P*, the primers SAP1P1 (5′-GGTTACGGAAAA**TCTAGA**AGATGGCCC-3′) and SAP1P2 (5′-TGTGT**GTCGAC**TTAGAAATGGAAGAGTGA-3′), derived from the published sequence (L12451) were used. The sequences of the *SAP4*, *SAP5*, and *SAP6* promoter regions were kindly provided by Klaus Schröppel (Institut für Klinische Mikrobiologie und Immunologie, Erlangen, Germany) and amplified with the primer pairs SAP4P1 (5′-CGGGAAGGC**TCTAGA**ATGTATACCC-3′) and SAP4P2 (5′-AACAT**GTCGAC**TTGAGTT-

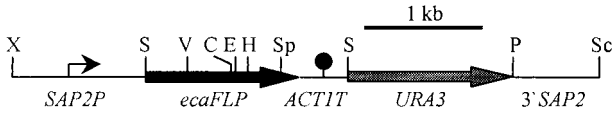

Fig. 1. Structure of the insert in plasmid pSFL213 carrying the *SAP2P-ecaFLP* fusion. Relevant restriction sites used for cloning are shown. C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sc, *Sac*I; Sp, *Spe*I; V, *Eco*RV; X, *Xba*I. For the construction of analogous fusions with other *SAP* genes, the corresponding fragments were substituted for the flanking *SAP2P* and 3′*SAP2* regions (see *Materials and Methods*).

GAACTTTGGATTAG-3′), SAP5P3 (5′-GTATAAATGC**TCTAGA**ATTCTGTTTGGCG-3′) and SAP5P2 (5′-ACATT**GTCGAC**TTGAGCTTAACTTTGGATTAG-3′), and SAP6P1 (5′-TCTCGGGAGGC**TCTAGA**ATGTATCA-3′) and SAP6P2 (5′-ACATT**GTCGAC**TTGAGCTTAACTTTAGATTAG-3′). To obtain the *SAP3* promoter, a clone carrying the *SAP3* gene was first identified by screening a *C. albicans* fosmid library (kindly provided by Stewart Scherer, Acacia Biosciences, Richmond, CA) with a probe from the *SAP3* coding region. A suitable subfragment of a positive clone (1H10) was cloned, and partial sequence analysis revealed that *SAP3* was located 1.1 kb downstream of the *CDR1* gene. The primers SAP3P1 (5′-CATTGTTA**TCTAGA**CGTGGTGG-3′), derived from the *CDR1* downstream sequence (X77589), and SAP3P2 (5′-AACAT**GTCGAC**TATATATGTGTATGTGTGTGTGG-3′) were used to amplify the *SAP3* promoter region. All *SAP* promoter fragments were cut at the introduced *Xba*I and *Sal*I sites (bold letters; nucleotide substitutions are underlined) and used to replace the *SAP2* promoter in pSFL11, pSFL31, pSFL41, pSFL51, and pSFL61, generating pSFL12, pSFL32, pSFL42, pSFL52, and pSFL62, respectively. The *Sal*I–*Spe*I fragment with *caFLP* sequences then was replaced in these plasmids by the corresponding *ecaFLP* fragment from pFLPT1234, resulting in pSFL13, pSFL33, pSFL43, pSFL53, and pSFL63. The inserts from these plasmids and pSFL213 were obtained by *Xba*I/*Sac*I digestion, gel-purified, and used to replace one of the *SAP* alleles in the ura-negative strain CFI1 by the corresponding *SAP-ecaFLP* fusion after electroporation (27) and selection of transformants on minimal agar plates without uridine.

Isolation of Chromosomal DNA and Southern Hybridization. Chromosomal DNA from *C. albicans* strains was isolated as described (28). Southern hybridization with enhanced chemiluminescence-labeled probes was performed with the ECL labeling and detection kit provided by Amersham Pharmacia, according to the instructions of the manufacturer.

***In Vivo* Experiments.** Eight- to twelve-week-old female BALB/c mice, which were used for i.v. and i.p. infection, or DBA/2 mice, which were used for oral infection, were purchased from Harlan (Borchen, Germany). To prepare the inoculum, *C. albicans* cells grown overnight in YPD broth at 37°C were washed twice in PBS (GIBCO) and resuspended in the same buffer. In all experiments, the inocula also were spread on MPA indicator plates to verify that no FLP induction had occurred before infection.

Infection of the oesophageal mucosa was produced after pretreating the animals 7 days and 24 h before infection with cyclophosphamide injected i.p. in a dosage of 150 mg/kg and 100 mg/kg, respectively. In addition, 24 h before infection, mice were treated orally with amoxycillin (10 mg per animal), gentamicin (8 mg per animal), and clindamycin (10 mg per animal). For oral infection, 1×10^8 blastoconidia were given by gavage. Three days after infection, mice were killed by cervical dislocation, and the oesophagus was removed aseptically and homogenized with Tenbroeck tissue grinders (Wheaton Scientific) in 5 ml of sterile distilled water supplemented with 5 μg/ml gentamicin. Aliquots of the homogenates were plated on indicator plates containing 1 μg/ml MPA and incubated at 30°C for 2 days to determine the percentage of MPA-sensitive cells, which gave rise to uniformly smaller colonies as compared with cells that contained the MPA^R marker and produced large colonies. Because there is sometimes a low background of cells that accidentally grow as smaller colonies of varying size (<2%; ref. 24), the MPA-sensitive phenotype was confirmed by restreaking on plates containing 10 μg/ml MPA in cases where the percentage of small colonies was only slightly higher than the unspecific background.

For induction of peritonitis, mice were infected i.p. with 5×10^7 blastoconidia in 0.5 ml of PBS. Thirty minutes 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 confirmed by microscopic examination) were recovered by first washing the peritoneal cavity to remove nonadherent cells. The organ then was cut out, homogenized in 10 ml of sterile distilled water, and plated. At 48 h postinfection, mesenteric lymph nodes and the infiltrated organs liver, pancreas, and spleen were cut out as a whole, homogenized, and plated as described above. The kidneys were removed separately and treated in the same way.

For i.v. infection, mice received either 2×10^5 or 4×10^5 blastoconidia in 0.2 ml of PBS by means of the lateral tail vein. Four days after infection, the mice were killed by cervical dislocation. The kidneys were removed aseptically and homogenized, and the percentage of MPA^S *C. albicans* cells was determined as described above.

Results

A *C. albicans* IVET with Enhanced Sensitivity. With an IVET that is based on genetic recombination as a reporter of gene expression, the activation of a gene can be detected in single cells whenever there is a sufficient concentration of the recombinase enzyme in a cell to result in excision of the marker, i.e., when the activity of the promoter is above a threshold level. Using a reporter strain that carried a *C. albicans*-adapted *FLP* gene (here referred to as *caFLP*) under control of the *SAP2* promoter, we previously could detect activation of the *SAP2* gene by monitoring FLP-mediated excision of an MPA^R marker, which rendered the cells MPA sensitive (24). After growth in *SAP2*-inducing medium, the majority (70–80%), but not all cells had lost the marker, indicating that, in some cells, the *SAP2* gene was not sufficiently activated to result in detectable FLP activity. Differences in *SAP2* expression within a population had previously also been detected by using *GFP* as a reporter gene (23); however, in this case virtually all cells fluoresced, although with different intensities. These observations suggested that the FLP-based IVET was not sensitive enough to detect low-level activation of a gene.

Recently, a mutated FLP recombinase, eFLP, has been described that carries four amino acid exchanges, P2S, L33S, Y108N, and S294P, which confer enhanced activity on the enzyme (25). To increase the sensitivity of our IVET for *C. albicans*, we introduced these four amino acid substitutions into caFLP. A reporter strain then was constructed that carried the *ecaFLP* gene under control of the *SAP2* promoter. The resulting strain S2FI5B differed from the previously described strain S2FI1 (24) only by the presence of *ecaFLP* instead of *caFLP* and carried the reporter fusion in the same *SAP2* allele (see also below).

FLP activity in strains S2FI1 and S2FI5B was monitored after a shift from *SAP2*-repressing medium into *SAP2*-inducing medium in which *SAP2* mRNA appears after 6–8 h of growth (24). As can be seen in Fig. 2, *ecaFLP* proved to be a more sensitive reporter of gene activation than *caFLP*, because FLP activity in strain S2FI5B was detected earlier than in strain S2FI1. In addition, *SAP2* activation was detected in all cells of strain S2FI5B because, after 12 h, the whole population had become MPA sensitive. In contrast, only 90% of the cells of strain S2FI1 had become MPA sensitive after 16 h, and this percentage was not further elevated even after 28 h (data not shown). Therefore, *SAP2* activation in *C. albicans* cells in which the gene was induced only at low levels could be detected with *ecaFLP* but not with *caFLP* as a reporter gene.

Construction of a Set of *C. albicans* Reporter Strains. To analyze the expression of individual members of the *SAP* gene family during infection, *ecaFLP* was fused to the promoters of the *SAP1-SAP6* genes and integrated into one of the corresponding *SAP* alleles

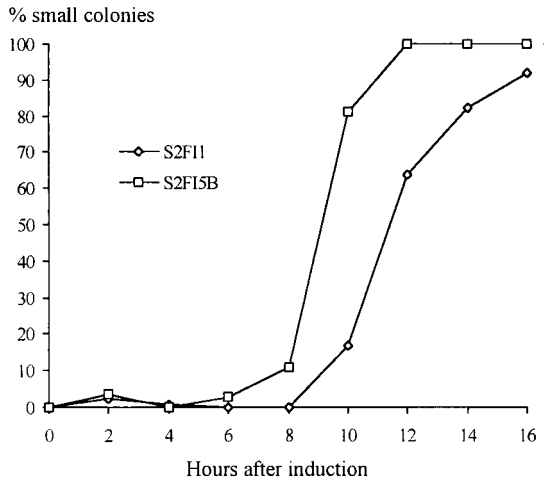

Fig. 2. FLP activity in *C. albicans* strains S2FI1 and S2FI5B carrying chromosomally integrated *caFLP* and *ecaFLP* genes, respectively, under control of the *SAP2* promoter. Cells were grown overnight in *SAP2*-repressing minimal medium and inoculated into *SAP2*-inducing medium YCB-BSA. FLP-mediated excision of the *MPA*^{R} marker was analyzed by determining the percentage of MPA^S cells at the indicated times.

of strain CFI1 that carries the deletable *MPA*^{R} marker. Southern hybridization analysis of the transformants confirmed that the fusions had been correctly integrated and that the other *SAP* genes remained unchanged in each case (Fig. 3). The two *SAP4* and the two *SAP5* alleles could be distinguished by a *Bgl*II restriction site polymorphism, and the *SAP1* and *SAP2* alleles by *Kpn*I and *Cla*I restriction site polymorphisms, respectively. In these cases, the two alleles were arbitrarily assigned a suffix (e.g., *SAP1-1* and *SAP1-2*), and two transformants carrying the reporter gene fusion in either of the two possible *SAP* alleles were selected. For *SAP3* and *SAP6*, no restriction site polymorphisms were found, and two randomly selected transformants were kept in each case. None of the strains had lost the *MPA*^{R} marker during selection on minimal plates and subsequent growth in YPD medium, demonstrating that, despite the enhanced sensitivity of the reporter system, all strains were stable during growth in standard laboratory media in which expression of *SAP* genes is repressed.

Differential Expression of *SAP* Genes During Infection of the Oesophageal Mucosa. The 12 reporter strains were used in a mouse model of oesophageal infection in which *C. albicans* invades into the epithelium but does not disseminate through the bloodstream. After 3 days of infection, heavy mycelial growth of the strains and mucosal invasion was observed. The fungal cells were recovered from the infected tissue, and the percentage of MPA-sensitive cells was determined. Fig. 4 shows that the *SAP5* and *SAP6* genes were strongly activated during infection of the oesophageal mucosa, whereas only low-level induction of *SAP1–SAP4* had occurred.

Stage-Specific Activation of *SAP* Genes During Intraperitoneal Infection. To monitor expression of the various *SAP* genes during systemic disease, a mouse model of *Candida* peritonitis was used, and the reporter strains were reisolated at various stages and from different sites of the infection. The *SAP5* gene was significantly activated already 30 min after inoculation in the peritoneal cavity, whereas the other *SAP* genes were induced in only a few cells (Fig. 5*A*). Remarkably, only yeast cells but no germ tubes or hyphae were detected at this early stage of the infection. After 4 h, *C. albicans* cells that adhered to the liver surface

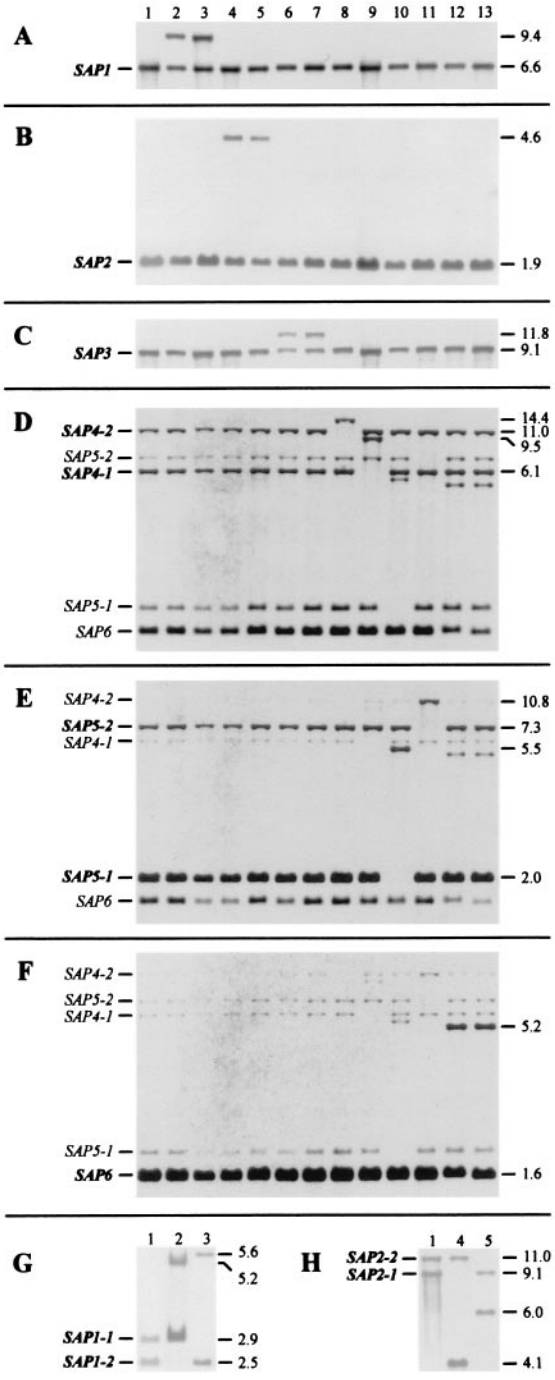

Fig. 3. Construction of *C. albicans* reporter strains carrying chromosomally integrated fusions of the promoter regions of *SAP1-SAP6* with *ecaFLP*. (*A–F*) Southern hybridizations of *Bgl*II-digested genomic DNA with the promoter fragments of the *SAP1–SAP6* genes as probes. To distinguish between integration into either of the two *SAP1* and the two *SAP2* alleles, *Kpn*I (*G*) and *Cla*I (*H*), digested DNA of the corresponding strains was used additionally. The fragments representing the wild-type *SAP* alleles are indicated in bold letters on the left side of the blots; the crosshybridizing *SAP4*, *SAP5*, and *SAP6* alleles also are indicated. The sizes of the wild-type fragments and those containing the reporter fusions are shown on the right side of the blots. Lanes: 1, CFI1 (parent strain); 2, S1FI2A (*sap1-2*::*SAP1P-ecaFLP*); 3, S1FI2B (*sap1-1*::*SAP1P-ecaFLP*); 4, S2FI5B (*sap2-1*::*SAP2P-ecaFLP*); 5, S2FI5G (*sap2-2*::*SAP2P-ecaFLP*); 6, S3FI2B (*sap3*::*SAP3P-ecaFLP*); 7, S3FI2C (*sap3*::*SAP3P-ecaFLP*); 8, S4FI2A (*sap4-2*::*SAP4P-ecaFLP*); 9, S4FI2B (*sap4-1*::*SAP4P-ecaFLP*); 10, S5FI2A (*sap5-1*::*SAP5P-ecaFLP*); 11, S5FI2B (*sap5-2*::*SAP5P-ecaFLP*); 12, S6FI2A (*sap6*::*SAP6P-ecaFLP*); 13, S6FI2B (*sap6*::*SAP6P-ecaFLP*).

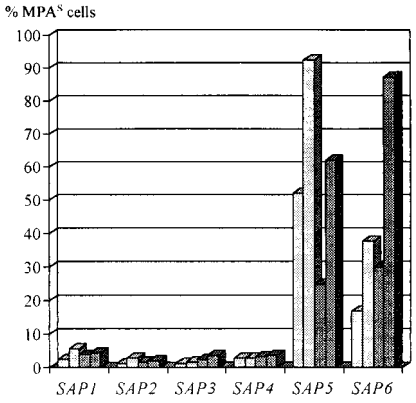

Fig. 4. Expression of individual *SAP* genes during oesophageal infection. For each of the reporter strains, two mice were orally infected, and *C. albicans* cells that had invaded the oesophageal epithelium were reisolated from homogenized tissue after 3 days of infection. Activation of the *SAP* genes in the reporter strains was analyzed by determining the percentage of MPA^S cells. The light gray columns show *SAP* induction in strains S1FI2A, S2FI5B, S3FI2B, S4FI2A, S5FI2A, and S6FI2A, and the dark gray columns show the results obtained with S1FI2B, S2FI5G, S3FI2C, S4FI2B, S5FI2B, and S6FI2B.

exhibited mycelial growth, and a large portion of the population had activated *SAP5* but not, or only at low levels, the other *SAP* genes (Fig. 5*B*). After 48 h, when the cells had invaded into the liver, a significant activation of *SAP6* and, as previously reported, *SAP2* had occurred in addition to *SAP5* (Fig. 5*C*). At this time point, the *C. albicans* cells also had disseminated by the bloodstream into the kidneys, and a *SAP* expression pattern similar to that seen after liver invasion was observed (Fig. 5*D*).

***SAP* Expression after Hematogenous Dissemination.** Because loss of the *MPA*^{R} marker is an irreversible event, down-regulation of a gene after a previous induction cannot be detected with the IVET used. To determine whether some *SAP* genes are induced only during the early stages of a systemic infection but are not expressed after *C. albicans* has gained access to the bloodstream, an additional model of disseminated candidiasis in which the mice are infected by the i.v. route was used. In this model, a different outcome of the infection is seen within a narrow range of the infectious dose (24). With an inoculum of 4×10^5 cells, a strong multiplication of *C. albicans* occurred after hematogenous dissemination, resulting in 10^6 to 10^7 colony-forming units that could be recovered from the kidneys. The expression of

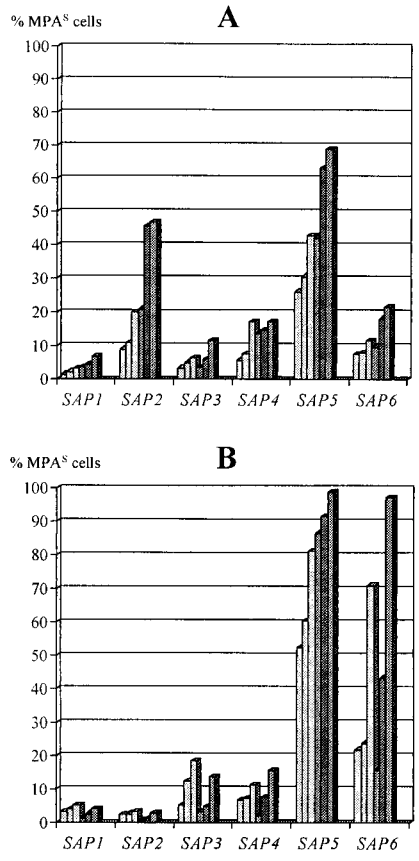

Fig. 6. Differential activation of *SAP* genes after hematogeneous dissemination. For each of the reporter strains, three mice were i.v.-infected with 4×10^5 cells (*A*) and three mice with 2×10^5 cells (*B*). *SAP* activation was determined in cells recovered from the kidneys after 4 days of infection. Strains are as described in the legend to Fig. 4.

SAP5, the first *SAP* gene induced after i.p. infection, was maintained after *C. albicans* had reached the bloodstream, because the gene also was activated after i.v. infection (Fig. 6*A*). In addition, *SAP4* and *SAP6* were significantly induced after dissemination to the kidneys, although at lower levels. The induction of *SAP2* at this late stage of the infection, which had been reported previously (24), could be confirmed in these

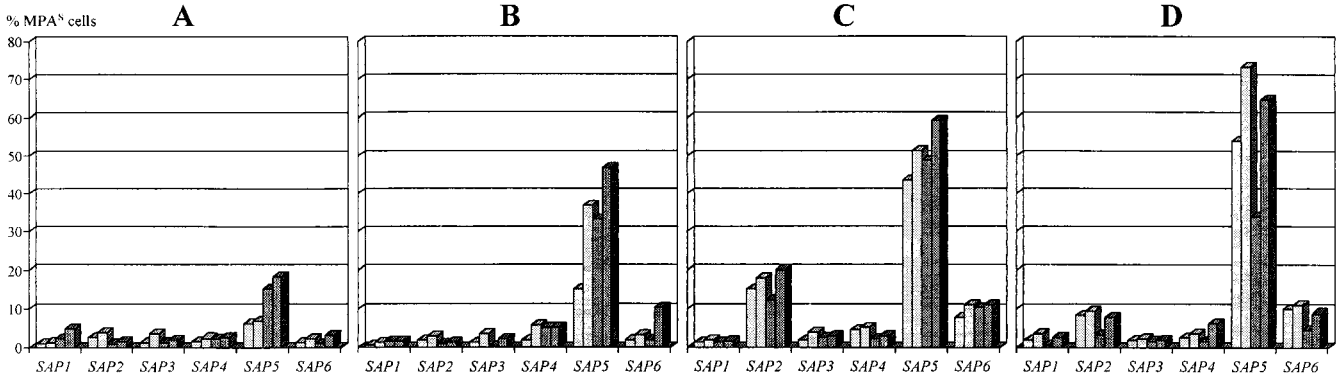

Fig. 5. Stage-specific induction of individual *SAP* genes during the course of i.p. infection. For each of the reporter strains, two mice were infected i.p., and *SAP* activation was determined in cells recovered by peritoneal lavage 30 min after inoculation (*A*), in cells adhering to the tissue after 4 h of infection (*B*), and in cells that had invaded the liver (*C*) or disseminated to the kidneys (*D*) after 48 h of infection. Strains are as described in the legend to Fig. 4.

experiments. Expression of *SAP1* and *SAP3* was more variable, because it was clearly detected in some animals but occurred only at very low levels in others. Most strikingly, the activation of some *SAP* genes was extremely dependent on the outcome of the infection. With a slightly lower inoculum dose (2×10^5 cells), the organ load in the kidneys was decreased by about 2 orders of magnitude as compared with the higher inoculum. In this case, *SAP2* expression was barely detectable, but other *SAP* genes were activated more strongly than with the higher infection dose that resulted in an overwhelming infection. Especially *SAP5* and *SAP6* had been induced in almost the whole population of infecting cells in some animals (Fig. 6*B*).

Discussion

The results of this study demonstrate that the recombination-based IVET for *C. albicans* is highly useful to analyze gene expression patterns at various stages of the fungus–host interaction. We have shown that *C. albicans* adapts to different host niches by the activation of appropriate subsets of *SAP* genes, according to the needs of the fungus in the course of an infection. It previously has been suggested that environmental parameters, like the pH of the host niche, determine which *SAP* gene is expressed, and that *SAP4*, *SAP5*, and *SAP6* are hyphae-specific genes (13, 14). Our results suggest that this is probably too simplistic a view and that the host signals governing the activation of specific *SAP* genes are more complex. *SAP5* and *SAP6*, but not the other *SAP* genes, were strongly activated during invasion of the oesophageal mucosa by *C. albicans* hyphae. *SAP5*, but not *SAP6*, also was induced immediately after inoculation in the peritoneal cavity, a host niche that is different from the oesophageal mucosa. Most strikingly, *SAP5* expression at this stage of the infection did not correlate with the presence of germ tubes or hyphae because only blastoconidia were observed in the peritoneal lavage. Therefore, the expression of *SAP5* may be induced by host signals that also result in hyphae formation, and both *SAP5* expression and mycelial growth then could contribute to tissue invasion. In contrast, *SAP6* activation was detected only when *C. albicans* hyphae also were observed in the infected tissue, and *SAP6* may indeed be a hyphae-specific gene.

The *SAP2* gene was significantly activated only in the late stages of infection, after spread to deep organs and concomitantly with tissue destruction as reported in a previous study (24). Because *SAP2* is activated *in vitro* when proteins are the sole nitrogen source, the Sap2 proteinase may allow *C. albicans* to thrive within the destroyed tissue by degrading host proteins for nutrient supply. Accordingly, no significant *SAP2* expression was detected after i.v. infection with a lower dose, which resulted in relatively few organisms reaching the kidneys, unable to effect the same tissue destruction as seen with a fulminant infection. It is very intriguing that the reverse effect was seen with the *SAP5* and *SAP6* genes that may be needed for tissue invasion. With lower numbers of cells attacking the deep organs, the majority of invading *C. albicans* cells apparently were forced to activate these weapons, whereas induction of the proteinases in only a part of the fungal population seems to be sufficient when an overwhelming infection takes place. After hematogenous dissemination, activation of additional *SAP* genes also was detected, although this varied among different animals. *SAP4* expression was consistently observed, but at lower levels than those seen for *SAP5* and *SAP6*, which are highly homologous to *SAP4*, the three genes forming a subgroup within the *C. albicans SAP* gene family (7). *SAP1* and *SAP3* were not activated to high levels in any of the infection models used; nevertheless, significant expression of these genes was detected in some animals. Interestingly, *SAP1* and *SAP3* are phase-specific genes in the *C. albicans* strain WO-1, where they are expressed only in the opaque phase but not in the white phase (29). Although strain SC5314, from which our reporter strains are derived, does not display the white-opaque switching, a similar phenomenon may result in a phase-variable expression of the *SAP1* and *SAP3* genes in this strain. However, the possibility remains that expression of these genes might not need strong promoter activity because low-level transcription could be compensated for by enhanced transcript stability or enhanced translation. In this case, a transcriptional activation of *SAP1* and *SAP3* that could be relevant *in vivo* might not be sufficiently strong to result in FLP activity.

C. albicans colonizes and infects more host niches in addition to those analyzed in our present study, for example the oral cavity or the vagina. Because the environmental conditions at these sites differ from other locations, *C. albicans* probably adapts to these niches by the expression of different sets of *SAP* and other virulence genes (16, 30, 31), which is supported by the results obtained with specific mutants (32). As a consequence of the differential expression of individual *SAP* genes at various stages of the infection, one would expect that the individual SAP isoenzymes differ with respect to their biochemical properties, allowing the fungus to use the proteinase that is best adapted to a certain environment and for a specific function. Purification of some of the proteinases has indeed shown differences, for example in their pH optimum (33). The recent successful expression of individual *SAP* genes in a heterologous host (15) should allow the dissection of the functional differences of the various isoenzymes and relate their properties to the *in vivo* expression pattern within the host.

We thank Klaus Schröppel for providing the sequences of the *SAP4–6* promoter regions. This study was supported by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (Grant O1 K1 8906-0) and the Deutsche Forschungsgemeinschaft (Grant MO 846/1-1). P.S. is the recipient of a grant from the Studienstiftung des deutschen Volkes.

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