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

TOSHIYUKI MIO,¹ TOSHIKO YAMADA-OKABE,² TOMIO YABE,^{1,3} TASUKU NAKAJIMA,³ MIKIO ARISAWA,¹ and HISAFUMI YAMADA-OKABE^{1*}

Department of Mycology, Nippon Roche Research Center, Kamakura 247,¹ Department of Hygiene, School of Medicine, Yokohama City University, Yokohama 236,² and Department of Applied Biochemistry, Faculty of Agriculture, Tohoku University, Sendai 981,³ Japan

Received 25 November 1996/Accepted 27 January 1997

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* null mutation. Although we never succeeded in generating the homozygous *C. albicans kre6* null mutant, the hemizygous *kre6* 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 -1,6glucan 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*

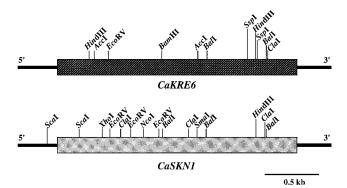


FIG. 1. Restriction maps of the *C. albicans KRE6* and *SKN1* cDNAs. Restriction maps of the *C. albicans KRE6* and *SKN1* cDNAs are illustrated. Thick bars represent the ORFs found in the *KRE6* and *SKN1* cDNAs.

^{*} Corresponding author. Mailing address: Department of Mycology, Nippon Roche Research Center, 200 Kajiwara, Kamakura, Kanagawa 247, Japan. Phone: 81-467-47-2213. Fax: 81-467-46-5320. E-mail: hisa fumi.okabe@roche.com.

2364 MIO ET AL.

J. BACTERIOL.

A aacaaccaccattcatatatatatatatatatattacttgataagacctcaaa	-1	ACATTAAATTTACGTATGGATGCTTATAAAAATCATAATTTATTCTATCGTTCAGGAATG T L N L R M D A Y K N H N L F Y R S G M	1260 420
ATGGGGTCTCAAAGAGATTTAACTICAAATAATCCTCATTTCCATATATCGAGTTCTCGT	60	GTACAAAGTTGGAATCAATTGTGTTATACTCAAGGTCATTTAGAAATTCTGGCTCGTTTA	1320
M A S Q R D L T S N N P H F H I S S S R	20	V Q S W N Q L C Y T Q G H L E I S A R L	440
AACAATACCAATGATGATTTCCAATTCTGATCATAATCCAAGAAATCCATTTGGTGATGAA	120	CCAAATTATGGTAATGTAACAGGGTTATGGCCTGGGTTATGGTCTATGGGGAATTTAGGT	1380
N N T N D D F N S D H N P R N P F G D E	40	PNYGNVTGLWPGLWSMGNLG	460
CCAGAAGAAGAAGAAGAATTAGATTCAGCATCATTITCATCCTCATCACAAAATAATCAA	180	AGACCAGGGTATTTGGGATCTACTGATGGGGTATGGCCATATTCTTACGATTCATGTGAT	1440
P E E E E E L D S A S F S S S S Q N N Q	60	R P G Y L G S T D G V W P Y S Y D S C D	
CCCCATCACCCTTTTGCTACATCAAATCAAAACC CTG AGCACTAATAATCTTCATAGTACC	240	GCCGGTATTACACCTAATCAATCTTCTCCTGATGGGATTTCTTATTTACCAGGTCAAAGA	480
P H H P F A T S N Q T S S T N N L H S T	80		1500
AACAGTATTCGAAATGAAGCTGAATATGGCAAACCATTCCAAGCTTATAGTGGCTACTAT	300	A G I T P <u>N</u> Q S S P D G I S Y L P G Q R	500
N S I R N E A E Y G K P F Q A Y S G Y Y	100	TTAAATGAATGTACATGTCCAGGTGAATTACATCCTAATGGAGGTGTTGGTAGAGGTGCCC	1560
TCAAATGGTGGTAGTTCA CTG TATTTAAATCAAGAAGGTGTGATTTCTGATGAAAGTAGA	360	L N K C T C P G E L H P N R G V G R G A	520
SNGGSSS S YLNQEGVISDESR	120	CCTGAAATTGATGTTATTGAAGTGAAGAGAGAGAGAGAGA	
CTATTATCATCOGGTAATAATAATAATAATAATAATAATCGAGATGTATCTAGTGTT L L S S G N N N N N N N N N R D V S S V	420 140	PEIDVIEGEVMTDSSGKKEN	540
GGTGGTGGTGGTGGTGGTGGTGGCAATAGATTGAATCCAACCAGTCCAGCAGAATTTGAT	480	TGTGGTGTTGCCTCTCAATCCTTACAATTGGCCCCTATGGATATTTGGTATATTCCTGAT C G V A S Q S L Q L A P M D I W Y I P D	560
G G G G G G S G D N R L N P T S P A E F D	160	TATAATTGGGTGGAAATCTACAATTTTCAGTTTCAACGATGAATACTTATACTGGTGGA Y N W V E I Y N F S V S T M N T Y T G G	1740
AGATATCCATCAATGGCAGGTTCAAGAGTAGTATCATCTACTTCATTAGTGTCGTTTAAT	540		580
R Y P S M A G S R V V S S T S L V S F N	180	CCAPTCCAACAAGCATTATCAGCAACAACCATGTTGAATGTTACATGGTATGAATTTGGT PFQQALSALSATTMLNVTWYEFG	1800
AATCCTTCCAATATGATGCGTAACCACCATCAACATCAACCTCCATCTCGGTTCATCTTCA	600		600
N P S N M M R N H H Q H Q P H L G S S S	200	GATAATGCCCATAATTICCAAACTTATGGTTATGAATATTTAAATGACCCTGAAACGGGT	1860
	660	D N A H N F Q T Y G Y E Y L N D P E T G	620
S P T S S M N N D S I S D K S T S P F	220	TATTTACGATGGTTTGTTGGTGATGATCCAACATTAACGGTTTATTCACAAGCTTTACAT Y L R W F V G D D P T L T V Y S Q A L H	1920 6 4 0
CCTAATGATTTCAGTCCATTGGTGGATACCCTGCTAGTCCATTCAGTCCATTGAGTATTGAT	720	CCAGATGGAAATATTGGTTGGAGACCTTTAAGTAAAGAACCAATGTCATTAATTTTAAAT	1980
PNDFSPFGGYPASSFPLSID	240	PDGNIGWRPLSKEPMSLILN	660
GAAAAAGAACCAGATGATTATTTGAATAATCCAGATCCAGTACAGGATGCTGAATACGAA	780	TIGGGATTICAAATAATIGGGCTTATATTGATTGGCCAAGTATATCTTTCCCCGTCACA	2040
E K E P D D Y L N N P D P V Q D A E Y E	260	L G I S N N W A Y I D W P S I S F P V T	
AAGAACCGATTTATGCACGATTTAAAGAACATGGATAAACGATCATTGGGTGGG	840 280	TTTAGAATCGATTATGTAAGAGTTTATCAACCACCAGATCAAATTAATGTTGGTTG	680 2100
GGATTTATTATTATTATTATAGCIGCATTAGCAGIGITTATCATTTTACCIGCATIGACT	900	F R I D Y V R V Y Q P P D Q I N V G C D	700
GFIILFIAALLAVFIILPALT	300	CCAACCGATTICCCAACTTATGATATTATICAACAACATTTGAATCTTTATGAAAATGCC	2160
TATTCTOGAGCTACTAACCCTTATCATCCAGAAAGTTACGAAGTTTTAACTAAATAT CTG Y <u>S G A T N</u> P Y H P E S Y E V L T K Y S	960 320	P T D F P T Y D Y I Q Q H L N L Y E N A	720
		<u>N</u> I T S F E D G G Y K F P K N S L I G C	740
Y P M L S A I R M N L V D P D T P E S A TATAAAAGAAGGCTAAAGATGGTAGTGGATGGGTATTAGITTTCAGTGATGAATTTGAT	340 1080	TAAaaaataaaagatggtactactattactactactacttgttttcatgatttttcttt * ttattaatgtttttggggttgataggtttgatttga	
Y K K A K D G S E W V L V F S D E F D	360	$\tt gtttttcatttgtttgttatttgattttgtattttttttt$	2400
GCTGAAGGTAGAACTTTTTTATGAAGGCGATGATCAATTTTTCACCGCTCCTGACATTCAT A E G R T F Y E G D D Q F F T A P D I H	1140 380	actacaagtactactactattaataatgcttcaataccatattatatgtgtatgttaagt	
TACGATGCCACTAAAGATTTAGAATGGTATGATCCAGATGCTGTAACCACAGCCAATGGA	1200	attaagtattattaagtaataagtataaagtagaaaatgtatttgaataaaatgatatat	
Y D A T K D L E W Y D P D A V T T A <u>N</u> G	400	attgctcaagtcttttttattatattttaaatattatattatttgttatagaatttttt	
		taaataaagettttatttatcaaaaaaaaaaaaaaaaaaa	2620

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),

B	1	cat	cat	ata	ata	tac	aat	tgg	cta	cta	aaa	act	taa	acti	ttt	aat	aca	act	taca	-1	A
	הע	100	Gan	עדיידיי	200	ኮጥል	יג גיד	TCA		GDA	ልልጥ		ദനന	ጉልሮ	CGA	ጥልርግ	TGA	AGG	ACAA	60	Т
M E		304		L	т	Y	N	A	P	K	I	ĸ	F	т	D	т	E	G	Q	20	с
		-	-					-		-	-										R
GAAG	AA	CAT	TT	TAT	rtr	CAA	TCG	GAG	таа	CAA	TTC.	AAC	CAA	TGA	TTT.	AAC	CAG	TCA	TGAC	120	
ΕE			F	Y	F	N	R	s	N	N	s	т	N	D	L	т	s	н	D	40	с
																					s
ICTI	'CA'	ГСA	ACT	rCA/	ACT.	ACA	AGA	TGC	CAA	TTC	CAG	AAG	ACA	AGC	CCC	ACC.	ACC	ACC	ACCA	180	
s s	;	s	т	Q	L	Q	D	А	N	s	R	R	Q	А	Ρ	Ρ	Ρ	P	Ρ	60	G
																					G
CATA	AT	CCA	TT	TC	IGA	CAA	TTC	CCA	TGA	ААА	TAG	TAC	TGA	ATC.	ATT	ATA	TCA	ATC	AGAA	240	
H N	1	Р	F	s	D	N	s	н	Е	N	s	т	Е	S	L	Y	Q	s	Е	80	G
																					E
	GA	rrı	CAT	FCA	ACC	ACT	ACT	TCA	TAA	TGA	TAG	таа	TAA	TAG	CAA	TAG	CAG	TAT	AGGC	300	
r F								н		D			N	s	N	s	s	I	G	100	С
	-	-		~	-																F
ኣልጥል	ልጥ	AGZ	CA	ACG	ኮልጥ	TCC	ATC	ACA	ACA	ACA	TGA	TAC	АСТ	GTC	ATT	ATA	TTC	AGC	ATCA	360	F
1 N		R	Q	R	I	P	S		0	Н	D	т	s	s	L	Y	s	A	S	120	~
• •	•		×	11	-	-	5	×	×		~	-	-	2		•	2		-		G
<u>י ריי</u>	גייי	ירי	201	ልጥሮ	مص	արա	'a Cim	ጥጥ	ጥልኦ	ւրդու	יייש	ልጥር	ልጥል	ጥ ር ግጥ	GGA	CAA	CCA	AGA	CGAA	420	G
2 CAA ? I		s s	T	S	P	L	V	s	N	F	Q	S	Y	s	D	N	Q	D	E	140	
	•	C)	-	5	£	ч	v	5	14	£.	×	5	1	5	2		×	~	-		0
، بېلىر	\	~~ 7	00	יי מיו	<u>م</u> ست	መ እ እ	CC 7	<u> </u>	ሞእር	הממי	marc	പ്പം	הממי	ጥጥ	תקע	ጥጥ እ	ጥልጥ	т <u>с</u> 2	ACAC	480	V
		R	GGG.	K	Y	N	0	N	T	N	R	S	S	s	N	Y	I	0	Н	160	
r 1	Ľ	ĸ	G	г	I	14	Ŷ	14	1	IN	r	5	5	5	14	1	-	Ŷ		100	C
			-			oma	~~~	ma c		mac				m	2000	0.00		maa	TGGA	540	I
										P	L	K	T	0	S	S.CAG	I	G	G	180	
5 1	,	т	s	Α	G	Y	D	R	Y	Р	Ц	r	т	Q	Б	2	т	G	G	100	C
														201	202	203		203	~ m ~	600	Ç
																			CATG	600	
S N	1	s	R	Ι	G	Г	s	S	S	s	Р	s	Q	Q	Q	Q	Q	Η	М	200	C
							_	-						-			mer	-		660	H
																			AATG	660	
ΥI	C	N	N	s	s	N	R	S	s	Y	s	Ρ	D	S	Α	т	D	L	М	220	Т
																					и
GTTI	FAT	GA	1AA	TGG		LTA													CTTA	720	
V 3	Ľ	Е	N	G	Е	F	S	Ρ	F	G	G	Y	Ρ	А	S	L	F	Ρ	L	240	P
																					N
AGTZ	ATT	'GA'	ſGA	AAA	AGA	ACC	CGA	ATGP	TTZ	\TT]									ATGCC	780	
s :	Γ	D	Е	К	Е	Ρ	D	D	Y	L	н	N	Ρ	D	Ρ	v	Q	D	А	260	г
																					S
GAA'	ΓΑΊ	'GA'	FAA	AAA	TCO	GTI	TTT	TATA	ATGI	ACTI	AAA	AAC	CAT	GGA	TAA	AGAA	ATC	TAT	GAAT	840	
E	Y	D	к	N	R	F	L	Y	D	Г	К	т	М	D	к	ĸ	Ş	М	N	280	G
																					E
GGA'	гтA	AT	IGC	ATT	TAT	TG	PAAT	IGTI	PTT1	raa1	TGC	TAT	TGC	TAT	ATT	TAT	'AA'	TT.	TACCA	900	~
G I				F															Р	300	Т
	-				-																F
0000		12.0		ma ~	maa	· ۳۰	· ۲۰۰۰	000			~~~~~~			<u>מי א</u> קות		1	UTC 7		TTTA	960	•
																				320	А
V I	ť	'T'	Y	T	G	Y	N	-P	ĸ	A	ĸ	IN	F	Е	N	Y	Ε	V	ч	J20	K
																					r
																			ATACT		_
r I	R	Y	s	Y	Ρ	R	L	S	А	I	R	т	s	L	I	D	Ρ	D	т	340	a
CCT	GA/	GA	IGC	CTI	TAT	TT	GA/	AATO	CTA	AAA2	ATGO	TG	AG/	ATC	GCC	CATI	rGG7	GT	TAGT	1080	С
P :	Е	D	А	L	F	W	к	s	ĸ	N	G	Е	Е	W	Ρ	L	v	F	S	360	
																					а
GAT	GAJ	\TT	TAA	TGC	CGI	AGG	3TAC	GAAC	CAT	PTT	ATG	AGG	GTG	ATGP	ATC2	AATT	TTT:	rca	CTGCA	1140	
																			A	380	
D	-	-			_										~						
D																					
	GA	ካጥጥ	ACA	TT	ATG:	ATG	CCAG	CTA	AAG	ATT	ragi	ATA	GTZ	ATGA	ATCO	CCGA	ATGO	CTG	гааст	1200	
																				1200 400	

۵C	ദദറ	י בידי	TCC	220	አጥጥ	ممد	mmm	100	ጥልጥ	CCA	TCC	mmm	ה א תיי	אאא	መርጉአ	TTCCC	יחיתי	አጣጣ	TTAT	1260
T	A	N	G	T	L	T	L	R	M	D	.16C	F	K	N	Н	G	L	F	Y	420
CG	ATC	AGG	GAT	GAT	TCA	AAG	TTG	GAA	TCA	ААТ	GTG	TTI	TAC	TCA	GGG	GAA	ATT	GGA	ATTT	1320
R	s	G	М	I	Q	s	W	N	Q	М	С	F	т	Q	G	ĸ	L	Е	F	440
~	.		م سر		~~~	000.0	100			~~~		7 mm		~~~		~~~~	3 000	~~~	AATG	1200
S	A A	AAA K	L	ACC P	G	GTA Y	G	N	I	CAC T		ATT F	w	GCC P	IGG G	GTT L	ATG W	GTC S	AA'IG M	1380 460
5	л	I.	П	r	G	1	G	IN	т	T	G	Ľ	vv	F	G	Ц	**	5	м	400
GG	ААА	TTT.	AGG	ICG	TCC.	AGG	GTA	TCT	TGC	CCT	GAC	TGA	AGG	TGT	TIG	GCC	АТА	TAC	TTAT	1440
G	N	L	G	R	Ρ	G	Y	L	А	s	т	Е	G	v	W	Ρ	Y	т	Y	480
																			TTTA	1500
D	S	С	D	Α	G	Ι	т	Ρ	N	Q	S	s	Ρ	D	G	I	s	Y	L	500
~~	200	m C 3	N N C				N MIC			.	100		100		maa		m aa	Taa	IGTT	15.00
P	G G	0	R	T.	N N	K	C C	TAC	C	P	AGG G	TGA E	AGC A	H	P	TAA N	R	G	V	1560 520
-	0	×		2			Ũ	-	C	-	Ŭ	-			•			0	•	520
GG	TAG	AGG	TGC	CCC	TGA	AAT	IGA	IGC.	ATT	GGA	AGG	GGA	ААТ	TCA	TGG	TGT	TAT.	AGG	TAGA	1620
G	R	G	А	Ρ	Е	I	D	А	L	Е	G	Е	I	н	G	v	I	G	R	540
																			TTTT	1680
v	s	Q	S	L	Q	v	A	Ρ	Y	D	Ι	W	Y	М	Ρ	N	Y	D	F	560
ĊT	גיית	יתר ה	TC 3 (י ה איז	T AC	ኮሞር	<u>አ</u> አሙ	A A C	<u>م</u> سم	ת ג ג	~~~~	T A C					100		CCAA	1740
L	E	I	H	N	S	S	I	T	L	M	N	T	Y		G		P		0	580
_	-						-	-	-		- 1	-	-	••	U	Ŭ	-	-	×	500
CA	AGC	TAT	TTC	GGG'	TGT.	AAC	AAT	GTT	AAA	IGT	TAC	ATG	GTA	TGA	ATA	IGG	TGA	TCA	ICAA	1800
Q	А	I	s	G	v	т	м	\mathbf{L}	N	v	т	W	Y	Е	Y	G	D	н	Q	600
																			ACGT	1860
н	N	F	Q	K	Y	G	Y	Ε	Y	L	N	D	D	Е	S	G	Y	L	R	620
тC	വനന	TTC:TT	TCC	TC A	~ a a	TCC	N N C*	ידיידיי	TAC	አአጥ	ריי היי		איישי		TATATA	<u>م</u> صم		יעע	IGGT	1920
W	F	V	G	D	N	P	T	F	T	I	Y	S	Q	AGC	L	H	P	N	G	640
													^		_		-		-	
AA	IGT	TGG	TTG	GAG	AAA	GTT	ACC	TAA	AGA	ACC	TTT.	AAG	TTT	GAT	TTT.	AAA	TTT	IGG	ГАТА	1980
N	v	G	W	R	к	L	Ρ	ĸ	Е	Ρ	L	s	L	I	L	N	F	G	I	660
	TAA N		TTGG W	GC.	Y Y	I'A'I' I			JCC. P				F	P	FTC. S	AAC T	TAT M	GAGI R	AGTC	2040 680
5	14	14	vv	А	1	т	υ	vv	P	5	Ъ	v	г	Р	5	Т	M	ĸ	v	680
GA	TTA	TGT	AAG	AGT	TTA'	I'CA/	ACC	TAA	AGA	FCA	ААТ	ГАА	TGT	IGG'	FTG	IGA	TCC	AAC	CGAT	2100
	Y	v		v	Y			к			I		v				Р	т	D	700
							FCA	ACA	ACA	PTT.					AAA'	IGT	TAA	FTT	AACA	2160
F	Ρ	т	Y	D	Y	Ι	Q	Q	н	г	N	v	Y	Q	N	v	N	L	т	720
AA K	F.	IGA/ E	AGA'. D			ΓΓΑ: Υ		F			ACA' H				rgg. G	-	I'TA	Aaa	cacc	2220
L	r	Б	D	G	G	ī	T	г	Р	r	п	r	ц	т	G	C				737
aa	ccc	ccci	tata	atti	taca	ata	ttt	ttai	tata	act	ttt	aaa	taga	acat	ttt	ca	ttca	atto	catt	2280
													31							
ca	ttg	tata	ataa	atte	gaa	tgaa	atti	ttt	ttag	gga	tat	att	tca	ttta	aato	cga	gtti	ttca	aaaa	2340
aa	aaa	aaaa	aaaa	aaa																2354

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

For screening, a 2.0-k0 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), $5 \times SSC$ (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 μ g 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 *SKNI* 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

ScKreбр ScSkn1p CaKreбр CaSkn1p		59 59
ScKre6p ScSkn1p CaKre6p CaSkn1p	52 : TNPFMGSDEESNARDG-E-SLSSSVHYQPQ-GSDSSLLHDNSRLDLSQNKGVSDYKGYY- 60 : DEDYNSPSGDDERRGANEYSSSSSINYNNDPNSDTSLLANEKNSPERNGQRMSDYKGYYA 60 : QPHHPFATSNQTSSTNNL-HS-TN-S-IRNEAEYGK-PFQAYSGYY-SNGGSSSYLNQ 59 : PPHNPFSDNSHENSTESLYQSETRFHQPLLHNDSNNSNSSIGNNRQRIPSQQHDTSSLYS	111
ScSkn1p CaKre6p	108:SR-NNS-RA-VSTANDNSFLQPPHRAIASSP-SLN-SNLSKNDIL- 120:KTNLJTSANNLNNHNNNYKNIISSSNDNSFASHLQPPDRNLPSHPSSNNMSSFSNNSLIK 112:EGVISDESRLLS-S-GNNNNNNNNRDVSSVGGGGGSGDNRLNPTSPAEFDRYPSMA 119:ASPIS-TSPLVS-NFQSYSDNQDEMTRGKYNQNTNRSSSNYIQHSPTSAGYDRYPLKT	166
ScSkn1p CaKre6p	148:SPPEFDRYPLVGSR-VT-SMTQ-L-NHHGRSP-TSSPGNESSASFSS-NPFLGEQDFS 180:SPPPFDRYPLVGTRHISAAQSQSQNLINEKKRANMTGSSSSAHDSSLSSTNLYMGEQDFS 167:GSRVVSSTSLVSFNNPSNMMRNHHQ-HQPHLGSSSSPTSSSMNNDSISDKSTSFPNDFS 175:QSSIGGSMSRIGL-SSSSPSQQQ-QQ-HMYDNNSSNRSSYSPDSATDLMVYE-NGEFS	199 239 225 228
ScSkn1p CaKre6p	200: PFGGYPASSFPIMIDEKEEDDYLHNEDPEEEARLDRREIDDFRYMDKRAASGIAGVLLL 240: PFGGYPASFFPITLDEKEDDDYIHNENVEEEAKLDRREVDOFKHMDRRSFLGLLGILFL 226: PFGGYPASSFPISIDEKEPDDYLNNEDPVQDAEVEKNREMHDLKNMIKRSLGGILGFIIL 229: PFGGYPASLFPISIDEKEPDDYLHNEDPVQDAEVEKNRELYDLKTMIKKSMNGLIAFIVM	299 285
ScSkn1p CaKre6p	260:FLAAIFIFIVLFALTFTAAI-DHESNTEEVTYLTQMQYPQISAIRTSIV 300:FMAGIFIFIVLFAITFSGVVYHHEHVHAANSAGSSSSNTTSKSLTEMQYPQLAAIRTTIV 286:FIAALAVFIILHALTYSGATNPYHPESYEVLTKMSYPMLSAIRMMIV 289:FLIAIAIFIILHVFTYTGY-NPKAKNFENYEVLTRMSYPRUSAIRTSUI	359 332
ScSkn1p CaKre6p	308: DFETEDTAKTREAMDGSKWELVFSDEFNAEGRTFYDGDDPYWTAPDVHYDATKDLEWYSF 360: DFDTFDSAKTRVAKDGSKWOLVFSDEFNAEGRTFYDGDDQFWTAPD I HYDATKDLEWYSF 333: DFDTFESAYKKAKDGSEWVLVFSDEFDAEGRTFYEGDDQFFTAPDI HYDATKDLEWYDF 337: DFDTFEDALFWKSKNGEEMPLVFSDEFNAEGRTFYEGDDQFFTAPDLHYDATKDLEWYDF	367 419 392 396
ScSkn1р CaKreбр	368: DASTTIVNGTIQLENDAFKNHGLYYESGMLQSWNKVCFIQGALEISANLENYCGEVSGLWPG 420: DAVITTINGTI, TLENDAFENHDLYYESGMVQSWNKLOFIEGALEVSANLENYCGEVTGLWPG 393: DAVITIANGTI, NLENDAFENHDLYYESGMVQSWNQLCYTQGHLEISAELENYGNVTGLWPG 397: DAVITIANGTI, NLENDAFENHGLFYESGMIQSWNQMCFIQGELEFSAELEGYCGNITGFWPG	427 479 452 456
ScSkn1p CaKre6p	428:LWIMGNLGRPGYLASTQGVWPYSMESCDAGITPNQSSPDGISYLPGQKLSICTODVEDHP 480:MWIMGNLGRPGYLASTQGVWPYSMEACDAGITPNQSSPDGISYLPGQKLSVCTODNEDHP 453:LWSMGNLGRPGYLGSTDGVWPYSMDSCDAGITPNQSSPDGISYLPGQRLNKCTOPGELHP 457:LWSMGNLGRPGYLASTEGVWPYTMDSCDAGITPNQSSPDGISYLPGQRLNKCTOPGEAHP	487 539 512 516
ScSkn1p CaKre6p		542 594 572 569
ScSkn1p CaKre6p	543: TMNTYAGGPFQQAVGAVSTLIVTWYEFGEYGGYFGKMAIEYINDDDNGYIRWFVGDTPTY 595: TMNTYAGGPFQQAVGAISTLIVTWYEFGEEAGYFGKMAIEYINDDDNGYIRWFVGENPTF 573: TMNTYTGGPFQQALGATTMLNVTWYEFGDNAHNFGTVGYEYINDPETGYLRWFVGDDPTL 570: LMNTYAGGPFQQAIGGVTMLNVTWYEYGDNAHNFGKMGYEYINDESGYLRWFVGDNPTF	654 632
ScSkn1p CaKre6p	603: TIHAKALHEDGNIGWRRISKEEMSITINLGISNNWAYIDWQYIFFFVVMSIDYVRIYQES 655: TILYATSLHESGNIDWRRISKEEMSATINLGISNNWAYIDWQYIFFFVTMSIDYVRLYQEK 633: TVYSQALHEDGNIGWRPLSKEEMSLTINLGISNNWAYIDWPSISFFVTFRIDYVRVYQEP 630: TIYSQALHENGNVGWRKLPKEELSLTLINFGISNNWAYIDWPSLVFESTMRVDYVRVYQEK	714 692
ScSkn1p CaKre6p	663:NAISVTCDESDYPTYDYIGSHINAFONANLITWEDAGYTFPRNIFTGKOTSSKFKLSS 715:GSTSITCDEEDYPTYDYIGSHINAYYNANLIDWEQAGYTFPRNIFTGCSSSKFSLS- 693:DQINVGCDETDEPTYDYIGOHINLYENANIISFEDGGYKFPRNSFIG-C 690:DQINVGCDETDEPTYDYIGOHINYYONVNLIKFEDGGYTFPRHKLIG-C	720 771 740 737

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

1640 medium supplemented with 50 μ g 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 per-formed 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-*XhoI* fragment of *KRE6* cDNA had been cloned in Bluescript (SK9⁺) with *Eco*RV and *Cla1* and then ligating with the 3.8-kb *Bam*HI-*Bg*/II fragment carrying the *hisG*-*URA3-hisG* module. For *C. albicans SKN1* gene targeting, pCASU was con-

structed by digesting pCAS1 in which the 1.0-kb XhoI-EcoRI fragment of SKN1 cDNA was cloned in Bluescript (SK9⁺) with *Ball* followed by ligation with the 3.8-kb *Bam*HI-*Bg*III 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 cCAKU were the cod pCASU were linearized by dioecting with Yhed and KonL 100 up of pCAKU and pCASU were linearized by digesting with Xbal and Kpnl, 100 μ g 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

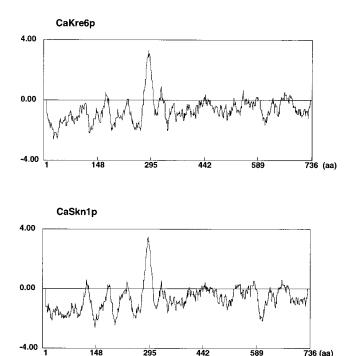


FIG. 4. Hydropathy plots of *C. albicans* Kre6p (CaKre6p) and Skn1p (CaSkn1p). Hydropathy profiles of Kre6p and Skn1p are calculated by using Kyte and Doolittle (22) parameters from the predicted amino acid sequences of the proteins. The number of amino acids (aa) is plotted on the *x* axis and hydropathy 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 *PacI-NspV* fragment of *KRE6* and a 0.8-kb *XhoI-SmaI* fragment of *SKN1* as the probes.

Fractionation and analysis of the cell wall \beta-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 *Bg*/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'TCTAAAAGATAGATCTATAATGT GGCTAG3' and 5'CCGGGCTGCAGAATTCTTTGTTTCAAAACC3'.

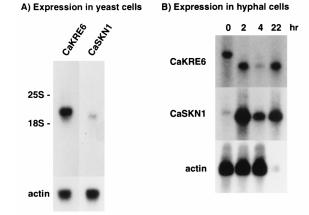
In order to replace the promoter of *C. albicans KRE6* with that of *HEX1*, pCAK1 harboring *KRE6* was digested with *Sma1* and *Bal1* and ligated with *C. albicans URA3* to generate pCAKU1. Then, *C. albicans KRE6* cDNA that was linked to the *HEX1* promoter was isolated by digesting pRC1708-CaKRE6 with *Xho1* and *Sma1* and was ligated at the *Bam*H1 cleavage site of pCAKU1. The resulting plasmid, pHCAKU1, was linearized by digestion with *Xho1* and *Apa11* 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



C) Morphology

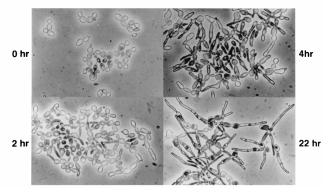
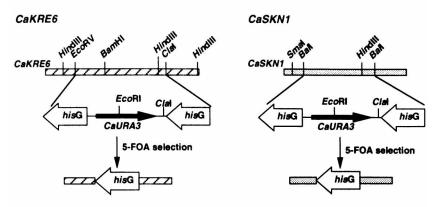


FIG. 5. Northern blotting of the *C. albicans KRE6* and *SKN1* mRNAs. Growing CAI4 cells were not induced (A) or induced to form hyphae (B) and were harvested at the indicated time after hyphal formation. Photographs of the cells after hyphal induction are shown in panel C.





B) Southern blotting

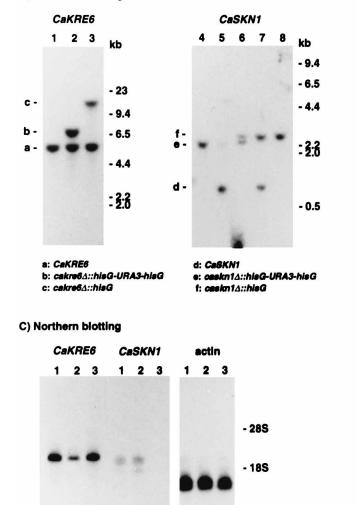


FIG. 6. Disruption of *C. albicans KRE6* (*CaKRE6*) and *SKN1* (*CaSKN1*). (A) The strategies for disruption of *KRE6* and *SKN1* are illustrated. (B) The correct integration of the *hisG* sequences into the *KRE6* or *SKN1* locus was confirmed by Southern blotting. Genomic DNAs extracted from strains CA14 (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 CA14 (lane 1), CaKRE6(+/-) (lane 2), and CaSKN1(-/-) (lane 3) were demonstrated by Northern blotting.

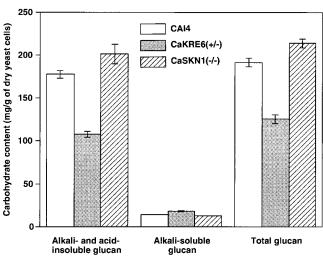


FIG. 7. Comparison of β -glucan levels in strains CAI4, CaKRE6(+/-) and CaSKN1(-/-). Cells of the indicated strains in mid-logarithmic phase were harvested, and the levels of alkali- and acid-insoluble, alkali-soluble, and total β -glucan were analyzed. β -Glucan levels are given in milligrams per gram (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 veast 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 $kre6\Delta$ 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 -1,6glucan 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 $kre6\Delta$, and homozygous $skn1\Delta$ mutant cells of *C. albicans^a*

	Molar ratio of glucose in samples													
Glucose	All	ali-soluble	glucan	Alkali- and acid-insoluble glucan										
linkage(s) ^b	CAI4	CaKRE6 (+/-)	CaSKN1 (-/-)	CAI4	CaKRE6 (+/-)	CaSKN1 (-/-)								
G1-	0.6	1.3	1.0	3.8	1.4	2.5								
$^{-3}G^{1-}$ $^{-6}G^{1-}$ $^{-6}_{-3}G^{1-}$	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								
-3G ^{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 $kre6\Delta$ [CaKRE6(+/-)] and homozygous $skn1\Delta$ 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 G¹⁻, $_{,3}$ G¹⁻, $^{-6}$ G¹⁻, and $_{,5}^{c}$ G¹⁻ 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.

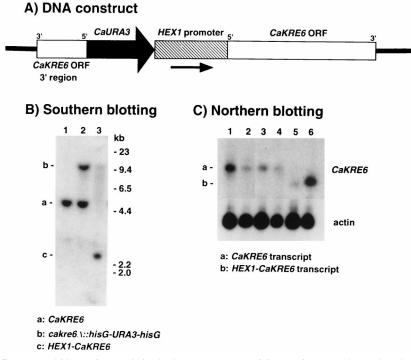


FIG. 8. Suppression of *C. albicans KRE6* (*CaKRE6*) transcription by the *HEX1* promoter. (A) Map of DNA used to replace the *KRE6* promoter with the *HEX1* promoter is illustrated. (B) The correct integration of the *HEX1-KRE6* DNA into the *KRE6* locus was confirmed by Southern blotting. Genomic DNAs extracted from strains CA14 (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 CA14 (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 GlcNAccontaining 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).

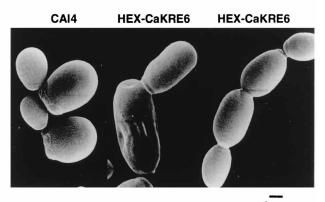
DISCUSSION

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 $kre6\Delta$ 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

A) Morphology





B) Susceptibility to calcofluor white

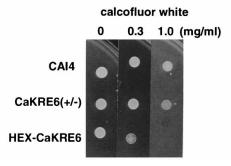


FIG. 9. Effects of suppression of *C. albicans KRE6* transcription on morphology and sensitivity to Calcofluor White. (A) Morphology of CAI4 and HEX-CaKRE6 cells that were cultured in glucose-containing medium were examined by scanning electron microscopy. (B) CAI4, CaKRE6(+/-), and HEX-CaKRE6 cells 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 $kre6\Delta$ 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 $kre6\Delta$ null mutation even after extensive transfection of pCAKU DNA into the hemizygous $kre6\Delta$ 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 ⁴²⁰R-V-S-G of *S. cerevisiae* Kre6p and ⁴⁷²R-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 ⁴⁴⁵N-V-T-G in Kre6p and ⁴⁴⁹N-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.

ACKNOWLEDGMENTS

We thank N. Kleckner (Harvard University) and A. Shinohara (Osaka University) for plasmid pNKY51, W. Fonzi (Georgetown University) for *C. albicans* CAI4, R. Cannon (Otago University) for plasmid pRC1708, and K. Kobayashi (Nippon Roche Research Center) for scanning electron microscopy.

This work was supported in part by grants in aid (glycotechnology program) from the Ministry of Agriculture, Forestry and Fisheries of Japan to T.N. and from the Ministry of Science, Technology and Education of Japan to T.Y.-O.

REFERENCES

- Alani, E., L. Cao, and N. Kleckner. 1987. A method for gene disruption that allows repeated use of URA3 selection in the construction multiply disrupted yeast strains. Genetics 116:541–545.
- Boone, C., M. Goebl, R. Puccia, A.-M. Sdicu, and H. Bussey. 1993. Yeast KRE2 defines a new gene family encoding probable secretory proteins, and is required for the correct N-glycosylation of proteins. Genetics 130:270–283.
- Boone, C., A. M. Sdicu, M. Laroche, and H. Bussey. 1991. Isolation from Candida albicans of a functional homolog of the Saccharomyces cerevisiae KRE1 gene, which is involved in cell wall β-glucan synthesis. J. Bacteriol. 173:6859–6864.
- Boone, C., S. S. Sommer, A. Hensel, and H. Bussey. 1990. Yeast KRE genes provide evidence for a pathway of cell wall β-glucan assembly. J. Cell Biol. 110:1833–1843.
- Brown, J. L., and H. Bussey. 1993. The yeast *KRE9* encodes an O glycoprotein involved in cell surface β-glucan assembly. Mol. Cell. Biol. 13:6346– 6356.
- Brown, J. L., Z. Kossaczka, B. Jiang, and H. Bussey. 1993. A mutational analysis of killer toxin resistance in *Saccharomyces cerevisiae* identifies new genes involved in cell wall (1-6)-β-glucan synthesis. Genetics 133:837–849.
- Brown, J. L., S. North, and H. Bussey. 1993. SKN7, a yeast multicopy suppressor of a mutation affecting cell wall β-glucan assembly, encodes a product with domains homologous to prokaryotic two-component regulators and to heat shock transcription factors. J. Bacteriol. 175:6098–6915.
- Bulawa, C. E., D. W. Miller, L. K. Henry, and J. M. Becker. 1995. Attenuated virulence of chitin-deficient mutants of *Candida albicans*. Proc. Natl. Acad. Sci. USA 92:10570–10574.
- Cannon, R. D., K. Niimi, H. F. Jenkinson, and M. G. Shepherd. 1994. Molecular cloning and expression of the *Candida albicans* β-N-acetylglucosaminidase (*HEX1*) gene. J. Bacteriol. 176:2640–2647.
- 10. Douglas, C. M., F. Foor, J. A. Marrinan, N. Morin, J. B. Nielsen, A. M. Dahl,

P. Mazur, W. Baginsky, W. Li, M. El-Sherbeini, J. A. Clemas, S. M. Mandala, B. R. Frommer, and M. B. Kurtz. 1994. The *Saccharomyces cerevisiae FKS1 (ETG1)* gene encodes an integral membrane protein which is a subunit of 1,3- β -D-glucan synthase. Proc. Natl. Acad. Sci. USA **91**:12907–12911.

- Drgonova, J., T. Drgon, K. Tanaka, R. Kollar, G. C. Chen, R. A. Ford, C. S. Chan, Y. Takai, and E. Cabib. 1996. Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. Science 272:277–279.
- Fleet, G. H. 1991. Cell walls, p. 199–277. In A. H. Rose and J. S. Harrinson (ed.), The yeasts, vol. 4. Academic Press, New York, N.Y.
- Furukawa, K., M. Tagaya, K. Tanizawa, and T. Fukui. 1993. Role of the conserved Lys-X-Gly-Gly sequence at the ADP-glucose-binding site in *Escherichia coli* glycogen synthase. J. Biol. Chem. 268:23837–23842.
- Gow, N. A. R., and G. W. Gooday. 1987. Cytological aspects of dimorphism in *Candida albicans*. Crit. Rev. Microbiol. 15:73–78.
- Hausler, A., L. Ballou, C. E. Ballou, and P. W. Robbins. 1992. Yeast glycoprotein biosynthesis: MNT1 encodes an α-1,2-mannosyltransferase involved in O-glycosylation. Proc. Natl. Acad. Sci. USA 89:6846–6850.
- Inoue, S. B., N. Takewaki, T. Takasuka, T. Mio, M. Adachi, Y. Fujii, C. Miyamoto, M. Arisawa, Y. Furuichi, and T. Watanabe. 1995. Characterization and gene cloning of 1,3-β-D-glucan synthase from *Saccharomyces cerevisiae*. Eur. J. Biochem. 231:845–854.
- Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153:163–168.
- intact yeast cells treated with alkali cations. J. Bacteriol. 153:163–168.
 18. Jiang, B., J. Sheraton, A. F. J. Ram, G. J. P. Dijkgraaf, F. M. Klis, and H. Bussey. 1996. *CWH41* encodes a novel endoplasmic reticulum membrane N-glycoprotein involved in β1,6-glucan assembly. J. Bacteriol. 178:1162–1171.
- Kang, M. S., and E. Cabib. 1986. Regulation of fungal cell wall growth: a guanine nucleotide-binding proteinaceous component required for activity of (1,3)-β-D-glucan synthase. Proc. Natl. Acad. Sci. USA 83:5808–5812.
- Kasahara, S., S. B. Inoue, T. Mio, T. Yamada, T. Nakajima, E. Ichishima, Y. Furuichi, and H. Yamada. 1994. Involvement of cell wall β-glucan in the action of HM-1 killer toxin. FEBS Lett. 348:27–32.
- 21. Kasahara, S., H. Yamada, T. Mio, Y. Shiratori, C. Miyamoto, T. Yabe, T. Nakajima, E. Ichishima, and Y. Furuichi. 1994. Cloning of the Saccharomyces cerevisiae gene whose overexpression overcomes the effects of HM-1 killer toxin, which inhibits β-glucan synthesis. J. Bacteriol. 176:1488–1499.
- Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157:105–132.
- Lussier, M., A. M. Sdicu, T. Ketela, and H. Bussey. 1995. Localization and targetting of the *Saccharomyces cerevisiae* Kre2p/Mnt1p α-1,2-mannosyltransferase to a medial-golgi compartment. J. Cell Biol. 131:913–927.
- Manuel, A., S. Santos, and M. F. Tuite. 1995. The CUG codon is decoded in vivo as serine and not leucine in *Candida albicans*. Nucleic Acids Res. 23:1481–1486.
- 25. Meaden, P., K. Hill, J. Wagner, D. Slipetz, S. S. Sommer, and H. Bussey.

1990. The yeast *KRE5* gene encodes a probable endoplasmic reticulum protein required for $(1\rightarrow 6)$ - β -D-glucan synthesis and normal cell growth. Mol. Cell. Biol. **10**:3013–3019.

- Mio, T., T. Yabe, M. Sudoh, Y. Satoh, T. Nakajima, M. Arisawa, and H. Yamada-Okabe. 1996. Role of three chitin synthase genes in the growth of *Candida albicans*. J. Bacteriol. 178:2416–2419.
- Mol, P. C., H.-M. Park, J. T. Mullins, and E. Cabib. 1994. A GTP-binding protein regulates the activity of (1-3)-β-glucan synthase, an enzyme directly involved in yeast cell wall morphogenesis. J. Biol. Chem. 269:31267–31274.
- Odds, F. C. 1987. Candida infection: an overview. Crit. Rev. Microbiol. 15:1-5.
 Derter C. C. L. L. Farrier, D. F. Nalazz, and L. H. Farrier, 1005. Description
- Parker, C. G., L. I. Fessler, R. E. Nelson, and J. H. Fessler. 1995. Drosophila UDP-glucose:glycoprotein glucosyltransferase sequence and characterization of an enzyme that distinguishes between denatured and native proteins. EMBO J. 14:1294–1303.
- Qadota, H., C. P. Python, S. B. Inoue, M. Arisawa, Y. Anraku, Y. Zheng, T. Watanabe, D. E. Levin, and Y. Ohya. 1996. Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-β-glucan synthase. Science 272:279–281.
- Ram, A. F. J., A. Wolters, R. T. Hoopen, and F. M. Klis. 1994. A new approach for isolating cell wall mutants in Saccharomyces cerevisiae by screening for hypersensitivity to calcofluor white. Yeast 10:1019–1030.
- Roemer, T., and H. Bussey. 1991. Yeast β-glucan synthesis: *KRE6* encodes a predicted type II membrane protein required for glucan synthesis *in vivo* and for glucan synthase activity *in vitro*. Proc. Natl. Acad. Sci. USA 88:11295– 11299.
- Roemer, T., and H. Bussey. 1995. Yeast Kre1p is a cell surface O-glycoprotein. Mol. Gen. Genet. 249:209–216.
- 34. Roemer, T., S. Delaney, and H. Bussey. 1993. SKN1 and KRE6 define a pair of functional homologs encoding putative membrane proteins involved in β-glucan synthesis. Mol. Cell. Biol. 13:4039–4048.
- 35. Roemer, T., G. Paravicini, M. A. Payton, and H. Bussey. 1994. Characterization of the yeast (1-6)-β-glucan biosynthetic components, Kre6p and Skn1p, and genetic interactions between the PKC pathway and extracellular matrix assembly. J. Cell Biol. 127:567–579.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Shepherd, M. G. 1985. Candida albicans: biology, genetics, and pathogenicity. Annu. Rev. Microbiol. 39:579–614.
- Sudoh, M., S. Nagahashi, M. Doi, M. Takagi, and M. Arisawa. 1993. Cloning of the chitin synthase 3 gene from *Candida albicans* and its expression during yeast-hyphal transition. Mol. Gen. Genet. 241:351–358.
- Surarit, R., P. K. Gopal, and M. Shepherd. 1988. Evidence for a glycoside linkage between chitin and glucan in the cell wall of *Candida albicans*. J. Gen. Microbiol. 134:1723–1730.