INFECTION AND IMMUNITY, Dec. 1997, p. 4909–4917 Vol. 65, No. 12
0019-9567/97/$04.00+0

A Gene Homologous to *Saccharomyces cerevisiae SNF1* Appears To Be Essential for the Viability of *Candida albicans*

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Received 27 May 1997/Returned for modification 26 June 1997/Accepted 15 September 1997

The *SNF1* gene of *Saccharomyces cerevisiae* (*ScSNF1*) is essential for the derepression of catabolic repression. We report here the isolation and characterization of an *SNF1* homolog from *Candida albicans* (*CaSNF1*) which is apparently essential for the viability of this organism. The putative amino acid sequence of *CaSNF1* has 68% identity with that of *ScSNF1* and can restore the *S. cerevisiae snf1*Δ mutant's ability to utilize sucrose. Disruption of one of the *CaSNF1* alleles resulted in morphological changes and decreased growth rates but did not modify the carbon source utilization pattern. Repetitive unsuccessful attempts to generate a *snf1*/*snf1* homozygote by disruption of the second allele, using various vectors and approaches, suggest the lethal nature of this mutation. Integration into the second allele was possible only when a full-length functional *SNF1* sequence was reassembled, further supporting this hypothesis and indicating that the indispensability of Snf1p prevented the isolation of *snf1*/*snf1* mutants. The mutant bearing two disrupted *SNF1* alleles and the *SNF1* functional sequence maintained its ability to utilize sucrose and produced stellate colonies with extensive hyphal growth on agar media. It was demonstrated that in a mouse model, the virulences of this mutant and the wild-type strain are similar, suggesting that hyphal growth in vitro is not an indicator for higher virulence.

Candida albicans is a yeast-like pathogen known to cause serious infections in immunocompromised patients (21). This diploid, asexual organism exhibits remarkable variation in many of its features, including colony morphology (3, 33), electrophoretic karyotype (30, 38, 39), carbon utilization pattern (25), and virulence (25).

One variant, *C. albicans* var. *stellatoidea* (*C. stellatoidea* type I), has received considerable attention due to its reduced virulence in animal model compared to the *C. albicans* reference strains (22, 39). *C. albicans* var. *stellatoidea* was initially identified by its inability to ferment or assimilate sucrose and its stellate colony morphology on blood agar medium (19). Further characterizations and comparisons with the reference *C. albicans* strain revealed a lower growth rate, higher sensitivity to UV radiation, and the inability to assimilate glycerol (22). Rarely, sucrose-positive revertants of *C. albicans* var. *stellatoidea* do emerge, exhibiting coreversion of many other characteristics. For example, the sucrose-positive revertants grow faster, exhibit increased virulence, and assimilate glycerol, suggesting the involvement of a global regulatory gene (39). Sucrose utilization by itself, however, appears to be unrelated to virulence in *C. albicans* since the *casuc1* mutant, which does not express sucrose-inhibitable α-glucosidase, and its revertant are equally virulent in mice (20, 22). These observations in *C. albicans* var. *stellatoidea* suggest that sucrose utilization may serve as a useful marker for the study of regulatory pathways which may be shared by cellular processes pertinent to pathogenicity of *C. albicans*.

The *Saccharomyces cerevisiae SNF1* gene (*ScSNF1*) encodes a serine/threonine protein kinase that is essential for the removal of catabolic repression (6). Alenza and Carlson first characterized the *SNF1* gene in several *S. cerevisiae* mutants unable to utilize sucrose as the sole carbon source (6). Its importance as a global regulator became evident when additional *snf1* mutants were isolated based on their inability to utilize ethanol (*ccr 1*) or glycerol and maltose (*cat 1*) (6). The protein Snf1p (72 kDa) interacts with the Mig1p-Ssn6p-Tup1p repressing complex and acts antagonistically to a protein phosphatase type I. This interaction relieves the inhibition of transcription of various target genes whenever glucose is not available to the cell (35). Snf1p also interacts with various transcriptional activators by facilitating their expression through specific target promoters (23, 24, 40). Moreover, it was recently suggested that *S. cerevisiae* Snf1p (ScSnf1p) interacts directly or indirectly in the RAS-cyclic AMP signal transduction pathway, further underscoring its importance (34, 36). Mutations in the *SNF1* gene affect multiple cellular processes such as glycogen accumulation (16), hypersensitivity to heat shock and starvation (34), peroxisome proliferation (32), and sporulation (18). Studies of *SNF1* homologs from humans (1), rats (4), plants (2, 14, 15, 26), and protozoan parasites (27) suggest that *SNF1* plays a crucial role in various stress response mechanisms as well as the regulation of metabolic pathways such as biosynthesis of cholesterol and other isoprenoid compounds (4).

In this study, we report the isolation and characterization of the *C. albicans SNF1* (*CaSNF1*) homolog. The heterozygote generated by the disruption of one of the *SNF1* alleles exhibited carbon utilization pattern identical to that of the wild-type strain but showed morphological variations and significantly reduced growth rate. Inability to disrupt the second allele, while integration of DNA at this site is readily accomplished, suggests that *C. albicans* SNF1p (CaSNF1p) is necessary for the viability of *C. albicans*. These observations contradict findings in *S. cerevisiae* and *C. glabrata*, both phylogenetically related to *C. albicans* (6, 28).

MATERIALS AND METHODS

Strains and plasmids. The strains used in this study and their genotypes are listed in Table 1. The plasmids used in this study were constructed as described below, and their features are listed in Table 2.

DNA and RNA purification. DNA from yeast cells was obtained as was previously described (8). A FastPrep FP120 centrifuge and FastRNA Red kit (Bio

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TABLE 1. Strains used in this study

Strain	Genotype	Source
C. albicans		
B311	Reference strain	ATCC 32354
SC5314	Clinical isolate, wild-type strain	W. Fonzi
CAI4	*ura3/ura3*	W. Fonzi
FD9	*ura3/ura3 SNF1/snf1::hisG-URA3-hisG*	This study
FD97, FD92	*ura3/ura3 SNF1/snf1::hisG*	This study
SD20	*ura3/ura3 snf1::cSNF1-URA3/snf1::hisG*	This study
S. cerevisiae		
MCY2916	*MAT*α *snf1Δ10 his3Δ200 ura3-52 lys2-801 leu2-3,112*	M. Carlson
MCY829	*MAT*α *his3Δ200 ura3-52 lys2-801*	M. Carlson

101, Vista, Calif.) were used to extract RNA from log-phase yeast cells. Bacterial plasmid DNA was isolated by using either an RPM or RPM-AFS kit (Bio 101).

Media and growth conditions. *C. albicans* and *S. cerevisiae* strains were maintained on YEPD agar (1% yeast extract, 2% peptone, 2% glucose) slants. Transformants were selected on YNB agar (yeast nitrogen base without amino acids; 6.7 g/liter; Difco) supplemented with either 2% glucose (YNBG), 2% sucrose (YNBS), or 2% glycerol (YNBGL) and other compounds essential for the growth of each mutant. Induction of hyphal growth on L-proline as a sole nitrogen source or nitrogen starvation was performed by using various growth media as described previously (11). The effect on hyphal growth induction of pH ranging between 4.7 and 8.0 was tested on YNBG, YNBS, L-proline, and low-ammonium sulfate agar (0.05 mM ammonium sulfate) adjusted to the appropriate pH. *Escherichia coli* was grown on Luria-Bertani broth or agar supplemented with 150 μg of ampicillin (Amersco, Solon, Ohio) per ml. Bacterial and yeast cells were grown at 37 and 30°C, respectively.

PCR. PCRs were performed with an MJ Research thermocycler, using *Taq* DNA polymerase and nucleotide mix from Boehringer-Mannheim Corp. (Indianapolis, Ind.). Two degenerative oligonucleotide primers compatible with the conserved regions of the *SNF1* gene (5′CGCAAGCTT[CT]CA[GA]CA[GA]AT[CAT]AT[CAT]AG3′ and 5′CGAAGCTTC[CT]TC[AG]TC[AG]TC[AG]AA[ACGT]GG3′) were used to amplify fragment of the *CaSNF1* homolog. Twenty-nine amplification cycles (melting at 94°C for 1 min; annealing at 50°C for 1 min; polymerization for 72°C and 1 min) were followed by an additional step of extended polymerization step (10 min). The PCR product which hybridized to the *ScSNF1* probe under low-stringency conditions (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate, 50°C) was cloned into the *Hin*dIII site of the pBluescript SK$^+$ vector (Stratagene, La Jolla, Calif.).

DNA transformation. *S. cerevisiae* was transformed by using a modified lithium acetate method (10, 17). *C. albicans* was transformed by electroporation as described by Varma et al. for *Cryptococcus neoformans* (37). Electrocompetent *E. coli* cells were transformed by electroporation as instructed by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.).

Cloning of the *C. albicans* cDNA and genomic DNA sequence. A cDNA library, previously constructed in the Uni-Zap XR vector (9), was screened with the PCR-generated *SNF1* DNA probe (600 bp). pBluescript phagemids were excised in vivo from positive plaques by using the R408 helper phage (Stratagene), and their inserts sequences were analyzed. One of these clones, termed pCSA2, was subjected to further investigations.

On the basis of Southern blot analysis of genomic DNA, a size-selected genomic library (3 to 5 kb) was constructed in the pBluescript SK$^+$ vector. This library was used to transform *E. coli*, and the transformants were screened with the *CaSNF1* cDNA probe. One of the clones, pGSA4, carrying a 4-kb insert, was used for further studies.

Construction of a complementation vector. The 4-kb *Eco*RI genomic fragment containing the *CaSNF1* sequence along with its flanking regions was cloned into plasmid YEp24. The resulted vector, pYGS4, was used to transform the *S. cerevisiae snf1* deletion mutant MCY2916 (Table 1). Transformants were selected for uracil prototrophy and/or sucrose utilization. Genomic DNAs obtained from the complemented *S. cerevisiae* cultures were used to transform *E. coli*. Plasmids rescued from these transformants were subjected to further characterization.

Construction of *CaSNF1* disruption vectors. (i) pSUR2. An *Eag*I-*Hpa*I fragment (1.2 kb) was deleted from plasmid pGSA4 and substituted with a 400-bp PCR-generated fragment containing 200 bp of the *CaSNF1* coding region and its 3′ flanking region (primer sequences were ATTAGCGTTAACTGGCACATG GAAATGCAG [*Eco*RI site underlined] and AATTAACCCTCACTAAAGGG [vector sequence]), yielding pΔGSA4. *Bgl*II linkers were inserted into the regenerated *Hpa*I site in order to clone the 4-kb *Bgl*II-*Bam*HI fragment containing the *hisG-URA3-hisG* cassette (7). The resulting plasmid, pSUR2, was digested with *Bam*HI and used to transform the *ura3* deletion mutant CAI4.

(ii) pSNUR1. An *Eco*RV-*Xba*I fragment containing the *C. albicans URA3* gene was treated with T4 polymerase and cloned into the *Hpa*I site of the previously described pΔGSA4.

(iii) pUR800. The *Eco*RV-*Xba*I fragment containing the *URA3* gene was cloned into the *Sma*I-*Xba*I site of pBluescript SK$^+$ vector. The 800-bp *Kpn*I-*Hpa*I fragment, which was deleted from the *CaSNF1* coding region during the construction of pSUR2, was cloned into the *Eco*RV-*Kpn*I sites flanking the *URA3* gene.

(iv) pTSU1. A 466-bp *Eco*RV-*Hin*cII fragment of the prokaryotic tetracycline resistance gene (Tet) was obtained from YIp5 (New England Biolabs, Beverly, Mass.). This fragment, to be used as a tag for Southern blot analysis, was cloned into the *Blp*I site of pCSA2 following a T4 DNA polymerase fill-in reaction. The *C. albicans URA3* gene was cloned into the *Nae*I site of the plasmid. The resulting plasmid contained the full-length cDNA sequence disrupted by a prokaryotic DNA fragment and flanked by a functional *C. albicans URA3* sequence.

(v) pMTSU1. In vitro amplification, using pTSU1 as a template and two oligonucleotide primers compatible with sequences within the *SNF1* coding region (TTACATATCTAGACACTTGGGCACAGGTC [*Xba*I site underlined] and TATCACTCGAGGTAACGTTGACAAGTCA [*Xho*I site underlined]), enabled the generation of a trimmed cDNA-Tet fragment (Fig. 1). The amplification product, consisting of 400- and 700-bp fragments of the *CaSNF1* coding region flanking the Tet tag, was cloned into Bluescript SK$^+$ vector harboring the *URA3* gene.

Determination of growth rate. Cells obtained from a fresh YNBG agar plates were used to inoculate 10 ml of YEPD and grown for 15 h at 37°C in a shaker (200 rpm). About 1 ml of this culture was used to inoculate 100 ml of prewarmed YEPD to reach an initial absorbance of 0.05 at 600 nm (Beckman DU-64 spectrophotometer). Cultures were grown at 37°C (200 rpm), and growth was monitored by optical density at 1-h intervals.

Determination of virulence. Cells (SC5314 and SD20 [Table 1]) were grown on YNBG agar for 24 h, washed with sterile saline, and resuspended at a concentration of 5×10^5 CFU/ml. Doses of 0.2 ml of cell suspension of either SC5314 or SD20 were injected into the lateral tail veins of 20 12-week-old, female BALB/c mice (18 to 20 g). To avoid erroneous estimations of cell number due to the mutants' hyphal growth, cell suspensions were vortexed for 30 s to minimize the number of cell chains, and the unseparated multiple cell compartments were counted as a single cell. Hemocytometer counts were confirmed by viable count on YEPD plates with variations of less than 10%. Mortality was monitored daily as presented in Fig. 7.

Patterns of utilization of carbon sources. An api20C diagnostic kit (BioMerieux Vitek Inc., Hazelwood, Mo.) was used to determine the carbon source utilization spectrum of the reference strain and the mutants. Cells were grown at 30°C for 96 h and monitored at 24-h intervals.

Nucleotide sequence analysis. Nucleotide sequences were determined by using Sequenase versions 2.0 (U.S. Biochemical, Cleveland, Ohio). Sequence analysis, comparisons, and database searches were performed with MacVector 3.0 (Oxford Molecular Group, Campbell, Calif.) and various other programs of the Genetics Computer Group (Madison, Wis.).

TABLE 2. Plasmids used in this study

Plasmid	Description
pCSA2	A 1,958-bp *CaSNF1* cDNA sequence cloned into the *Eco*RI-*Xho*I sites of pBluescript SK$^+$ vector
pGSA4	A 4-kb *CaSNF1* genomic sequence cloned into the *Eco*RI site of pBluescript SK$^+$ vector
pΔGSA4	A 1.2-kb fragment was deleted from the *SNF1* coding region in pGSA4 and substituted with a 400-bp PCR-generated fragment containing the 3′ *CaSNF1* coding region and its flanking region
pYGS4	*S. cerevisiae* complementation vector; a 4-kb genomic fragment cloned into plasmid YEp24
pSUR2	A 7-kb fragment containing the *CaSNF1* gene disrupted with the *hisG-URA3-hisG* cassette and its flanking genomic sequences cloned in pBluescript SK$^+$ vector
pSNUR1	Similar to pSUR2 but lacks the *hisG* sequences flanking the *C. albicans URA3* gene
pUR800	The 800-bp coding region fragment deleted from the *CaSNF1* sequence on pSUR2, cloned flanking the *C. albicans URA3* gene in pBluescript SK$^+$ vector
pTSU1	Full-length *CaSNF1* cDNA sequence disrupted with the prokaryotic Tet fragment and *C. albicans URA3* gene cloned in pBluescript SK$^+$ vector
pMTSU1	Partial *SNF1* cDNA sequence disrupted with the prokaryotic Tet fragment and *C. albicans URA3* gene cloned in pBluescript SK$^+$ vector

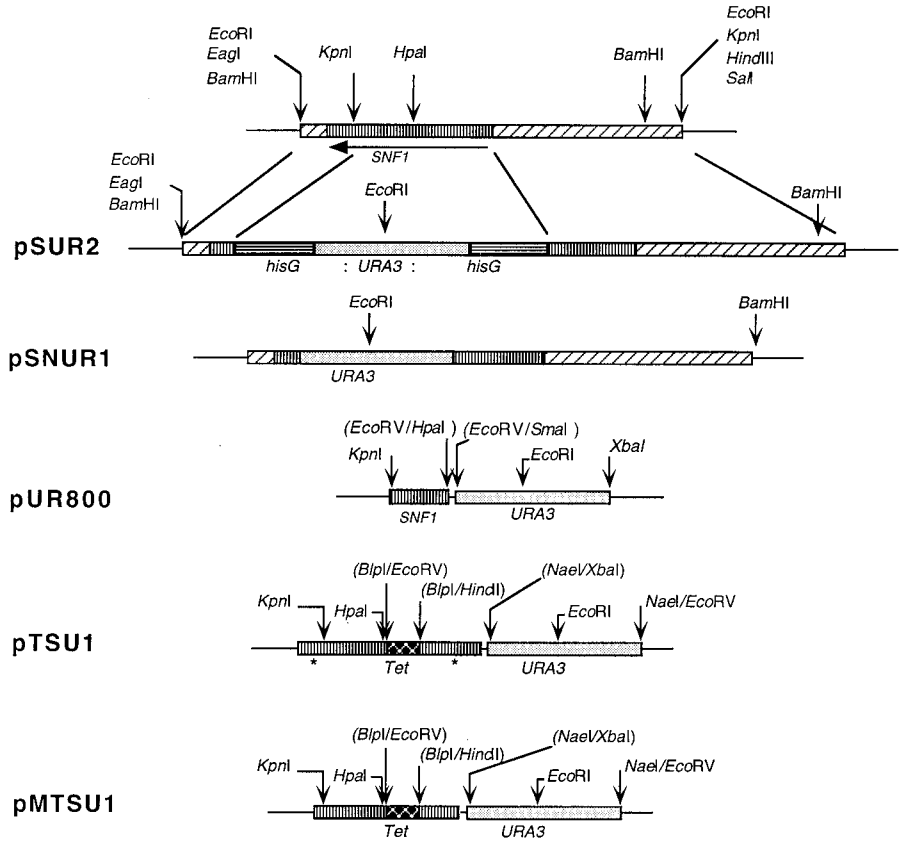

FIG. 1. Partial restriction map of the *SNF1* genomic clone and the vectors used in this study. Upper panel, the 4-kb *Eco*RI genomic fragment obtained from *C. albicans* B311, containing the *SNF1* gene coding region (▥) and its noncoding flanking regions (▨). pSUR2 is a gene replacement vector in which 800 bp of the coding region were replaced by the *hisG-URA3-hisG* cassette (▤). pSNUR1 is similar to pSUR2; the *hisG* sequences flanking the *URA3* gene were eliminated. pUR800 contains the 800-bp fragment of the coding region deleted from the *SNF1* sequence in pSUR2 and the *URA3* gene. pTSU1 is a pop-in/pop-out vector containing the full-length *CaSNF1* cDNA sequence disrupted with a fraction of the prokaryotic Tet gene (▩) and the *URA3* gene. pMTSU1 is similar to pTSU1 but harbors only a partial cDNA sequence obtained by in vitro amplification. The locations of oligonucleotide primers are marked with asterisks.

Nucleotide sequence accession number. The genomic nucleotide sequence data reported in this paper were submitted to the National Center for Genome Research with accession no. L78129.

RESULTS

Isolation of the *CaSNF1* homolog. Two degenerative oligonucleotide primers were derived from conserved regions of the *SNF1* sequence of *S. cerevisiae* and its plant and mammalian homologs (4, 15, 26). In vitro amplifications using the *C. albicans* B311 genomic DNA template yielded several products of various molecular weights. A 600-bp PCR product hybridized with the *ScSNF1* probe under low-stringency conditions (2× SSC–0.1% sodium dodecyl sulfate, 50°C) and was cloned into the *Hin*dIII site of pBluescript SK^+. Comparisons of the cloned fragment with the *ScSNF1* gene nucleotide sequence revealed a 69% similarity.

Isolation of the *CaSNF1* cDNA. A *C. albicans* B311 cDNA library (3.1×10^5 PFU) was screened with the *CaSNF1* PCR-generated probe to identify 30 positive plaques (9). Phagemids excised from 12 randomly chosen clones were found to each carry an insert of about 2 kb, similar to the size of the *SNF1* transcript (see Fig. 7). Nucleotide sequence analysis of one of these clones, pCSA2, revealed the presence of a 1,958-bp-long fragment, encoding a deduced polypeptide of 616 amino acids. Comparisons with the putative ScSnf1p amino acid sequence revealed 68% identity, while only 69% similarity was observed at the nucleotide sequence level.

Isolation of the *CaSNF1* genomic sequence. Southern blot analysis of *C. albicans* B311 genomic DNA performed with the *CaSNF1* cDNA probe revealed the existence of a single copy of this gene in a haploid genome equivalent and that both alleles were present on 4-kb *Eco*RI DNA fragments (data not shown). A size-selected genomic library was constructed, and about 1,300 independent clones were screened to isolate six identical clones each carrying a 4-kb insert. Nucleotide sequence analysis revealed the presence of a 621-amino-acid open reading frame of *CaSNF1* and its noncoding flanking regions. Comparisons with the *ScSNF1* nucleotide sequence revealed 69% similarity, while 68% identity was found at the protein level. The presumed kinase catalytic domain, a 300-amino-acid stretch at the amino terminus, is highly conserved in all the homologs compared (Fig. 2). A partial restriction map of the genomic *CaSNF1* clone is shown in Fig. 1.

It is noteworthy that a stretch of histidine residues, found at the amino terminus of the Snf1p homologs of *S. cerevisiae* (5) and *C. glabrata* (28), also exists in the *C. albicans* homolog. This unique nickel binding site, absent in higher organisms, is not essential for the derepression of catabolic repression in *S. cerevisiae* (5), and its functional role is unknown. The *C. albicans* Snf1p sequence contains a 12-amino-acid stretch (C KIVNVIEKANK) with no such similarity to other Snf1p sequences. Although it does not resemble any of the known protein motifs included in the data base (MOTIFS), it may have a functional or structural significance in *C. albicans.*

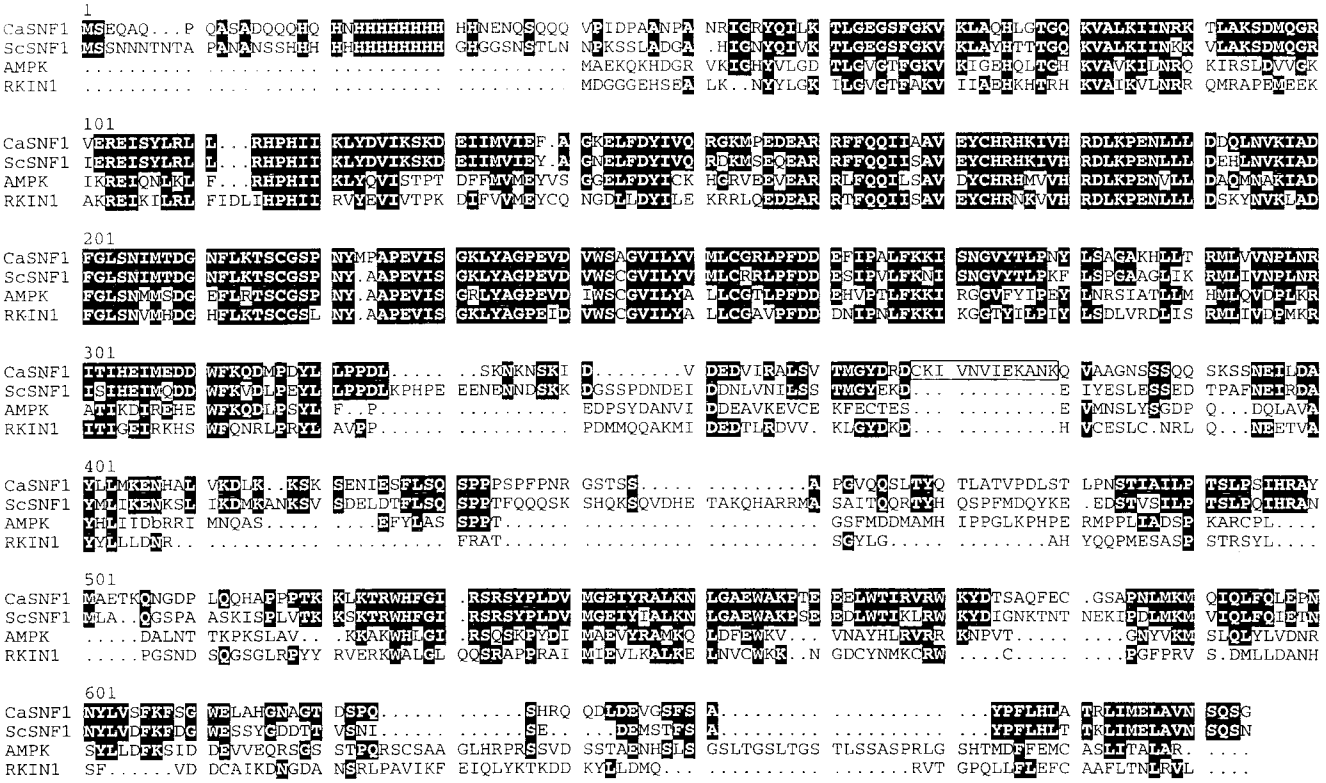

FIG. 2. Alignment of the putative CaSNF1p amino acid sequence (CaSNF1) with its homologs obtained from *S. cerevisiae* (ScSNF1) (6), rat (AMPK) (4), and rye (RNKIN1) (2). The unique 12-amino acid-stretch found solely in *C. albicans* is marked with a box.

As was previously shown for *ScSNF1*, the *CaSNF1* sequence is constitutively expressed. Northern hybridization was performed with the *SNF1* cDNA probe and total RNA obtained from wild-type *C. albicans* cultures grown in either YNBG, YNBS, or YNBGL. Analysis of the hybridization revealed similar amounts of *SNF1* transcript (about 2.0 kb) in all three cultures, indicating a pattern of constitutive expression (data not shown).

Complementation of a *ScSNF1* deletion mutant. The pYGS4 vector containing the *CaSNF1* gene and its flanking regions was used to transform the *snf1* deletion mutant of *S. cerevisiae* (strain MYC2916), which is unable to utilize sucrose. Transformants were first selected for uracil prototrophy on YNBG agar and then tested for sucrose utilization. All of the randomly chosen transformants (a total of 150) grew on YNBS agar, demonstrating complementation of the *snf1* mutation (Fig. 3). Crude DNA extracted from 33 complemented yeast clones was used to transform *E. coli*. The plasmids rescued from the bacterial transformants exhibited restriction patterns identical to that of pYGS4. Attempts to select *S. cerevisiae* transformants simultaneously for uracil prototrophy and sucrose utilization on YNBS agar resulted in a more than 100-fold decrease in the number of transformants.

Creation and characterization of *SNF1*/*snf1*::*hisG* heterozygote. The *ura*-blast method, first applied to *C. albicans* by Fonzi et al. (7), was used to disrupt the first *CaSNF1* allele. The *Bam*HI-digested vector pSUR2 was used to transform *C. albicans ura3* deletion mutant CAI4 (Table 1). None of the over 500 uracil-positive transformants tested was defective in sucrose utilization. Southern blot analysis of *Eco*RI-digested genomic DNA hybridized with the *CaSNF1* cDNA probe showed structural modifications of the *SNF1* loci in about 30% of the transformants (Fig. 4a, lanes A and B). This observation indicated that gene replacement through homologous recombination resulted in disruption of one of the *SNF1* alleles. PCR performed with various oligonucleotide primers and Southern blot analysis with either the *hisG* or the *URA3* probe supported the same conclusion (data not shown). The heterozygotes exhibited an about 39% lower growth rate on either YEPD, YNBG, or YNBS compared with the parental strain. The het-

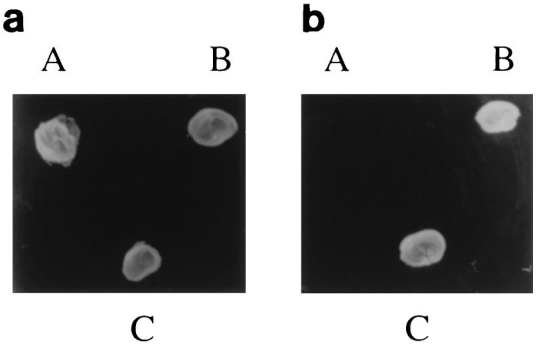

FIG. 3. Complementation of an *S. cerevisiae snf1* deletion mutant with the *CaSNF1* homologous sequence. A, *S. cerevisiae* MCY2916 *snf1* deletion mutant transformed with YEp24 to regain *URA3* prototrophy; B, *S. cerevisiae* MCY829 *SNF1* reference strain; C, *S. cerevisiae* MCY2916 *snf1* deletion mutant complemented with pYGS4. Cells were grown at 30°C on either YNBG (a) or YNBS (b) supplemented with leucine, histidine, and lysine as required for the growth of these strains.

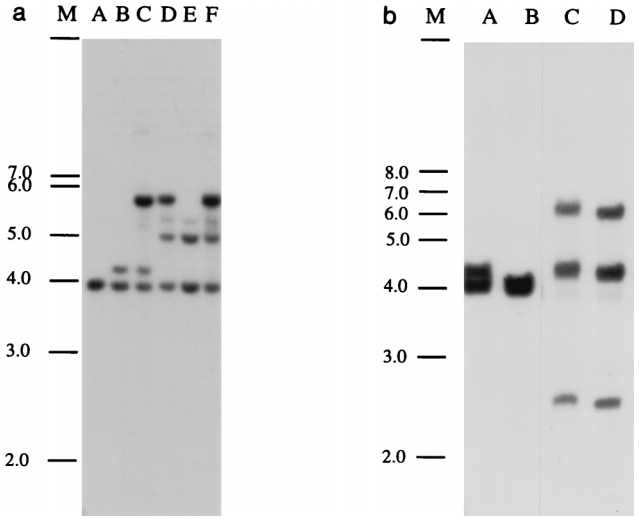

FIG. 4. Southern blot hybridization of *Eco*RI-digested DNA with the cDNA *CaSNF1* probe. (a) Lane A, *ura3* deletion mutant of *C. albicans* (CAI4); lane B, *SNF1*/*snf1* heterozygote (FD97) obtained by disruption with the pSUR2 vector; lanes C to F, randomly chosen clones of FD97 transformed with pMTSU1 exhibiting either ectopic insertion (lane C) or repeated insertion into the previously disrupted allele (lanes D to F). All transformants tested retained the intact *SNF1* allele. (b) Lane A, FD97 heterozygote; lane B, *ura3* deletion mutant of *C. albicans* (CAI4); lanes C and D, randomly chosen clones of FD97 transformed with pTSU1, exhibiting insertion into the intact *CaSNF1* sequence of FD97.

erozygote produced significantly longer and more abundant cell chains compared to the parental strain, and colonies were rough and tended to penetrate the agar. A YEPD-grown, late-stationary-phase heterozygote culture (FD9 [Table 1]) was plated on 5-fluoro-orotic acid–YNB agar medium to select the uracil auxotrophic *SNF1*/*snf1* clones. Two of the uracil auxotrophic heterozygote clones, FD92 and FD97, were used to disrupt the second *CaSNF1* allele.

Disruption of the second *SNF1* allele. Four different integrative vectors were used to disrupt the second *CaSNF1* allele. Each attempt was followed by a sucrose utilization test of approximately 500 independent transformants and a Southern blot analysis of about 60 clones. The heterozygote's transformation efficiency (150 to 170 transformants/μg of DNA) was approximately 30% lower than that observed for the *ura3* mutant (200 to 210 transformants/μg of DNA).

The vector pSUR2, initially used to replace the first *CaSNF1* allele, was also used to disrupt the second homolog in FD97 and FD92. All transformants tested grew on sucrose as a sole carbon source. Southern blot analysis revealed that insertions had occurred either in the previously disrupted allele or in ectopic sites in the genome, leaving the second *SNF1* allele intact.

The second vector, pSNUR1, was designed to reduce the homology with the *hisG* sequence in the previously disrupted allele. None of the transformants obtained with pSNUR1 lost the ability to utilize sucrose. Southern blot analysis revealed the presence of an intact *SNF1* allele in each of the transformants tested. Comparison with the results obtained with pSUR2 revealed a 10% decrease in the rate of insertions into the previously disrupted allele, and a similar 30% reduction in transformation efficiency was observed compared with the wild-type strain.

The third vector, pUR800, contained the 800-bp fragment (*Hpa* I-*Kpn* I [Fig. 1]) which had previously been deleted from the *CaSNF1* coding region in pSUR2. This vector, designed to target a unique sequence of the intact allele for homologous recombination, was used to transform the *SNF1*/*snf1* heterozygote. None of the transformants tested exhibited a sucrose-negative phenotype. Southern blot analysis confirmed that the second *SNF1* allele remained intact in all transformants.

The fourth vector, pMTSU1, constructed as a pop-in/pop-out vector (29), contained a truncated *CaSNF1* cDNA sequence. The vector was linearized at the *Hpa*I site within the *CaSNF1* coding region to increase the likelihood of homologous recombination and used to transform strains FD97 and FD92. Transformations were also performed with the circular form of the same vector. None of the transformants tested (>500) lost the ability to utilize sucrose, and as has been previously observed, the wild-type allele was preserved in all clones analyzed by Southern blot hybridization (Fig. 4a).

To demonstrate that an insertion into the yet intact allele is technically feasible and is prevented only by the requirement for a functional *SNF1* allele, a similar pop-in/pop-out vector, pTSU1, was designed. The heterozygote FD97 was transformed with either the linearized (at the *Hpa*I site) or the circular form of pTSU1, and the transformants were selected for uracil prototrophy. This vector carries the full-length cDNA sequence disrupted by a portion of the prokaryotic Tet gene. pTSU1 integration into the yet intact *SNF1* allele of the heterozygote should result in the disruption of the native sequence and the assembly of a faultless or Tet-disrupted full-length *SNF1* sequence flanked by the *URA3* gene and the vector sequence either up- or downstream. Such an integration may disengage promoters, enhancers, or other regulatory elements from the structural *SNF1* sequence and thereby affect its expression. Southern blot analysis indicated that in over 30% of the transformants, the integration occurred in the second *SNF1* allele regardless of whether linear or circular DNA was used (Fig. 4b and 5). PCR analysis using oligonucleotide primers compatible with either the *SNF1* coding region, its 3′ and 5′ flanking regions, or the pBluescript vector sequences demonstrated that all transformants bear complete *SNF1* sequences flanked by the native 5′ region and, in the 3′ region, the *URA3* gene and the entire vector sequence (data not shown).

Five independent transformants, each carrying the full-length reassembled *SNF1* sequence, were grown to stationary phase in YEPD medium to allow excision of the integrated sequence (pop-out) under nonselective conditions (29). Uracil auxotrophs were isolated on 5-fluoro-orotic acid–YNBG agar (total of 221 colonies) and analyzed by colony hybridization to determine the nature of the structural modification resulting in the *URA3* sequence excision. The Tet tag probe hybridized with only four of these clones, indicating that in the vast majority of the clones, the entire pTSU1 vector was excised and the original *SNF1* allele was reassembled. Further analysis of the four Tet-positive clones indicated the presence of an intact *SNF1* sequence, most likely created by chromosomal rearrangement before or after excision of the *URA3* sequence.

Phenotypes of the clones harboring the reassembled functional *SNF1* sequence. The carbon utilization patterns of five independent uracil prototrophs bearing the reassembled functional *SNF1* sequence were identical to those of both the wild-type strain SC5314 and the heterozygote FD97. However, these mutants differed significantly from the wild type by producing rough colonies with hyphal margins that tended to penetrate into the agar media of either YEPD or YNBG at 30 or 37°C. Microscopic observations revealed extensive hyphal and pseudohyphal growth with occasional budding yeast cells (Fig. 6). Hyphal growth was further enhanced by nitrogen starvation (YNBG–0.05 mM ammonium sulfate) (11) but was not affected by pH ranging between 4.7 and 8.0. Hyphal growth

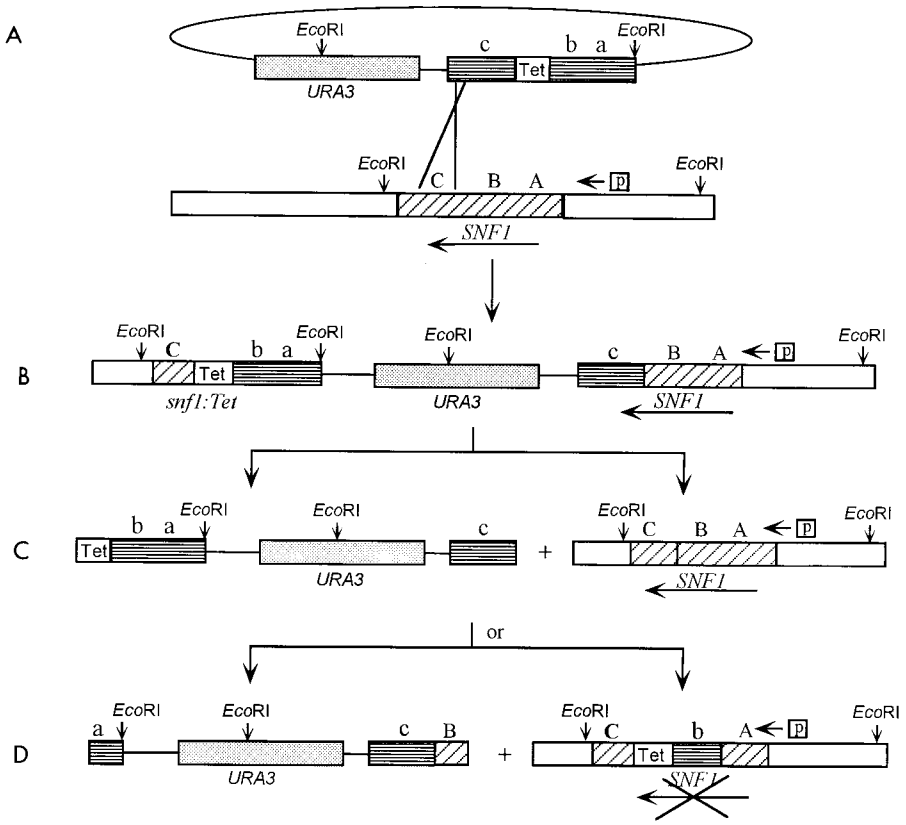

FIG. 5. Integration of the pTSU1 vector into the intact *CaSNF1* allele of the heterozygote mutant FD97. Homologous recombination between the two *SNF1* coding regions resulted in disruption of the chromosomal copy and assembly of a functional copy with modified 3′ flanking region (A and B). Two possible outcomes of the pop-in/pop-out experiment by homologous recombination and reassembly of the *SNF1* are diagrammed (C and D). Cells represented by the cartoon in panel D may be nonviable, and only those cells represented in panel C were recovered in our experiments.

was not as evident when the cells were grown in liquid YEPD medium. The growth rates of a pTSU1 mutant clone (SD20), a heterozygote (FD97), and a wild-type strain (SC5314) were determined in YEPD medium at 37°C. The average generation time of FD97 was longer (103 ± 6 min) than that observed for SD20 (84 ± 4 min) or the reference strain SC5314 (74 ± 4 min). The RNA level of *SNF1* in SD20 and FD97 was slightly lower than that in the wild-type *SNF1* strain (CAI4). Interestingly, an additional transcript about 2.6 kb was clearly visible in SD20 (Fig. 7).

Virulence of clones harboring a cDNA insertion in their *SNF1* genes. Hyphal growth is generally accepted as an important feature of *C. albicans* that contributes to its virulence. To analyze whether the mutant's hyphal growth contributes to its pathogenicity, we compared its virulence in an animal model to that of the wild-type strain. Mortality of BALB/c mice injected intravenously with 10^5 CFU of SD20 or SC5314 is shown in Fig. 8. No significant difference in lethality was observed between mice infected with the wild-type and with the mutant clone (SD20).

DISCUSSION

This report describes the isolation and characterization of the *SNF1* gene homolog of *C. albicans* and provides evidence suggesting that CaSNF1p is essential for viability. A high degree of similarity was observed between the deduced amino acid sequence of CaSNF1p and other Snf1p homologs. The ability of the *CaSNF1* gene to restore sucrose utilization of an *S. cerevisiae snf1* deletion mutant indicates functional resemblance between CaSNF1p and the *S. cerevisiae* serine/threonine kinase. The complemented *S. cerevisiae* clones exhibited a shorter generation time while growing on YEPD or defined media (data not shown). This observation indicated that additional growth defects that may have been a consequence of the *snf1* mutation were also restored. Attempts to simultaneously select transformants for sucrose utilization and uracil prototrophy were unsuccessful, primarily due to the reduced number of transformants obtained, as had been previously observed upon complementation with the *C. glabrata SNF1* homologous gene (28). This phenomenon may be a result of the stress imposed on the *S. cerevisiae* transformants by double selection. On the other hand, in accordance with the constitutive level of expression observed in Northern blot analysis, it may indicate that a basal level of Snf1p is required to initiate the metabolic pathway of sucrose utilization.

Several different approaches were undertaken to disrupt both alleles of the *SNF1* gene in *C. albicans*. Integration of the vector pSUR2, followed by excision of the *URA3* gene, resulted in disruption of the first *SNF1* allele and uracil auxotrophy of the heterozygote. Although no modifications were detected in the heterozygote's carbon utilization pattern, indicating the recessive nature of the *snf1* mutation, a significant decrease in growth rate as well as morphological changes were observed. These phenotypic alterations suggest that some other unknown cellular function(s) may simultaneously be affected by either the reduced gene dosage or the loss of expression of this particular allele.

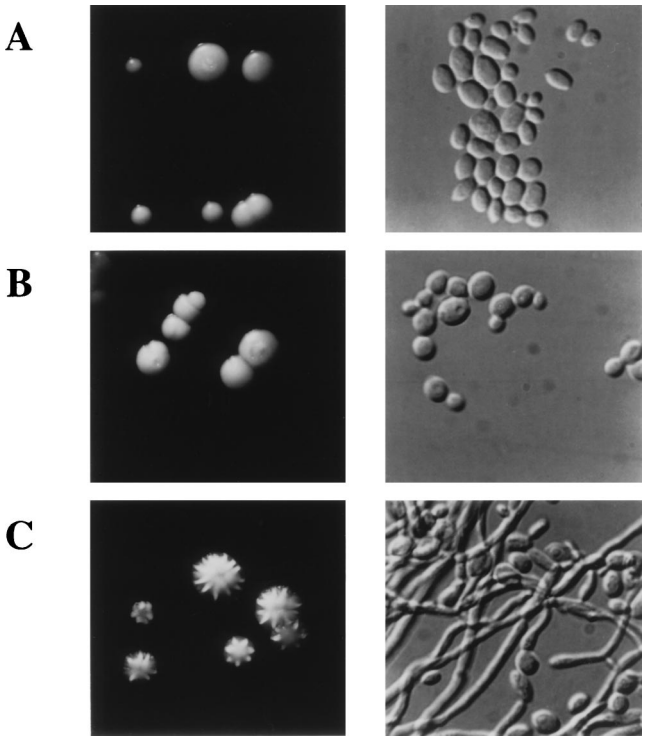

FIG. 6. Colony and microscopic morphology of the wild-type *C. albicans* and *snf1* mutants. (A) Wild-type strain; (B) FD97 (*SNF1*/*snf1*::*hisG*) heterozygote; (C) pTSU1 transformant SD20 (*snf1*::*cSNF1*/*snf1*::*hisG*). The transformants were grown on either YNBG agar (left) or in YNBG broth (right) at 30°C for 48 h.

The vector used for the first allele replacement, pSUR2, failed to disrupt the second *SNF1* allele of the heterozygote. Additional vectors designed to circumvent possible technical obstacles, by the removal of the *hisG* sequences (pSNUR2), by targeting the integration by a unique sequence (pUR800), or using the previously adequate pop-in/pop-out approach (pMTSU1) (31), did not succeed in integration into the second *CaSNF1* allele. Such failures may reflect the lethal nature of a *snf1* mutation in *C. albicans* and less likely resulted from unresolved technical difficulties. In the absence of conditional mutants or inducible expression systems unaffected by glucose

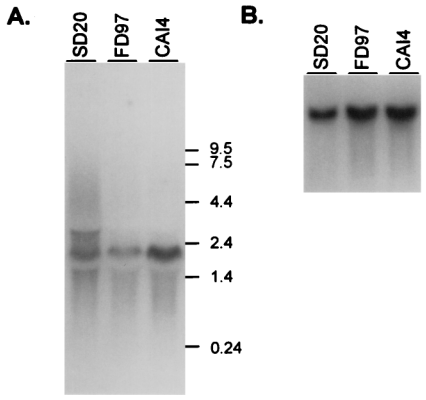

FIG. 7. Analysis of *SNF1* expression. (A) RNAs were isolated, fractionated in formaldehyde agarose gels, transferred to nylon membranes, and hybridized with a probe of *SNF1* cDNA. (B) The same blot was stripped and hybridized with actin gene as a control. Sizes are indicated in kilobases.

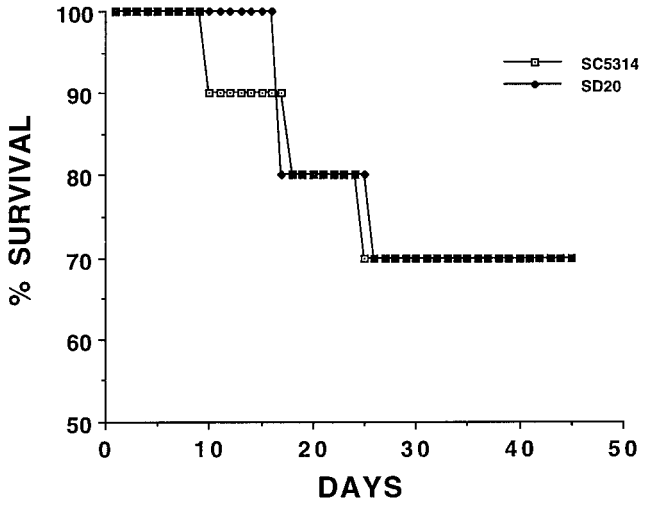

FIG. 8. Virulence of the *C. albicans* wild-type strain (SC5314) and the pTSU1 transformant (SD20) in mice. Either SC5314 or SD20 (10^5 CFU of each) was used to inject (intravenously) 20 mice. Mortality was monitored daily for 45 days.

repression, a unique vector (pTSU1) was designed to distinguish between these two hypotheses. Insertion of this vector into the heterozygote genome via homologous recombination resulted in disruption of the intact allele and created a functional *SNF1* sequence sufficient for providing the cells with the vital Snf1p (Fig. 4b and 5). Southern blot analysis using the *SNF1* cDNA and the Tet sequence probes as well as PCR analysis indicated that the predicted insertion occurred in about 30% of the transformants, resulting in the reassembly of a functional *CaSNF1* copy. It is noteworthy that 5′ region of the *SNF1* sequence flanking the Tet insertion in pTSU1 is slightly longer than that of 3′ region (Fig. 5). Failure of pTSU1 to integrate into the 5′ region of the resident *SNF1* allele, therefore, cannot be biased due to the length of homologous sequences. The relatively high abundance of pTSU1 insertion into the second allele clearly showed that the lack of *snf1*/*snf1* transformants was not due to technical obstacles and negated the assumption that the double deletants were too few to be detected. Furthermore, since excision of the disrupting vector always resulted in the reassembly of the functional sequence (Fig. 4b and 5), it is most likely that a selection had driven this process. A similar vector harboring partial cDNA sequence, pMTSU1, failed to integrate into the intact *SNF1* sequence, probably due to its inability to generate a full-length functional *SNF1* sequence. These observations further support the notion that Snf1p plays a vital role in viability of *C. albicans.*

The pTSU1 transformant (SD20) harboring two disrupted *snf1* alleles and a reconstructed functional *SNF1* sequence exhibited various phenotypic modifications, including a shorter generation time than the parental heterozygote, a tendency to produce hyphae, and stellate colony morphology. Although hyphal growth is generally regarded as an important factor contributing to the pathogenicity of *C. albicans* (12, 13, 25), we could not demonstrate higher virulence of these transformants in mice. These results indicated the existence of multiple mechanisms of induction and formation of hyphal morphology in *C. albicans* and that the extensive hyphal growth observed in vitro in SD20 most likely reflects a general stress response. Moreover, this observation indicates that hyphal growth in vitro does not necessarily result in higher mortality of experimental animals.

The altered phenotype of the pTSU1 transformants may result from the disengagement of a downstream regulatory component(s) due to the insertion of the *URA3* gene and vector sequences. This possibility is supported by the altered expression pattern of *SNF1* in SD20 (Fig. 7). These elements may be required for the fine-tuning of *SNF1* gene expression and/or allelic differential expression at different stages of cell cycle. A faulty expression pattern of the reassembled *SNF1* sequence is probably adequate for providing the cell with the vital Snf1p function(s) but may fail to fully accomplish other, yet unknown roles of this kinase. Differential levels of expression of two similar members of the *SNF1* gene family have previously been demonstrated in barley (14, 15). Although their functional identity was not thoroughly investigated, it was suggested that tissue specificity as well as a developmental signal affected the expression of each *SNF1* copy (14, 15). Similarly, the two *CaSNF1* alleles may be expressed differentially or in a synchronized fashion with the progress of cell cycle or in response to diverse environmental conditions. It is interesting that a higher incidence of budding and the inability of cells to halt growth and division upon entering the stationary phase were documented for *S. cerevisiae* strains harboring *snf1* point mutations (34). Similar defect may account for the tendency to produce cell chains in FD97 and hyphae in SD20.

Some similarities can be found between the phenotypic modifications reported for *C. albicans* var. *stellatoidea* and the SD20 mutants, including extensive hyphal growth, distinct stellate colony morphology, and reduced growth rate. Our preliminary results indicate that the chromosomal rearrangement, a characteristic of *C. albicans* var. *stellatoidea*, affected the *SNF1* gene loci, as evidenced by electrophoretic karyotyping (data not shown). We are currently studying whether this genomic rearrangement accounts for the *C. albicans* var. *stellatoidea* phenotype.

Although in *S. cerevisiae* the *snf1* mutation is not lethal, the mutants exhibit pleiotropic defects (5). In barley, the constitutive expression of a *SNF1* antisense transcript was shown to almost entirely eliminate growth of transgenic plants and was suggested to be lethal due to a developmentally essential role associated with this gene (14). The evidence presented in this study emphasizes the substantial regulatory role of the *SNF1* gene in *C. albicans* and suggests that it is essential for viability. It is unclear, however, whether CaSNF1p has additional essential regulatory roles or that a surrogate regulatory pathway, which exist in other organisms, was lost. Additional studies on the role(s) of CaSNF1p may provide novel information regarding regulatory mechanisms and the function of this indispensable regulatory component.

ACKNOWLEDGMENTS

We thank M. Carlson and W. Fonzi for providing the strains and plasmids used in this study. We thank M. Carlson, R. Jiang, and A. Varma for critically reading the manuscript and Huei-Fung Tsai for help with the artwork for figures.

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Editor: T. R. Kozel