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Isolation of the *Candida albicans* Homologs of *Saccharomyces cerevisiae KRE6* and *SKN1*: Expression and Physiological Function

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Cell wall β-glucan in a pathogenic fungus, *Candida albicans*, is highly branched with β-1,3 and β-1,6 linkages. We have isolated the *C. albicans* cDNAs for *KRE6* and *SKN1*, the genes required for β-1,6-glucan synthesis in *Saccharomyces cerevisiae*. The results of Northern blot analysis revealed that *C. albicans KRE6* was expressed at a higher level than *SKN1* in the yeast phase, while *SKN1* expression was strongly induced upon induction of hyphal formation. In addition, the *C. albicans KRE6* and *SKN1* mRNAs but not the actin mRNA were shortened during the yeast-hypha transition. Unlike *S. cerevisiae*, more than 50% of cell wall glucan was β-1,6 linked in *C. albicans*. Neither β-1,3-glucan nor β-1,6-glucan was affected by the homozygous *C. albicans skn1*Δ null mutation. Although we never succeeded in generating the homozygous *C. albicans kre6*Δ null mutant, the hemizygous *kre6*Δ mutation decreased the *KRE6* mRNA level by about 60% and also caused a more than 80% reduction of β-1,6-glucan without affecting β-1,3-glucan. The physiological function of *KRE6* was further examined by studying gene regulation in *C. albicans*. When *KRE6* transcription was suppressed by using the *HEX1* promoter, *C. albicans* cells exhibited the partial defect in cell separation and increased susceptibility to Calcofluor White. These results demonstrate that *KRE6* plays important roles in β-1,6-glucan synthesis and budding in *C. albicans*.

A recent increase in the incidence of fungal infection is one of the serious problems in medical care of immunocompromised patients, and *Candida albicans* is the most common pathogen that causes deep mycosis in humans (28). *C. albicans* cells grow as diploids without a sexual cycle and undergo morphological conversion from yeast to mycelium upon exposure to several stimuli (14, 37). Since this morphogenetic transition is often accompanied with changes in the cell wall components and virulence, there seems to be a close relation between the cell wall composition and virulence.

β-Glucan is a major component of the fungal cell wall and can possess β-1,3 linkages (β-1,3-glucan) or β-1,6 linkages (β-1,6-glucan) (12). *Saccharomyces cerevisiae* β-1,3-glucan synthase consists of two subunits, the membrane-localized catalytic subunit and the GTP-binding regulatory subunit (19, 27). Although *S. cerevisiae* may possess more than two β-1,3-glucan synthase isozymes, recent studies revealed that *FKS1* (also called *GSC1*) and its homolog, *FKS2* (also called *GSC2*), encode the catalytic subunits of β-1,3-glucan synthase (10, 16) and that GTP-bound Rho1p is physically associated with Fks1p to function as the regulatory subunit of the enzyme (11, 30).

Several genes responsible for β-1,6-glucan synthesis are identified from the *S. cerevisiae kre* mutants that are resistant to K1 killer toxin (2, 4–7, 25, 32, 34). Of these genes, *KRE5* encodes the protein that harbors endoplasmic reticulum retention signal (25) which shares significant sequence similarity with UDP-glucose:glycoprotein glucosyltransferase of *Drosophila melanogaster* (29). *KRE6* and the multicopy suppressor for the *kre6*Δ null phenotype, *SKN1*, code for the homologous proteins that contain the putative UDP-glucose-binding consensus sequence (32, 34) and localize in the Golgi subcompartment, where Kre2p functions as α-1,2-mannosyltransferase for protein O glycosylation (15, 23, 35). These facts suggest that *KRE5*, *KRE6*, and *SKN1* are required for early steps in β-1,6-glucan synthesis. It has also demonstrated that Kre1p and Kre9p are O-glycosylated cell surface proteins and contribute to the production of β-1,6-glucan polymers (5, 33).

Much remains to be learned about β-glucan synthesis in *C. albicans*. Analysis of the *C. albicans* cell wall revealed the existence of the covalent linkage between chitin and β-1,6-glucan (39). More recently, Boone et al. (3) isolated the *C. albicans KRE1* gene by functional complementation of the *S. cerevisiae kre1*Δ null mutation and suggested that *S. cerevisiae*

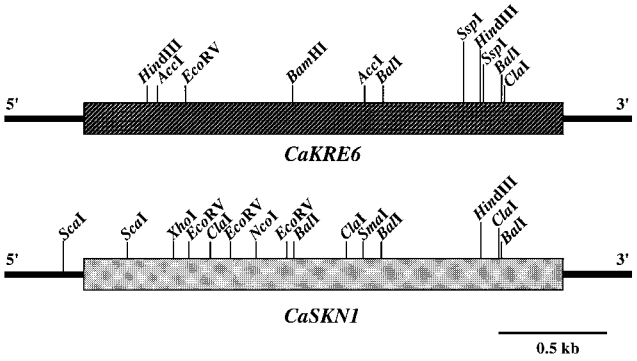

FIG. 1. Restriction maps of the *C. albicans KRE6* and *SKN1* cDNAs. Restriction maps of the *C. albicans KRE6* and *SKN1* cDNAs are illustrated. Thick bars represent the ORFs found in the *KRE6* and *SKN1* cDNAs.

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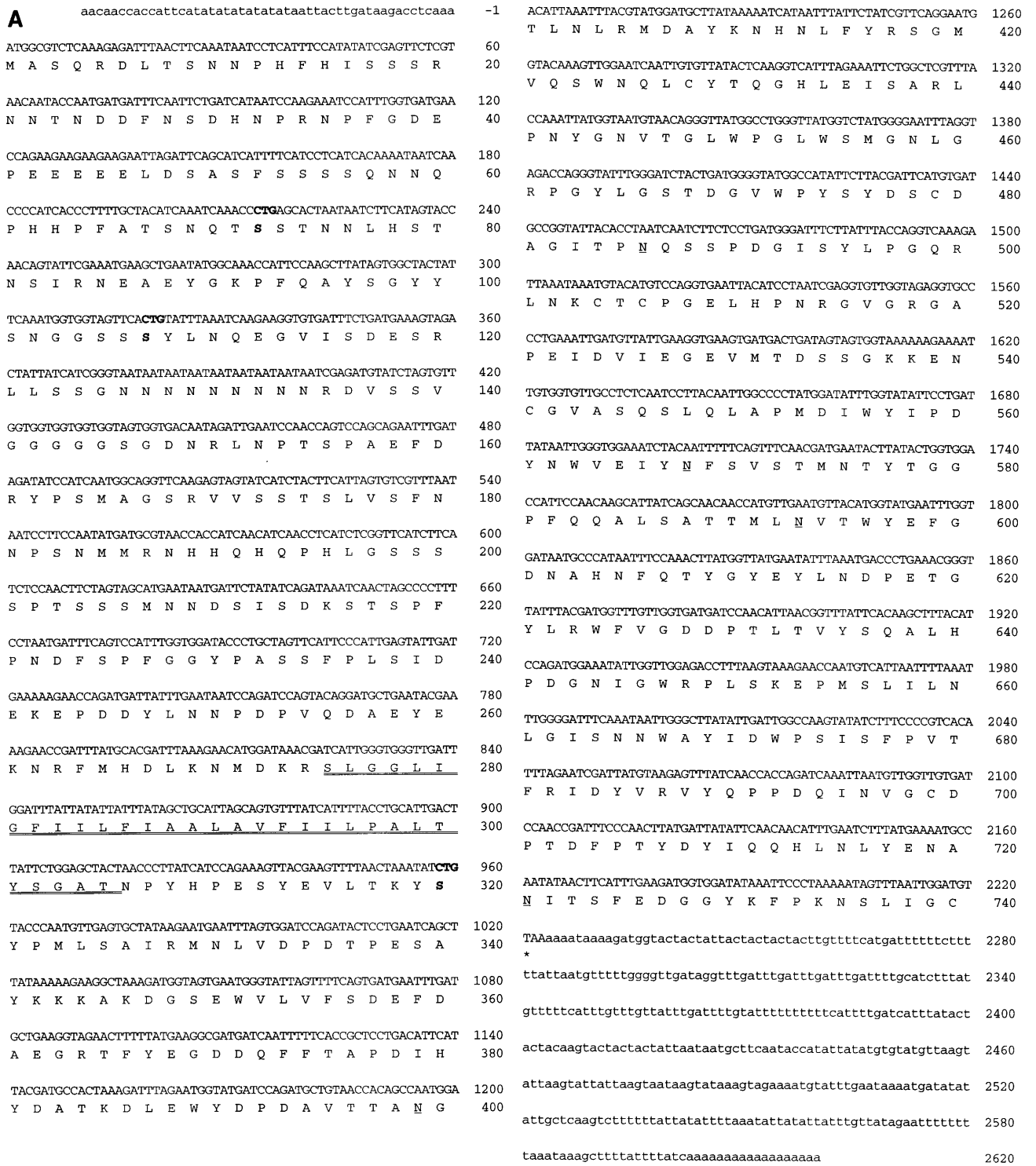

FIG. 2. Nucleotide sequences of *C. albicans KRE6* and *SKN1* cDNAs and expected amino acid sequences of Kre6p and Skn1p. Nucleotide sequences of the *KRE6* (A) and *SKN1* (B) cDNAs together with the deduced amino acid sequences of Kre6p and Skn1p are indicated. The potential transmembrane domains in Kre6p and Skn1p are underlined. According to the report by Manuel et al. (24), the CTG codon in bold letters is decoded as serine instead of leucine.

and *C. albicans* have similar mechanisms of β-1,6-glucan synthesis. Here we report that β-1,6-glucan is the major component of *C. albicans* cell wall and that the *C. albicans KRE6* gene plays important roles in cell wall assembly and budding.

MATERIALS AND METHODS

Generation of a cDNA library and screening. Poly(A)$^+$ RNA was isolated from growing *C. albicans* cells (IFO 1060) by oligo(dT) column chromatography (21, 36). cDNA was synthesized with the ZAP cDNA synthesis kit (Stratagene),

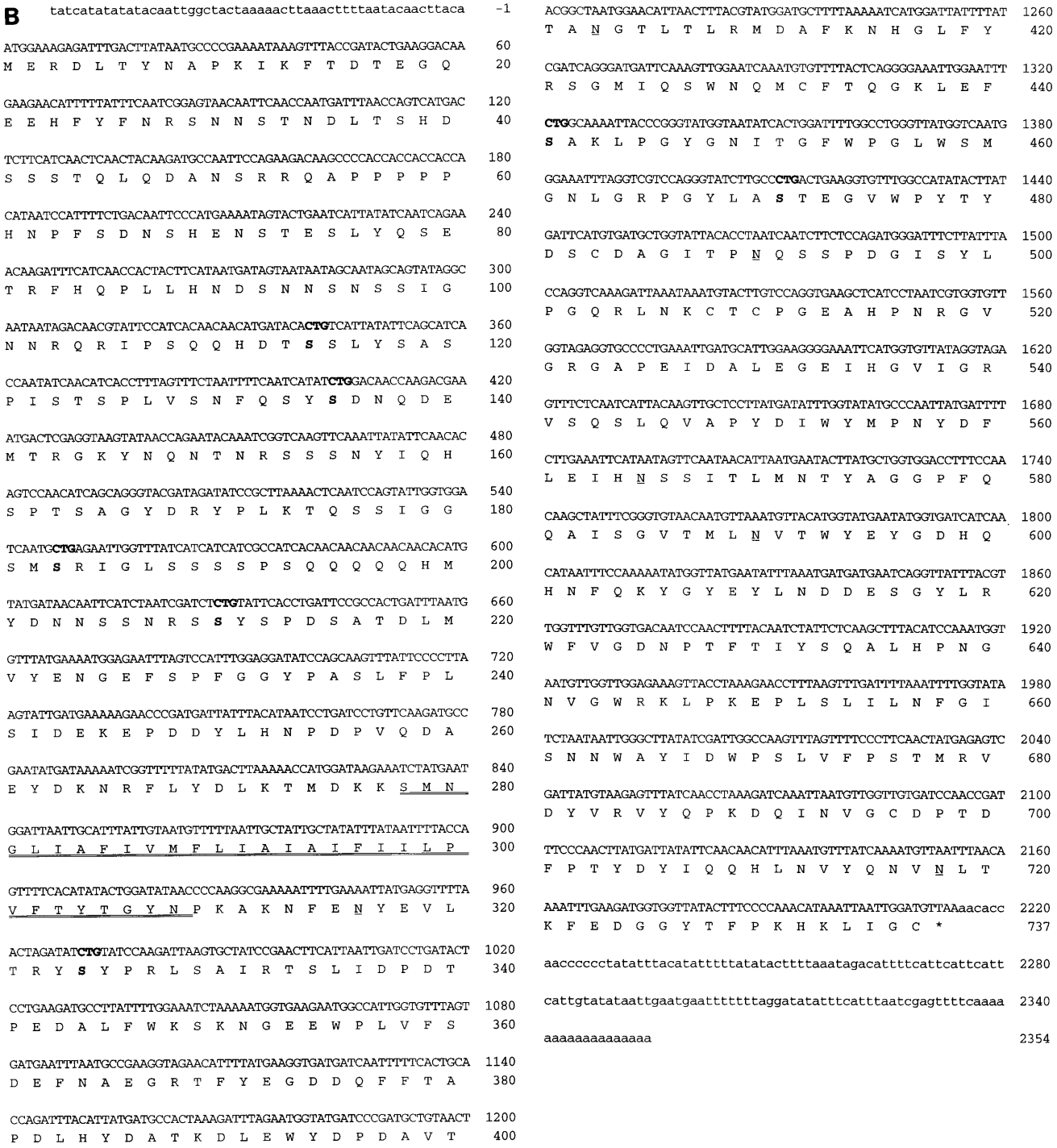

FIG. 2—*Continued.*

ligated with the Uni-Zap XR vector (Stratagene), and packaged in vitro with Gigapack II packaging extract (Stratagene).

For screening, a 2.0-kb DNA fragment of *S. cerevisiae KRE6* that was amplified by PCR (20) was used for the probe. Hybridization was performed in a buffer containing 50 mM sodium phosphate (pH 6.5), 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt's solution, 0.1% (wt/vol) sodium dodecyl sulfate (SDS) and 25% (vol/vol) formamide at 42°C for 18 h. Plasmid DNA (Bluescript SK^+) that harbored the *C. albicans KRE6* or *SKN1* cDNA was recovered from the phage by an in vivo excision protocol with the helper phage. DNA sequencing was carried out as described elsewhere (36).

Southern and Northern blotting. Twenty-five micrograms of genomic DNA and 10 μg of poly(A)$^+$ RNA were extracted as described previously (21) and used for blotting. Hybridization was carried out under stringent conditions in a buffer containing 50 mM sodium phosphate (pH 6.5), 5× SSC, 1× Denhardt's solution, 0.1% (wt/vol) SDS, and 50% (vol/vol) formamide at 42°C for 18 h. Unless otherwise specified, the probes used were the entire coding regions of *C. albicans KRE6* and *SKN1* and exon 2 of *ACT1*.

Induction of hyphal formation. Hyphal formation was induced by the method of Bulawa et al. (8). Growing *C. albicans* cells in YPD (yeast extract-peptone-dextrose) medium were washed twice with RPMI 1640, transferred to RPMI

FIG. 3. Sequence alignment of Kre6p and Skn1p. Amino acid sequences of *C. albicans* Kre6p (CaKre6p) and Skn1p (CaSkn1p) were compared with *S. cerevisiae* Kre6p (ScKre6p) and Skn1p (ScSkn1p) by using the Genetyx (version 8.0) program. Amino acids that are identical among the four proteins are boxed. Dashes indicate gaps introduced to optimize alignment.

1640 medium supplemented with 50 μg of uridine per ml, and further cultured at 37°C for the times indicated in Fig. 5.

Disruption of *C. albicans KRE6* and *SKN1* genes. Gene disruption was performed by the *ura* blaster protocol (1, 26). For *C. albicans KRE6* disruption, pCAKU was generated by digesting pCAK1 in which the 1.5-kb *Eco*RI-*Xho*I fragment of *KRE6* cDNA had been cloned in Bluescript (SK9$^+$) with *Eco*RV and *Cla*I and then ligating with the 3.8-kb *Bam*HI-*Bgl*II fragment carrying the *hisG-URA3-hisG* module. For *C. albicans SKN1* gene targeting, pCASU was constructed by digesting pCAS1 in which the 1.0-kb *Xho*I-*Eco*RI fragment of *SKN1* cDNA was cloned in Bluescript (SK9$^+$) with *Bal*I followed by ligation with the 3.8-kb *Bam*HI-*Bgl*II fragment carrying the *hisG-URA3-hisG* module. Thus, the 1.5-kb *Eco*RV-*Cla*I region of *KRE6* and the 0.6-kb *Bal*I-*Bal*I region of *SKN1* were replaced by *hisG-URA3-hisG* in pCAKU and pCASU, respectively. After pCAKU and pCASU were linearized by digesting with *Xba*I and *Kpn*I, 100 μg of each DNA was transfected into *C. albicans* CAI4 (*ura3*Δ::*imm434*/*ura3*Δ::*imm434*) cells by the lithium acetate method (17). Before the second round of

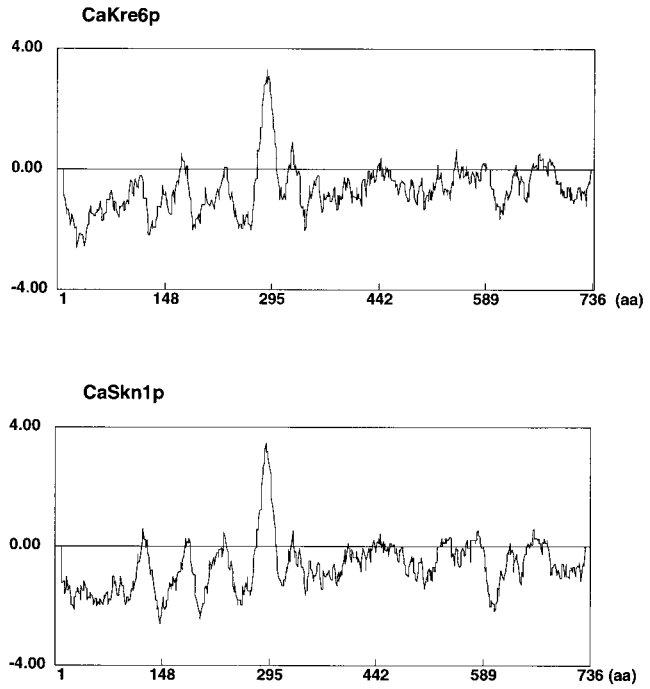

FIG. 4. Hydropathy plots of *C. albicans* Kre6p (CaKre6p) and Skn1p (CaSkn1p). Hydropathy profiles of Kre6p and Skn1p are calculated by using Kyte and Doolittle (22) parameters from the predicted amino acid sequences of the proteins. The number of amino acids (aa) is plotted on the *x* axis and hydropathy is plotted on the *y* axis of each graph.

transformation, the *URA3* gene was excised by 5-fluoroorotic acid (5-FOA) as described previously (26). The correct integration of *hisG-URA3-hisG* into the *KRE6* or *SKN1* locus was confirmed by Southern blotting using a 0.8-kb *Pac*I-*Nsp*V fragment of *KRE6* and a 0.8-kb *Xho*I-*Sma*I fragment of *SKN1* as the probes.

Fractionation and analysis of the cell wall β-glucan. Cell wall β-glucan was extracted from the *C. albicans* cells that were grown to late logarithmic phase in YPD medium, and the ratio of β-1,3 and β-1,6 linkages was determined by methylation analysis as described earlier (20).

Regulation of *C. albicans KRE6* expression. In order to control the expression of *C. albicans KRE6*, a plasmid in which *KRE6* was ligated to the *HEX1* promoter was constructed. For the directional cloning of the *KRE6* cDNA in pRC1708, an *Eco*RI site was generated just before the translation initiation site of the *HEX1* open reading frame (ORF) by PCR, and a *Bgl*II site located just after the translation termination site of the *HEX1* ORF was converted into an *Xho*I site in pRC1708 (9). Then the entire ORF of *HEX1* in pRC1708 was replaced with that of *KRE6* to generate pRC1708-CaKRE6. Primers used for generating *Eco*RI sites in the *HEX1* promoter of pRC1708 were 5′TCTAAAAGATAGATCTATTAATGT GGCTAG3′ and 5′CCGGGCTGCAGAATTCTTTGTTTCAAAACC3′.

In order to replace the promoter of *C. albicans KRE6* with that of *HEX1*, pCAK1 harboring *KRE6* was digested with *Sma*I and *Bal*I and ligated with *C. albicans URA3* to generate pCAKU1. Then, *C. albicans KRE6* cDNA that was linked to the *HEX1* promoter was isolated by digesting pRC1708-CaKRE6 with *Xho*I and *Sma*I and was ligated at the *Bam*HI cleavage site of pCAKU1. The resulting plasmid, pHCAKU1, was linearized by digestion with *Xho*I and *Apa*II and introduced into *C. albicans* CAI4 cells in which one of the *KRE6* alleles had been already flanked by the *hisG* sequences. The transfectants were selected for uracil auxotrophy in the medium containing 2% (wt/vol) *N*-acetylglucosamine (GlcNAc), and the cells bearing the *HEX1* promoter at the remaining intact *C. albicans KRE6* allele were designated HEX-CaKRE6. Induction and repression of *C. albicans KRE6* expression were performed by culturing the HEX-CaKRE6 cells in medium containing 2% (wt/vol) GlcNAc and 2% (wt/vol) glucose, respectively. In some experiments, the concentrations of Calcofluor White indicated in Fig. 9 were added to the medium.

Nucleotide sequence accession numbers. Nucleotide sequences of the *C. albicans KRE6* and *SKN1* cDNAs will be available in DDBJ, GenBank, and EMBL databases under accession numbers D88490 and D88491, respectively.

RESULTS

Cloning of *C. albicans KRE6* and *SKN1* cDNAs. Since *S. cerevisiae* Kre6p has a potential UDP-glucose-binding sequence, it seems to be an important protein for the catalytic activity of β-1,6-glucan synthase (32). Therefore, we sought to clone the *C. albicans* homolog of *S. cerevisiae KRE6*. By screening a *C. albicans* cDNA library with *S. cerevisiae KRE6* DNA as a probe, two types of cDNAs that were highly related to *S. cerevisiae KRE6* and *SKN1* were identified, and therefore they were designated *C. albicans KRE6* and *SKN1*, respectively (Fig. 1).

The *C. albicans KRE6* and *SKN1* cDNAs contained ORFs of 2,223 and 2,214 bp that could encode proteins of 82.5 and 80 kDa, respectively (Fig. 2). Although the N-terminal regions of the Kre6 and Skn1 proteins of *S. cerevisiae* and *C. albicans* were rather different, the overall sequence identities of *C. albicans* Kre6p were 55.5% with *S. cerevisiae* Kre6p and 50.5% with *S. cerevisiae* Skn1p, and those of *C. albicans* Skn1p were 53.2% with *S. cerevisiae* Kre6p and 52.2% with *S. cerevisiae* Skn1p (Fig. 3). As in *S. cerevisiae* Kre6p and Skn1p, *C. albicans* Kre6p and Skn1p had no obvious signal sequence at the N termini but contained one potential transmembrane domain within the N-terminal halves, suggesting that they are type II membrane proteins (Fig. 4). In addition, two basic amino acid residues just in front of the transmembrane domain and all potential six asparagine-linked glycosylation sites present in the C-terminal halves of *S. cerevisiae* Kre6p and Skn1p were also conserved in *C. albicans* Kre6p and Skn1p. We also isolated the genomic DNAs encompassing the 5′ noncoding regions of *C. albicans KRE6* and *SKN1* and confirmed that neither ORF could be extended further upstream due to the appearance of stop

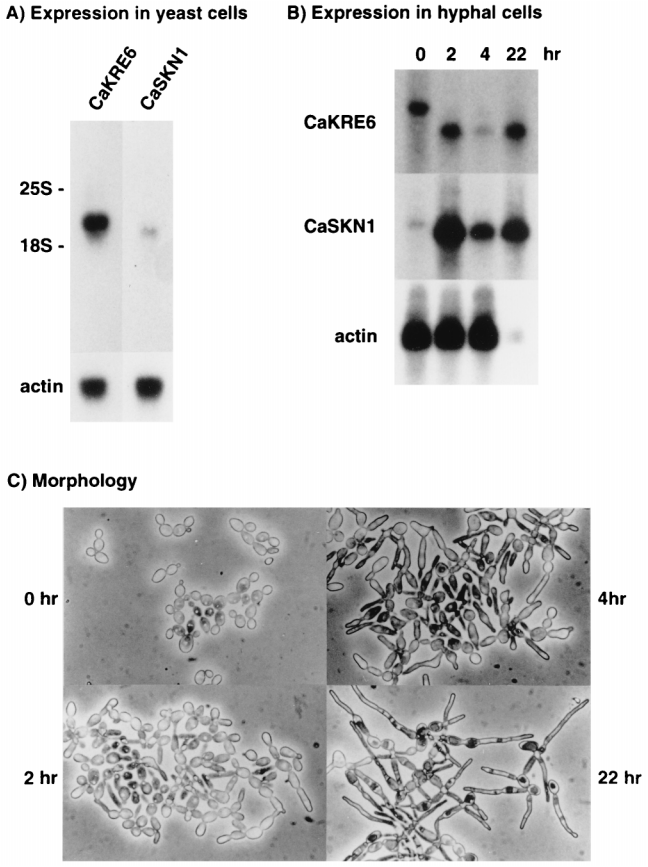

FIG. 5. Northern blotting of the *C. albicans KRE6* and *SKN1* mRNAs. Growing CAI4 cells were not induced (A) or induced to form hyphae (B) and were harvested at the indicated time after hyphal formation. Photographs of the cells after hyphal induction are shown in panel C.

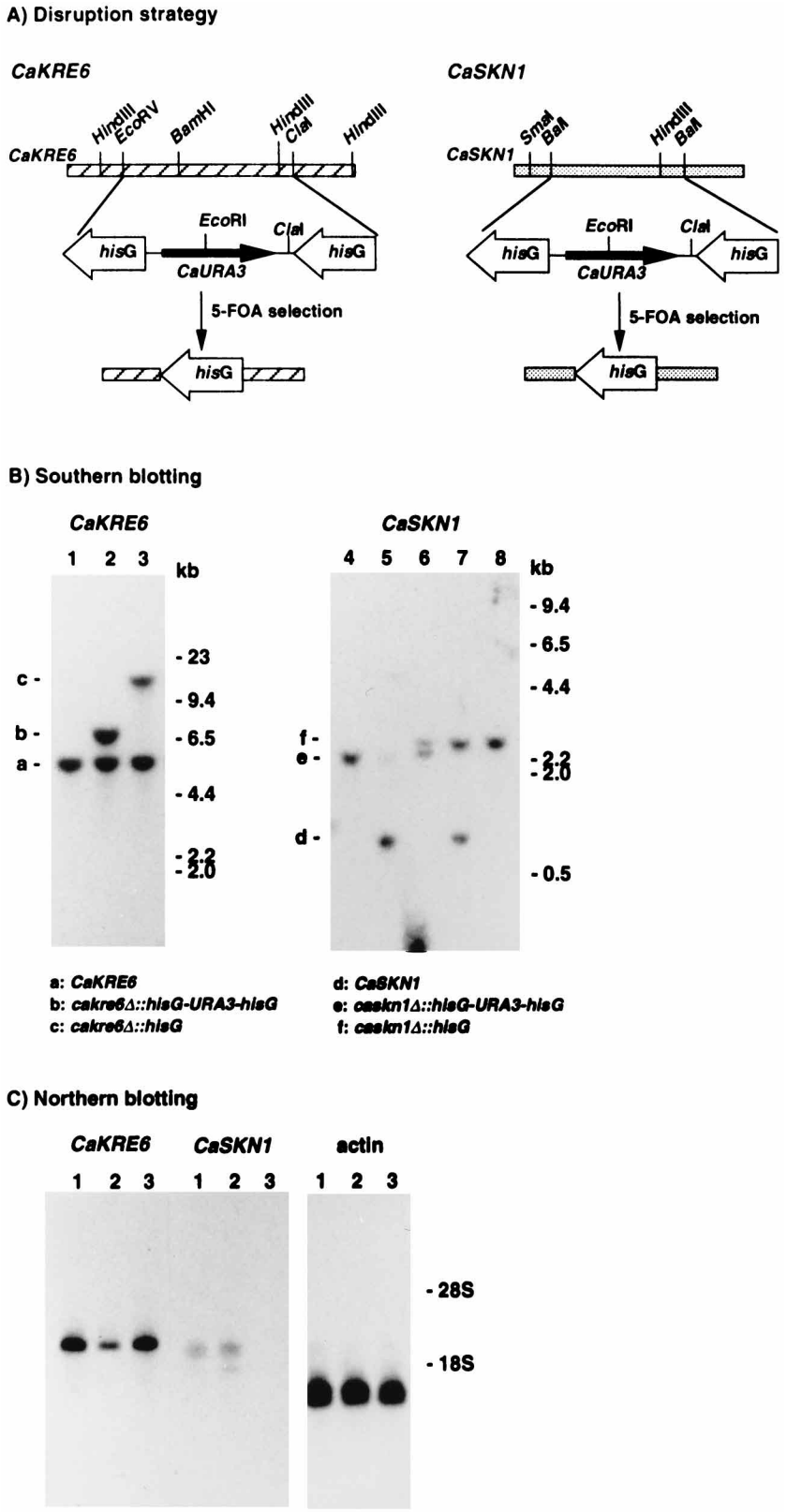

FIG. 6. Disruption of *C. albicans KRE6* (*CaKRE6*) and *SKN1* (*CaSKN1*). (A) The strategies for disruption of *KRE6* and *SKN1* are illustrated. (B) The correct integration of the *hisG* sequences into the *KRE6* or *SKN1* locus was confirmed by Southern blotting. Genomic DNAs extracted from strains CAI4 (lanes 1 and 4), CaKRE6(+/−) (lanes 2 and 3), CaSKN1(+/−) (lanes 5 and 6), and CaSKN1(−/−) (lanes 7 and 8) before (lanes 1, 2, 4, 5, and 7) and after (lanes 3, 6, and 8) 5-FOA selection were digested with *Eco*RI (for the *KRE6* probe) or *Eco*RI and *Xho*I (for the *SKN1* probe). Bands derived from the *KRE6* alleles and *SKN1* alleles are indicated by a to f. (C) The mRNA levels of *KRE6*, *SKN1*, and actin in strains CAI4 (lane 1), CaKRE6(+/−) (lane 2), and CaSKN1(−/−) (lane 3) were demonstrated by Northern blotting.

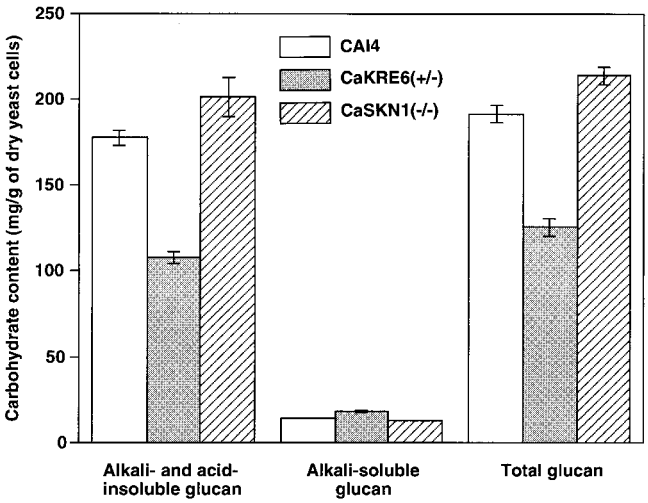

FIG. 7. Comparison of β-glucan levels in strains CAI4, CaKRE6(+/−) and CaSKN1(−/−). Cells of the indicated strains in mid-logarithmic phase were harvested, and the levels of alkali- and acid-insoluble, alkali-soluble, and total β-glucan were analyzed. β-Glucan levels are given in milligrams per gram (dry weight) of yeast cells.

codons and also that there is no indication of the existence of exons and introns (data not shown).

Northern blotting revealed that *C. albicans KRE6* and *SKN1* were expressed as 2.4- and 2.3-kb mRNAs, respectively. These sizes agreed well with the lengths of the ORFs (Fig. 5A). The level of the *C. albicans KRE6* mRNA was 20 to 30 times higher than that of the *C. albicans SKN1* when cells were in the yeast phase (Fig. 5A). When growing CAI4 cells were transferred to RPMI 1640 medium, more than 90% of the cells underwent hyphal growth within 4 h, and almost all cells exhibited a typical hyphal morphology by 22 h (Fig. 5C). Induction of hyphal growth strongly increased the amount of the *C. albicans SKN1* mRNA, and the level of the *SKN1* mRNA remained high for at least 22 h. The *C. albicans KRE6* mRNA level was more or less the same between yeast and hyphal cells, although the actin mRNA level was significantly lowered at 22 h (Fig. 5B). It should be also noted that both the *C. albicans KRE6* and *SKN1* mRNA levels were transiently decreased at 4 h, and this transient decrease of the mRNA level was not observed in the actin mRNA (Fig. 5B). The higher expression of *C. albicans SKN1* in cells that were induced to form hyphae prompted us to search for the sequence of the 5′ noncoding region of the *SKN1* locus, but there was no particular known sequence except for potential TATA boxes. Interestingly, induction of hyphal growth reduced the sizes of the *C. albicans KRE6* and *SKN1* mRNAs (Fig. 5B). However, the decrease in sizes of the *C. albicans KRE6* and *SKN1* mRNAs was not the general consequence of morphogenetic transition from yeast to hypha because the size of the actin mRNA remained constant even after hyphal induction (Fig. 5B).

Involvement of *C. albicans KRE6* in β-1,6-glucan synthesis. Since *KRE6* is involved in β-1,6-glucan synthesis of *S. cerevisiae*, it is highly conceivable that *KRE6* and *SKN1* are also required for β-glucan synthesis in *C. albicans*. We have addressed this possibility by disrupting the *C. albicans KRE6* and *SKN1* genes. The results of Southern blotting revealed that the *hisG-URA3-hisG* sequences were correctly integrated into the *C. albicans KRE6* or *SKN1* locus and that *URA3* was eliminated by the 5-FOA treatment (Fig. 6B). Although both alleles of *C. albicans SKN1* were flanked by the *hisG* sequences, we never obtained the clones harboring the homozygous *kre6*Δ null mutation despite the extensive second-round transformation, raising the possibility that *KRE6* is essential for the growth of *C. albicans*.

There was no significant difference in morphology, growth rate, ability to form hyphae, and susceptibilities to antifungal agents such as echinocandin B among parental CAI4, the hemizygous *kre6*Δ null mutant [CaKRE6(+/−)], and the homozygous *skn1*Δ null mutant [CaSKN1(−/−)] strains. We also examined whether cell wall β-glucan synthesis was affected by the disruption of *C. albicans KRE6* and *SKN1*. Strains CaSKN1 (−/−) and CAI4 contained similar levels of β-glucan in the alkali-insoluble and -soluble fractions (Fig. 7). However, the amount of β-glucan in the alkali-insoluble fraction decreased remarkably in CaKRE6(+/−), while that in the alkali-soluble fraction with the same mutation increased slightly (Fig. 7).

Methylation analyses of the fractionated β-glucans revealed that a much higher percentage of β-1,6 linkages was present in the *C. albicans* cell wall β-glucan than in *S. cerevisiae* cell wall β-glucan. More than half of the alkali-insoluble glucan was β-1,6 linked (Table 1), whereas about 90% of the alkali-insoluble glucan was β-1,3 linked in *S. cerevisiae* (12, 20). The remarkable reduction of the alkali-insoluble β-glucan in strain CaKRE6(+/−) was mainly due to the decrease in β-1,6-glucan; the hemizygous *C. albicans kre6*Δ mutation decreased β-1,6-glucan in the alkali-insoluble fraction by about 80% but did not affect β-1,3-glucan in the alkali-insoluble or -soluble fraction (Table 1). Furthermore, there was a correlation between the levels of β-1,6-glucan and *C. albicans KRE6* mRNA. In CaKRE6(+/−), the amount of *C. albicans KRE6* mRNA was about 40% of that of CAI4, whereas the levels of the *SKN1* and actin mRNAs were almost the same as those of CAI4 (Fig. 6C). These results demonstrate that *KRE6* is involved in β-1,6-glucan synthesis in *C. albicans* and that *SKN1* is functionally redundant.

Suppression of *C. albicans KRE6* expression affected budding. In order to gain more insights into the physiological role of *C. albicans KRE6*, we have developed a system to regulate *KRE6* expression. This was achieved by generating a *C. albicans* strain in which one of the *KRE6* alleles was flanked by the *hisG* sequences but another was replaced with that linked to the *HEX1* promoter so that *KRE6* gene expression was controlled by GlcNAc and glucose (Fig. 8A). Southern blotting revealed that the 4.6-kb wild-type *C. albicans KRE6* allele was

TABLE 1. Molar ratios of β-glucans in wild-type, hemizygous *kre6*Δ, and homozygous *skn1*Δ mutant cells of *C. albicans*[a]

Glucose linkage(s)[b]	Molar ratio of glucose in samples					
	Alkali-soluble glucan			Alkali- and acid-insoluble glucan		
	CAI4	CaKRE6 (+/−)	CaSKN1 (−/−)	CAI4	CaKRE6 (+/−)	CaSKN1 (−/−)
G^{1-}	0.6	1.3	1.0	3.8	1.4	2.5
$_{-3}G^{1-}$	7.6	3.9	8.8	14.7	14.1	10.6
$^{-6}G^{1-}$	2.2	2.8	1.8	20.6	3.0	12.2
$^{-6}_{-3}G^{1-}$	1.0	1.0	1.0	1.0	1.0	1.0
$_{-3}G^{1-}/^{-6}G^{1-}$ ratio	3.39	1.40	4.98	0.71	4.72	0.87

[a] One-gram samples of fractionated β-glucan from the parental strain CAI4 and the hemizygous *kre6*Δ [CaKRE6(+/−)] and homozygous *skn1*Δ null [CaSKN1(−/−)] mutant strains were methylated, hydrolyzed, and acetylated. The methylated alditol acetates were analyzed, and the molar ratios of glucose with the indicated linkages are shown.

[b] G^{1-}, $_{-3}G^{1-}$, $^{-6}G^{1-}$, and $^{-6}_{-3}G^{1-}$ represent 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylglucose, 2,3,4-tri-*O*-methylglucose, and 2,4-di-*O*-methylglucose, respectively.

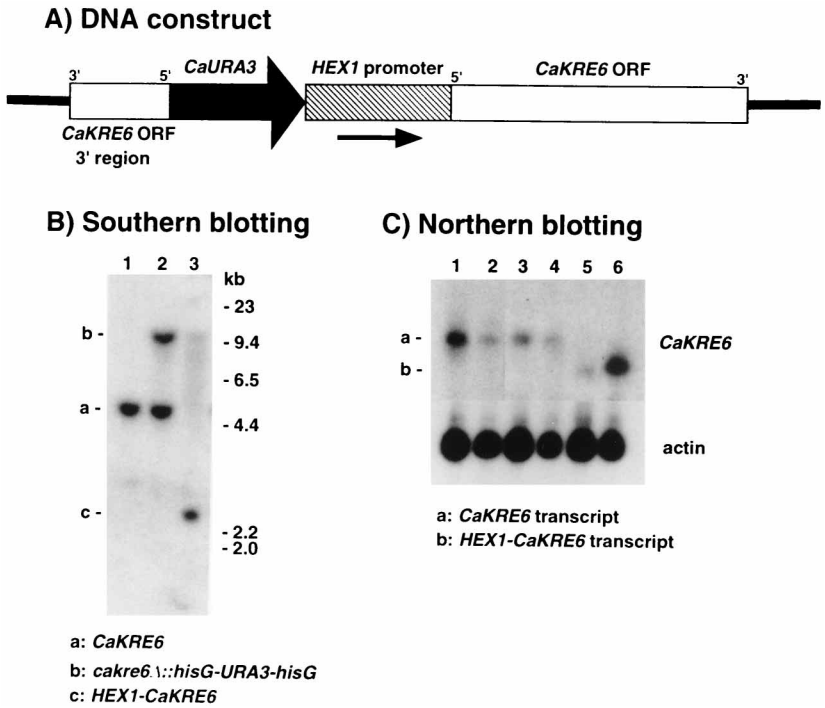

FIG. 8. Suppression of *C. albicans KRE6* (*CaKRE6*) transcription by the *HEX1* promoter. (A) Map of DNA used to replace the *KRE6* promoter with the *HEX1* promoter is illustrated. (B) The correct integration of the *HEX1-KRE6* DNA into the *KRE6* locus was confirmed by Southern blotting. Genomic DNAs extracted from strains CAI4 (lane 1), CaKRE6(+/−) (lane 2), and HEX-CaKRE6 (lane 3) were digested with *Eco*RI. Bands that were derived from the intact *KRE6* allele, the *kre6*Δ::*hisG-URA3-hisG* allele, and the *HEX1-KRE6-URA3* allele are labeled a, b, and c, respectively. (C) The mRNA levels of *KRE6* and actin in strains CAI4 (lanes 1 and 2), CaKRE6(+/−) (lanes 3 and 4) and HEX-CaKRE6 (lanes 5 and 6) in the presence of glucose (lanes 1, 3, and 5) or GlcNAc (lanes 2, 4, and 6) were demonstrated by Northern blotting.

converted into the 2.7-kb *HEX1-KRE6* allele in the HEX-CaKRE6 strain (Fig. 8B). Since *URA3* was also connected to the *HEX1-C. albicans KRE6* DNA, we used strains CAI4 and CaKRE6(+/−), which still contained single copies of *URA3* in the original *URA3* locus and the disrupted *KRE6* locus, respectively, as control strains in the following experiments. The HEX-CaKRE6 cells grew in either glucose- or GlcNAc-containing medium, although the *KRE6* and actin mRNA levels declined in the presence of GlcNAc. As mentioned earlier, the hemizygous *C. albicans kre6*Δ mutation alone decreased the *KRE6* mRNA level by about 60%. Cultivation of HEX-CaKRE6 in glucose-containing medium further decreased the level of the *C. albicans KRE6* mRNA; the *KRE6* mRNA level in HEX-CaKRE6 was about one-fifth that of CaKRE6(+/−) in glucose-containing medium (Fig. 8C). In that circumstance, the HEX-CaKRE6 cells exhibited a partial defect in cell separation (Fig. 9A). However, it should be also noted that morphology and growth of hyphae were not significantly affected by *C. albicans KRE6* suppression.

As some of the *S. cerevisiae* mutants that were hypersensitive to Calcofluor White were defective in β-1,6-glucan synthesis (18, 31), we also examined whether the suppression of *C. albicans KRE6* transcription affected susceptibility to Calcofluor White. As expected, strain HEX-CaKRE6 was more susceptible to Calcofluor White when cultured in glucose-containing medium, while the hemizygous *kre6*Δ mutation only slightly increased the Calcofluor White sensitivity (Fig. 9B).

DISCUSSION

We have isolated the cDNAs for *C. albicans* homologs of *S. cerevisiae KRE6* and *SKN1*. Although there was no obvious change in the amount of cell wall β-glucan in the homozygous *C. albicans skn1*Δ null mutants, the hemizygous *kre6*Δ mutation resulted in a more than 80% reduction of the cell wall β-1,6-glucan. Thus, only *C. albicans KRE6* is active and *SKN1* is redundant in terms of β-1,6-glucan synthesis. The finding that *C. albicans SKN1* expression was significantly induced upon induction of hyphal growth allowed us to speculate that *SKN1* is required for hyphal growth. However, the efficiency of hyphal formation was not affected by the disruption of *C. albicans SKN1*, leading us to the conclusion that the increased expression of the *SKN1* mRNA would not be essential even for the hyphal growth of *C. albicans*. On the other hand, in *S. cerevisiae*, *SKN1* is capable of suppressing the *kre6*Δ null phenotype in a dosage-dependent manner. Disruption of either *KRE6* or *SKN1* is not lethal, so *KRE6* and *SKN1* are expected to be functionally complementable (34). We could not address the possibility that *C. albicans SKN1* is able to suppress the *kre6*Δ null mutation due to the failure to recover strains containing a disruption of both alleles of *KRE6* in *C. albicans*.

Induction of hyphal growth shortened *C. albicans KRE6* and *SKN1* mRNAs, while actin mRNA was more or less the same size in yeast and hyphal cells. In addition, the sizes of mRNAs of other cell wall genes such as the chitin synthase gene (*C. albicans CHS3*) (38) and the β-1,3-glucan synthase gene (*C. albicans GSC1*) (data not shown) were not significantly altered by hyphal induction. These results raise the possibility that there are at least two transcription start sites in the *C. albicans KRE6* and *SKN1* genes and that they are used in a yeast- or hypha-specific manner. In fact, there are several potential TATA boxes in the 5′ noncoding regions of *C. albicans KRE6* and *SKN1*. Thus, it would be of interest to identify the hyphal

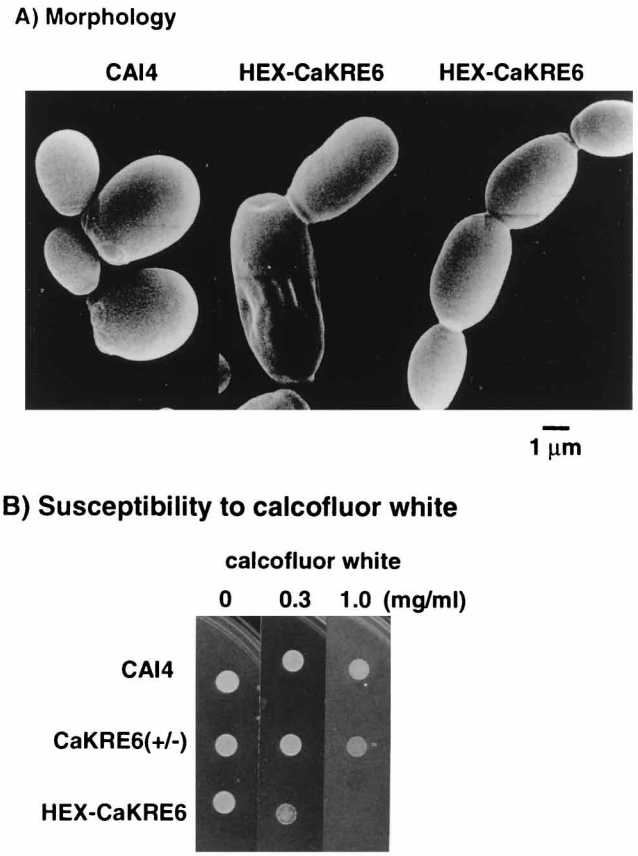

FIG. 9. Effects of suppression of *C. albicans KRE6* transcription on morphology and sensitivity to Calcofluor White. (A) Morphology of CAI4 and HEX-CaKRE6 cells that were cultured in glucose-containing medium were examined by scanning electron microscopy. (B) CAI4, CaKRE6(+/−), and HEX-CaKRE6 cells were cultured in the presence of the indicated concentrations of Calcofluor White.

growth-responsive elements in the *C. albicans KRE6* and *SKN1* promoters to understand the mechanism behind the yeast- or hypha-specific transcription.

On the other hand, we have exploited the controllable gene expression system in *C. albicans* by using the *HEX1* promoter. Cultivation of strain HEX-CaKRE6 in glucose-containing medium decreased the *C. albicans KRE6* mRNA level by about 80% compared to the hemizygous *kre6*Δ mutant whose *KRE6* mRNA level was already about 40% of the parental CAI4 level. When *KRE6* expression was repressed, *C. albicans* cells did not separate completely after mitosis, suggesting that the wild-type level of β-1,6-glucan is required for the completion of budding. Moreover, *C. albicans KRE6* repression conferred an increased susceptibility to Calcofluor White. This is consistent with the results reported for *S. cerevisiae*. The *cwh48* strain, a mutant that is hypersensitive to Calcofluor White, has a *kre6* allele. The introduction of the intact *KRE6* gene into the *cwh48* mutant restored the wild-type sensitivity to K1 killer toxin, whose acceptor was identified as β-1,6-glucan (31).

As mentioned earlier, we never achieved the homozygous *C. albicans kre6*Δ null mutation even after extensive transfection of pCAKU DNA into the hemizygous *kre6*Δ mutant cells, and therefore, we expected that *KRE6* would be an essential gene. As the HEX-CaKRE6 cells grew in the glucose-containing medium, the glucose-repressed level of *C. albicans KRE6* mRNA in the HEX-CaKRE6 cells must be sufficient to keep the cells alive. More strict repression of *C. albicans KRE6* will be necessary to define the essentiality of the *KRE6* gene.

In *S. cerevisiae*, more than 90% of the cell wall β-glucan is β-1,3 linked and β-1,6-glucan is less than 10% of the total β-glucan (12, 20). However, the results of methylation analysis of *C. albicans* cell wall β-glucan revealed that about 60% of the alkali- and acid-insoluble β-glucan is β-1,6 linked and that 10 to 20% of the alkali-soluble glucan is also β-1,6 linked. Thus, the proportion of β-1,6-glucan in the *C. albicans* cell wall is considerably higher than that in *S. cerevisiae*.

It was reported that amino acid sequences ^{420}R-V-S-G of *S. cerevisiae* Kre6p and ^{472}R-V-T-G of *S. cerevisiae* Skn1p shared limited sequence similarities to the substrate-binding motif of glycogen synthase, K/R-X-G-G (13, 32). We also searched for such a sequence in *C. albicans* Kre6p and Skn1p and found the potential candidates ^{445}N-V-T-G in Kre6p and ^{449}N-I-T-G in Skn1p. However, because the basic amino acid in K/R-X-G-G of glycogen synthase is essential for binding to the substrate (13), another biochemical approach should be used to address the possibility that *C. albicans* Kre6p and Skn1p bind to UDP-glucose and function as β-1,6-glucan synthase.

Previously, Boone et al. (3) isolated the *C. albicans* homolog of *S. cerevisiae KRE1* and demonstrated that *C. albicans KRE1* functionally complemented the loss of susceptibility of the *S. cerevisiae kre1*Δ null mutant to K1 killer toxin. Identification of the *KRE6* and *SKN1* homologs in *C. albicans* further supports the idea that the mechanism by which the *KRE* genes are involved in β-1,6-glucan synthesis is generally conserved in yeast cell wall biosynthesis.

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