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Isolation from *Candida albicans* of a Functional Homolog of the *Saccharomyces cerevisiae KRE1* Gene, Which Is Involved in Cell Wall β-Glucan Synthesis

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The *KRE1* gene of *Saccharomyces cerevisiae*, *sacKRE1*, appears to be involved in the synthesis of cell wall β-glucan. *S. cerevisiae* strains with mutations in the *KRE1* gene produce a structurally altered cell wall (1→6)-β-glucan, which results in resistance to K1 killer toxin. We isolated the *canKRE1* gene from *Candida albicans* by its ability to complement a *kre1* mutation in *S. cerevisiae* and confer sensitivity to killer toxin. Sequence analysis revealed that the predicted protein encoded by *canKRE1* shares an overall structural similarity with that encoded by *sacKRE1*. The *canKRE1* protein is composed of an N-terminal signal sequence, a central domain of 46% identity with the *sacKRE1* protein, and a C-terminal hydrophobic tract. These structural and functional similarities imply that the *canKRE1* gene carries out a function in *C. albicans* cell wall assembly similar to that observed for *sacKRE1* in *S. cerevisiae*.

Cell wall β-glucans are common among fungi and plants but absent from mammals, and the enzymes responsible for β-glucan biosynthesis have been recognized as potential targets for specific antifungal agents. Studies of the ascomycete *Saccharomyces cerevisiae* have identified three genes whose products are involved in (1→6)-β-D-glucan assembly (4, 13) and are required for normal cell growth and cell wall structure. An important unanswered question from such work was whether *S. cerevisiae* genes implicated in β-glucan synthesis are also present in pathogenic fungi. We have addressed this possibility, using the opportunistic pathogen *Candida albicans*, an imperfect yeast of uncertain but possibly close relationship to *S. cerevisiae* (3). Cloning, sequencing, and expression work indicates that *C. albicans* has genes that resemble those of *S. cerevisiae* with various degrees of identity, including some which can functionally complement mutant defects in *S. cerevisiae* (6, 11, 17). A recent report showed that one gene for the cell wall biosynthetic enzyme chitin synthase demonstrated both sequence similarity and functional homology (1).

An argument for the possible similarity of the genes involved in β-glucan synthesis comes from structural studies which show that the walls of both *S. cerevisiae* cells and *C. albicans* cells are qualitatively similar in possessing both (1→3)- and (1→6)-β-glucan. However, there are significant quantitative differences both in the proportions of these polymers and in their structures (7). In general, there are considerably more (1→6)-linked residues in the walls of the yeast form of *C. albicans* than in those of *S. cerevisiae*. The most abundant cell wall glucan of *S. cerevisiae* is alkali insoluble and has a degree of polymerization of approximately 1,500 glucopyranosyl residues, composed principally of linear (1→3)-linkages, 3% of which are branched through a (1→6)-linkage. In contrast, the alkali-insoluble glucan polymer in the yeast form of *C. albicans* contains from 43 to 53% (1→6)-β-linkages and from 30 to 39% (1→3)-β-linked glucose residues with some 7% branch points (7). *S. cerevisiae* also produces an acid-soluble glucan which has an estimated degree of polymerization of 140 and is composed of predominantly linear (1→6)-linked glucose residues with 14% (1→3)-branch points and some internal (1→3)-linked residues (4, 12). An equivalent acid-soluble (1→6)-glucan with a very similar structure is also found in the *C. albicans* cell wall (7).

In this study, we screened two *C. albicans* genomic libraries contained on *S. cerevisiae* multicopy vectors for DNA that could complement *kre1* mutants of *S. cerevisiae*. The *KRE1* gene of *S. cerevisiae* is involved in cell wall (1→6)-β-glucan synthesis (4). Disruption of the *KRE1* locus causes a reduction in the level of this glucan to about 60% of wild-type levels. The (1→6)-β-D-glucan from the cell wall of *kre1* null mutants is a smaller polymer than the wild type and contains fewer (1→6)-linked glucopyranosyl residues. Disruptions of *KRE1* confer complete resistance to the K1 killer toxin of *S. cerevisiae*. K1 toxin displays a lectinlike affinity for linear (1→6)-β-glucan and must bind to the walls of sensitive yeast cells in order to initiate the killing process. A model for the action of the *KRE1* gene product is that it mediates the addition of linear side chains of (1→6)-linked glucose units to a highly branched (1→6)- and (1→3)-linked glucan backbone. These *KRE1*-dependent side chains are required for toxin binding, and in their absence cells are toxin resistant (4). As described above, *C. albicans* contains a cell wall (1→6)-β-glucan fraction that resembles that of *S. cerevisiae* (7). In addition, the cell wall of the yeast form of *C. albicans* binds killer toxin with an affinity similar to that of the *S. cerevisiae* cell wall (22). We reasoned that if a homolog of the *S. cerevisiae KRE1* gene existed in *C. albicans*, it might function in *S. cerevisiae* to complement a *kre1* mutant and render such cells sensitive to killer toxin. We report here on a *C. albicans* gene that appears to be functionally and structurally related to the *KRE1* gene from *S. cerevisiae*.

MATERIALS AND METHODS

Strains, media, and plasmids. The *S. cerevisiae* strains used were 11A (*matα kre1-1 ura3*), 3 (*matα glc1 his3*

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kre1::*HIS3* [4]), and T158C/S14a (*MATa*/*MATα his4c-864*/*HIS4 ade2-5*/*ADE2* [KIL-k1]). *C. albicans* WO-1 was obtained from P. T. Magee, University of Minnesota, St. Paul.

Growth conditions and media (yeast extract-peptone-dextrose [YEPD], complete and Halvorson's) were as described previously (5, 21). Transformation was carried out by the lithium acetate technique of Ito et al. (10).

The *C. albicans* genomic library in pEMBLYe23 was kindly provided by P. T. Magee and was prepared by using partial *Hin*dIII-*Bam*HII digestion of genomic DNA from strain WO-1 inserted into the *Bam*HI site of pEMBLYe23 (2). A second *C. albicans* library was prepared from strain WO-1. Genomic DNA was isolated from a dense overnight culture of cells grown in 500 ml of YEPD. DNA was prepared as described by Rose et al. (16), without sucrose gradient enrichment of high-molecular-weight DNA. After being extracted with chloroform-isoamyl alcohol (24:1), DNA was precipitated with 95% ethanol and spooled onto glass rods. The spooled DNA was dissolved in 5 ml of low TE (10 mM Tris [pH 7.5], 1 mM EDTA), extracted with phenol-chloroform (1:1), reprecipitated with ethanol, dried, and dissolved in 2 ml of low TE. Samples of genomic DNA were partially digested with *Sau*3A and size fractionated by electrophoresis through a 1% agarose gel. DNA fragments approximately 5 to 10 kb in size were purified by using Gene Clean (Bio 101) and were ligated into YEp352 (9). YEp352 was digested with *Bam*HI and treated with phosphatase (Pharmacia) prior to ligation with genomic DNA.

pFL44 is a yeast 2μm-based multicopy shuttle vector which contains the pUC19 polylinker and the *URA*3 selectable marker. It was obtained from F. Lacroute, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France (4). The yeast expression vectors pVT102-U and pVT105-U were obtained from T. Vernet (19).

DNA manipulations. p771 is a pUC19-derived plasmid that contains the prepro-α-factor amino-terminal leader and signal cleavage site fused in frame with the *sacKRE1* open reading frame (deleted for the DNA encoding the first 24 amino acids, which includes the *sacKRE1* leader) (4). pML1 was derived from p771 by digestion with *Hin*cII, followed by isolation of the vector-containing fragment and religation. pML2 was prepared by religating the vector-containing fragment described above in the presence of a nonphosphorylated *Xba*I linker (5′-TGCTCTAGAGCA-3′; New England Biolabs). pML3 was derived from pML2 after digestion with *Xba*I and *Spe*I and religation of the vector-containing fragment. pML6 was derived from p771 by digestion with *Hin*cII and *Sna*BI and religation of the vector-containing fragment. pML4, pML5, and pML7 were constructed by digestion of pML1, pML3, and pML6, respectively, with *Bgl*II and *Hin*dIII, followed by ligation of the fragments containing *sacKRE1* DNA into *Bam*HI- and *Hin*dIII-digested pVT102-U. pVT102-U is a 2 μm-based expression vector which contains the *S. cerevisiae ADH1* promoter next to the *Bam*HI site of the polylinker (19).

Cloning the *C. albicans KRE1* gene. Strain 11A (4) was transformed with the pEMBLYe23 *C. albicans* bank, and the uracil prototrophs were selected. Transformants were screened for sensitivity to killer toxin by using methylene blue staining as described previously (4).

DNA sequencing. Subclones of the *C. albicans KRE1* genomic DNA were made in Bluescript vectors. Plasmids with subclones were transformed into *Escherichia coli* UT580, and single-stranded DNA was made by using M13K07 as a helper phage (19). Sequencing was done by the dideoxy procedure (18) and was done for both strands by using the Sequenase kit (U.S. Biochemicals, Cleveland, Ohio) with α-^{35}S-dATP (Amersham Canada, Ltd., Oakville, Ontario, Canada) as a labelled substrate. DNA primers were either Bluescript specific or synthesized to be complementary to regions of the *canKRE1* DNA sequence.

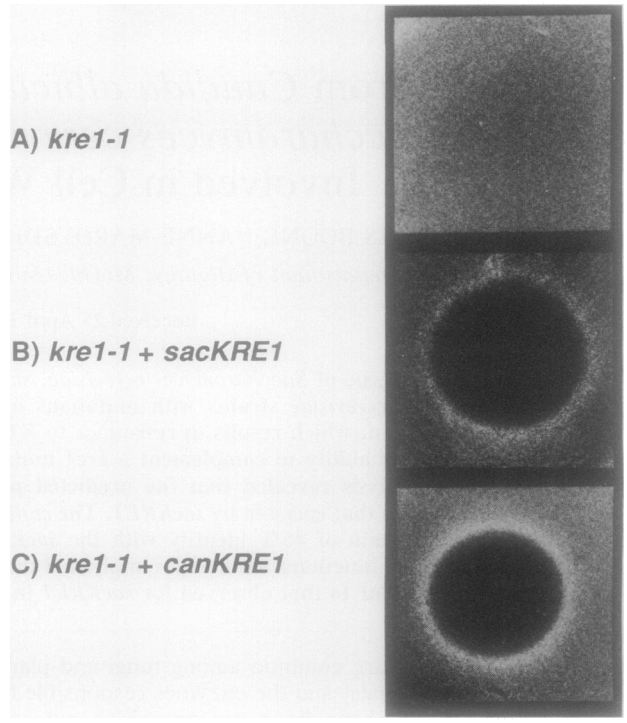

FIG. 1. Complementation of killer toxin resistance of *kre1* mutant cells by *sacKRE1* and *canKRE1*. Killer toxin was spotted to selective agar medium seeded with *S. cerevisiae kre1-1* strain 11A (A) or strain 11A transformed with *sacKRE1* (B) or *canKRE1* (C). The strain *kre1-1* is toxin resistant and grows in the presence of K1 killer toxin. Strain 11A transformed with *sacKRE1* is sensitive to the toxin and forms a zone of killing around the point of application of the toxin. Similarly, strain 11A transformed with *canKRE1* shows a zone of killing, although these cells are less sensitive and form a smaller zone than the one in panel B, indicating partial complementation by this gene.

Glucan determination. Yeast cultures were grown on selective minimal medium to stationary phase. Total glucan levels were determined following alkali extraction, and (1→6)-β-glucan levels were determined following (1→3)-β-glucanase digestion and dialysis, as described elsewhere (4).

RESULTS

Cloning of the *canKRE1* gene. A *C. albicans* library in the vector pEMBLYe23 was screened for DNA that would complement an *S. cerevisiae kre1* mutant and restore sensitivity to killer toxin, as described in Materials and Methods. Approximately 15,000 transformants were screened, and 4 independent transformants that conferred partial sensitivity on a strain harboring the *kre1-1* mutation, which normally produces a fully resistant phenotype (Fig. 1), were found. These complementing plasmids were extracted from *S. cerevisiae* cells, amplified in *E. coli*, and retransformed into a *kre1* mutant of *S. cerevisiae*, in which they were capable of conferring partial sensitivity to killer toxin (Fig. 1). The complementing plasmids were also able to complement

strain 3 carrying a null mutation of the *KRE1* gene. Restriction analysis of the inserts indicated that each plasmid contained a 9.2-kb DNA fragment. These inserts fell into two related groups distinguished by restriction fragment length polymorphisms (see Fig. 2). Since *C. albicans* is a diploid, it is likely that the two distinct inserts represented allelic genomic fragments from homologous chromosomes.

Complementation of resistance to killer toxin is a sensitive but indirect measure of synthesis of (1→6)-β-D-glucan. To directly determine the ability of the *canKRE1* gene to synthesize (1→6)-β-D-glucan in a *kre1* mutant, we examined the glucan level of strain 11A harboring the *kre1-1* allele transformed with *canKRE1-2* on plasmid YEp352. The following levels of (1→6)-β-D-glucan were found (in micrograms per milligram [dry weight] ± 1 standard deviation): *kre1-1* mutant, 16.0 ± 2.5; *kre1-1* transformed with *can KRE1-2*, 26.8 ± 1.7; and *kre1-1* transformed with the YEp352 vector alone, 19.8 ± 1.7. These figures compare with an average value of 42.0 ± 4.7 for *kre1-1* transformed with *sacKRE1* on centromeric or multicopy plasmids (4). Values for total β-glucan were also determined for the strains and transformants described above; they were not significantly different, with an average level of 134 ± 18 μg/mg (dry weight). Thus, while the level of (1→6)-β-D-glucan in *canKRE1* transformants appears to be higher than that in the *kre-1* mutant, it is less than the wild-type level seen with the *sacKRE1* gene.

Genomic Southern analysis and subcloning. Genomic Southern hybridizations were made to directly test whether the DNA fragments isolated were from *C. albicans*. A 2.0-kb *Eco*RI-*Eco*RV probe from *canKRE1-2* was used and hybridized at high stringency to *Hin*dIII-*Bam*HI and *Hpa*I-*Hin*dIII fragments of digested genomic DNA from *C. albicans* (data not shown). The fragments detected were consistent with the presence of both allelic forms of the cloned DNA in the genome of *C. albicans*. No cross hybridization at high stringency with this probe on digested genomic DNA of *S. cerevisiae* was seen. In further experiments, a probe containing the *S. cerevisiae KRE1* gene hybridized to digested *S. cerevisiae* genomic DNA but failed to hybridize to digested genomic DNA of *C. albicans*.

A series of subclones of the pYe23:KRE1.2 insert DNA were made and tested for their ability to complement an *S. cerevisiae kre1* mutant. The results are shown in Fig. 2B, in which it can be seen that the smallest fragment capable of allowing complementation was the 1.9-kb *Eco*RI-*Eco*RV region. Deletion of the terminal *Hin*dIII fragments of *KRE1-2* abolished complementing activity. A similar result was found with pYe23:KRE1.1, in which deletion of the approximately 2-kb terminal *Hin*dIII region led to loss of the complementing activity (Fig. 2A).

DNA sequence and predicted open reading frame of *can KRE1*. Within the region necessary for functional complementation, there is an open reading frame of 130 amino acid residues. The first ATG of this open reading frame is 67 bp downstream from a TATAA box, and a yeast transcription start site, TCAA (8), lies 48 bp from the TATAA box, at position −16 (Fig. 3). The predicted protein sequence of molecular weight 13,857 has at its N-terminal end a signal sequence and putative sites for signal cleavage that obey the Von Heijne rules (20). Such sites are shown in Fig. 3, with Ala-15–Ser-16 or Ala-20–Ala-21 being candidates. Following the signal cleavage sites is a region that has significant homology with the *sacKRE1* protein. The region encompasses some 61 amino acid residues from *canKRE1* (30 to 91) and contains 28 residues identical with *sacKRE1* (46% identity) (Fig. 4). This similarity increases to 79% if a further 20 conserved residues are taken into account. The remaining C-terminal protein sequence of *canKRE1* shows only slight identity with that of *sacKRE1* but resembles it structurally in having a C-terminal hydrophobic sequence (starting at Leu-115). Just prior to the hydrophobic sequence is an asparagine residue, Asn-109, with a potential site for an N-linked glycosyl attachment. A search of the GenBank data base revealed no other protein sequences with similarity to that of *canKRE1*.

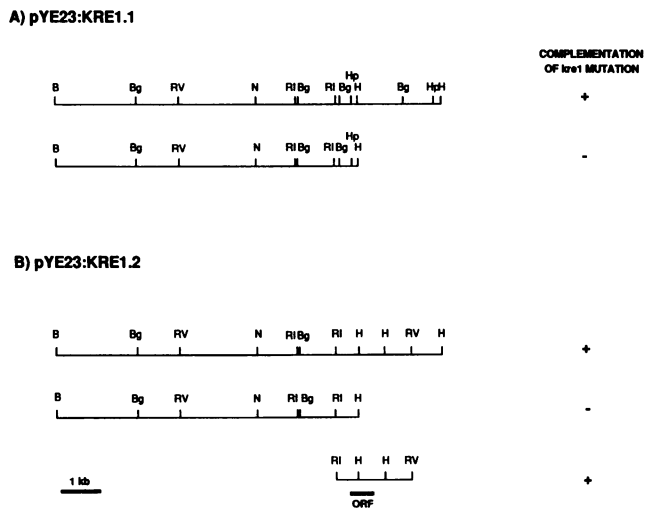

FIG. 2. Restriction map of DNA in the *canKRE1* region and localization of the gene by complementation. The two allelic forms (*canKRE1-1* and *canKRE1-2*) of this region obtained as complementing inserts in the pEMBLYe23 vector are shown at the tops of panels A and B, respectively. Note the restriction site polymorphisms at the *Hpa*II, *Hin*dIII, *Bgl*II, and *Eco*RV sites in the fragments. Subclones are shown below, with their ability to complement the *kre1-1* mutation. Also shown is the position of the *canKRE1-2* open reading frame (ORF) as determined from DNA sequencing. Restriction site abbreviations: B, *Bam*HI; Bg, *Bgl*II; RV, *Eco*RV; N, *Nsi*I; RI, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I.

Internal deletions of *sacKRE1* retain partial function. To examine the possible role of the large serine-threonine-rich *KRE1* domain absent from the *canKRE1* homolog, we made some internal deletions of this region in the *sacKRE1* gene and examined their abilities to complement a *kre1* mutant. These deletions contain the α-factor signal sequence fused to a *KRE1* signal sequence deletion or to a progressive set of more extreme deletions to the *Hin*cII (pML4), *Spe*I (pML5), or *Sna*BI (pML7) sites, which remove 61, 177, and 230 codons from the *sacKRE1* open reading frame, respectively (Fig. 5). Replacement of the *KRE1* signal sequence with that of the α-factor had no effect on *KRE1* activity, as previously reported (4). Both the *Hin*cII and the *Spe*I deletions in the nonconserved serine-threonine-rich coding region of *sac KRE1* retained considerable partial function in conferring killer toxin sensitivity, compared with the wild type (Fig. 5), but with a progressive loss of sensitivity as the deletion increased in size. However, further deletion into the conserved coding region from the *Spe*I to the *Sna*BI sites led to complete loss of activity. Because the expression vectors for these constructs would be expected to overproduce the *sacKRE1* product, we may overestimate the residual functions of the deletions. Nevertheless, such a result emphasizes that the nonconserved serine-threonine-rich region is

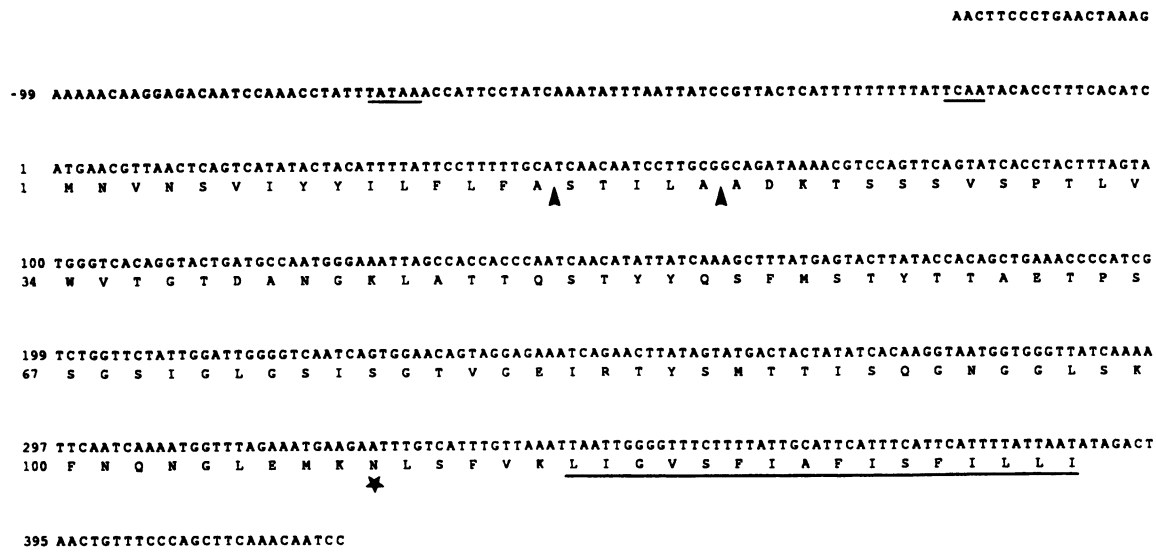

FIG. 3. DNA sequence and predicted open reading frame of *canKRE1-2*. A potential TATAA box and a transcript start site (TCAA) are underlined upstream of the initiating Met codon. The predicted open reading frame is shown in the one-letter amino acid code. Candidate sites for signal cleavage are indicated by arrowheads, and the possible glycosyl attachment site at Asn-109 is indicated by an asterisk. The C-terminal hydrophobic domain is underlined. The region with homology to the *sacKRE1* protein is from *canKRE1* protein amino acid residues 30 to 91 and is shown in detail in Fig. 4.

not essential for *sacKRE1* product activity and can explain why the smaller *canKRE1* gene is able to partially complement *kre1* mutations in *S. cerevisiae*.

Partial complementation and a search for other homologs. The observed partial complementation suggested that the *canKRE1* gene might be one of a family of related genes in *C. albicans*, another of which was functionally more closely related to the *sacKRE1* gene and not present in the genomic library screened initially. To explore this possibility, we made an explicit search for other functional homologs of *sacKRE1* in *C. albicans*. A second genomic library of *C. albicans* DNA was prepared (see Materials and Methods) and screened for fragments that complemented the *kre1-1* mutation in *S. cerevisiae*. Approximately 13,000 transformant colonies were screened, and 5 complementing colonies were identified. Restriction analysis of the five different-size complementing DNA inserts indicated that three contained the *canKRE1-1* allele and two contained the *canKRE1-2* allelic form. Including the earlier cloning of the *canKRE1* gene on four occasions, we have isolated this gene nine times as seven independent DNA fragments from two separately prepared genomic libraries of *C. albicans*. This suggests that *canKRE1* is probably the only gene in these *C. albicans* libraries able to complement the *kre1* mutation in *S. cerevisiae* when present on a 2-μm plasmid.

DISCUSSION

The isolation of genes from *C. albicans* by functional complementation of mutations in *S. cerevisiae* has been a successful strategy (1, 6, 11, 17). The isolation of at least a partially functional homolog of *sacKRE1* from *C. albicans* suggests that it may have a function related to that of *sacKRE1* and implies that it may be possible to isolate other *C. albicans* genes involved in glucan synthesis in this way. In this regard, we have recently isolated a fragment of *C. albicans* DNA that can partially complement null mutations in *KRE6*, another *S. cerevisiae* gene involved in β-glucan synthesis (4, 15). The structural differences between the wall glucans of the two yeasts, which include a higher overall proportion of (1→6)-β-glucopyranosyl residues in *C. albicans*, may imply that the function of the *canKRE1* gene is somewhat different from that of *sacKRE1* and that the null phenotype might be more severe in *C. albicans* than in *S. cerevisiae*. The basis of the partial complementation by *canKRE1* remains unclear, but it appears unlikely that there are other more active genes in *C. albicans* which are able to complement *kre1* mutations, as we have repeatedly isolated alleles of *canKRE1* in our screens. The *C. albicans* gene appears to be only partially functional in *S. cerevisiae*, even when present on a multicopy plasmid. This defect could be

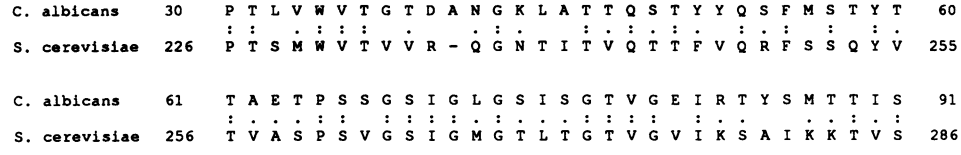

FIG. 4. Alignment of regions of the amino acid sequences of the *KRE1* gene products from *C. albicans* and *S. cerevisiae*. Identical residues are connected by double dots and constitute 28 of 61 residues (46%). Similar residues are indicated by a single dot and occur in 20 of 61 residues (33%). One gap, marked by a hyphen, has been introduced between residues 235 and 236 of the *S. cerevisiae* sequence to maximize alignment.

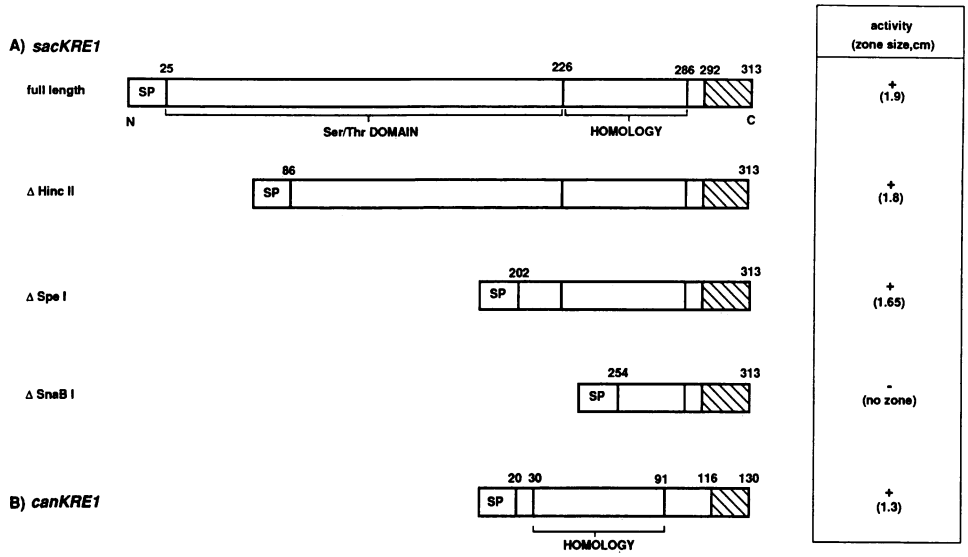

FIG. 5. Schematic structural and functional comparison of the *sacKRE1* product and comparison of internal deletions of this protein with the *canKRE1* product. (A) The full-length *sacKRE1* product, with the α-factor signal peptide at its amino-terminal end, followed by a domain from residues 25 to 226 which contains 50% Ser-Thr residues. From residues 226 to 286, there is a region of homology between the *sacKRE1* and *canKRE1* proteins, shown in detail in Fig. 4. The sequence terminates with a hydrophobic domain (hatched box). The Δ*Hin*cII, Δ*Spe*I, and Δ*Sna*BI sites of progressive internal deletions in the *sacKRE1* gene that retain the α-factor signal peptide are indicated, and they are contained on plasmids pML4, pML5, and pML7, respectively. (B) The full-length *canKRE1* product, as described in detail in the legend to Fig. 3. The region of homology with the *sacKRE1* product is indicated, as is the C-terminal hydrophobic domain (hatched box). In the box to the right, the phenotypes of the genes, on the basis of sensitivity to killer toxin when transformed into a resistant *kre1-1* mutant that gives no toxin zone (see Fig. 1), are indicated. +, a toxin zone size dependent on the gene; −, no killer toxin zone. Actual zone sizes are shown in parentheses.

through a promoter that is poorly recognized or through a failure of the *canKRE1* product to correctly function in glucan assembly in *S. cerevisiae*. The fact that the *canKRE1* gene is capable of complementing a *KRE1* null allele demonstrates that it can function without interacting with residual *sacKRE1* product.

A comparison of the *canKRE1* product with its *sacKRE1* counterpart shows the predicted protein to be smaller but to have an overall conservation of basic structure. Both predicted proteins have an N-terminal signal sequence, a region of identity between the two proteins, and a C-terminal hydrophobic domain. These similarities suggest that the *canKRE1* product, like that of *sacKRE1*, is a protein which enters the secretory pathway. The presence of a hydrophobic domain at the C termini of both proteins may imply that these regions are functionally important in both genes. This C-terminal hydrophobic domain in *sacKRE1* has been proposed to be a potential membrane insertion domain or to be the site for the attachment of a glycosylphosphatidylinositol membrane anchor (4, 14), and a mutation in it considerably reduces *sacKRE1* function.

The larger *sacKRE1* protein has an N-terminal region of the mature protein, absent from *canKRE1*, that is high in Ser-Thr residues and which contains a 15-amino-acid residue repeat. This region, which constitutes approximately 64% of the *sacKRE1* protein, is apparently highly O glycosylated and appears to be nonessential, as it can be deleted with only partial loss of function of the protein. The *sacKRE1* product contains no sites for asparagine-linked glycosyl attachment, whereas the *canKRE1* product contains one such site (Fig. 3).

The precise role of *KRE1* in β-glucan synthesis remains unknown. However, it appears to be a member of a set of *KRE* genes required for *S. cerevisiae* cell wall glucan assembly. The finding of a *C. albicans* gene with structural and functional similarities to the *S. cerevisiae KRE1* gene suggests the possibility that *S. cerevisiae* and *C. albicans* share a similar set of genes for the production of cell wall glucan. This observation is important from a practical point of view. Studies of glucan synthesis in *S. cerevisiae* should be instructive in identifying essential genes involved in the assembly of the *C. albicans* cell wall whose products would provide targets for antifungal compounds.

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