Yeast KRE Genes Provide Evidence for a Pathway of Cell Wall β-Glucan Assembly

Charles Boone, Steve S. Sommer,* Andreas Hensel, and Howard Bussey

Department of Biology, McGill University, Montreal, Quebec, Canada H3A 1B1; and * Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota 55905

Abstract. The Saccharomyces cerevisiae KRE1 gene encodes a Ser/Thr-rich protein, that is directed into the yeast secretory pathway, where it is highly modified, probably through addition of O-linked mannose residues. Gene disruption of the KRE1 locus leads to a 40% reduced level of cell wall (1→6)-β-glucan. Structural analysis of the (1→6)-β-glucan fraction, isolated from a strain with a krel disruption mutation, showed that it had an altered structure with a smaller average polymer size. Mutations in two other loci, KRE5 and KRE6 also lead to a defect in cell wall (1→6)-β-glucan production and appear to be epistatic to KRE1. These findings outline a possible pathway of assembly of yeast cell wall (1→6)-β-glucan.

β-Glucans, homopolymers of glucose, are an abundant class of polysaccharides that includes cellulose, and appears to serve structural, functional, and morphological roles at the cell surface of fungi, bacteria, and plants (Fleet and Phaff, 1981; Sharp et al., 1984; Ikon de Iannino and Ugalde, 1989; Kato, 1981). Despite their widespread occurrence, there has been surprisingly little work to address the basis of cell wall glucan biosynthesis at the genetic and molecular level in eukaryotes. In vitro enzymatic reactions resulting in glucan synthesis have been defined and partially characterized for several systems (Kang and Cabib, 1986; Aloni et al., 1982), although components of the synthetic machinery have eluded purification. The isolation of mutants defective in the production of cell wall glucan should define genes that encode biosynthetic enzymes as well as other products, for example those that regulate glucan synthesis or generate glucan precursors. A mutant approach has been valuable in understanding the synthesis of such other cell wall polysaccharides, as mannan (Ballou, 1982) and chitin (Silverman et al., 1988; Bulawa et al., 1986).

Mixed linked β-glucans consisting of glucopyranosyl residues joined through (1→3) and (1→6)-linkages are common to fungi belonging to the Ascomycetes, Basidomycetes, and Oomycetes (Wessels and Sietema, 1981). Fractionation studies of the Saccharomyces cerevisiae cell wall demonstrated the presence of several glucan subclasses, which could be structurally distinguished by polymer length and the ratio of (1→3) to (1→6)-β-D-linkages (Fleet and Manners, 1976). Much of the yeast cell wall glucan is isolated from whole cells as an alkali insoluble fraction that was found to contain two distinct types of polymers. The most abundant alkali insoluble glucan consists predominantly of repeating units of linear (1→3)-β-linked residues, 3% of which are branched through a (1→6)-β-linkage (Manners et al., 1973a). This glucan has a degree of polymerization estimated to be 1,500 and has been proposed to determine the shape and stability of the yeast cell wall (Zlotnik et al., 1984). The other alkali-insoluble glucan has a degree of polymerization estimated to be 140 and contains residues that are predominantly connected through linear (1→6)-β-linkages (Manners et al., 1973b). This glucan will be referred to as (1→6)-β-glucan, although in addition to linear (1→6)-β-linked units it is composed of some linear (1→3)-linked residues and a relatively high proportion of (1→3, 1→6)-linked branched residues (14%). Yeast (1→6)-β-glucan accounts for ~20% of the alkali insoluble glucan or 3% of the total cellular dry weight.

The K1 killer toxin of S. cerevisiae provides a selection scheme for the isolation of mutants defective in (1→6)-β-D-glucan production. This toxin is a protein secreted by killer yeast strains which kills sensitive (nonkiller) strains. K1 toxin displays a lectin-like affinity for linear (1→6)-β-D-glucan and must bind to the wall of sensitive yeast in order to initiate the killing process (Bussey et al., 1979). Mutations in the KRE1 gene result in killer toxin resistance and are associated with an abnormal production of the cell wall (1→6)-β-glucan (Hutchins and Bussey, 1983).

We describe here that the KRE1 gene encodes a protein directed into the yeast secretory pathway. The (1→6)-β-glucan fraction which remained in a krel mutant yeast strain had an altered structure with a smaller average polymer size and suggests that (1→6)-β-glucan is synthesized in a stepwise manner. We address this possibility through the isolation of additional killer resistant mutants, some of which are required for (1→6)-β-glucan biosynthesis and appear to be epistatic to KRE1. Gene products required for fungal cell wall biosynthesis have been recognized as potential targets for specific antifungal antibiotics and the KRE genes are discussed in this context.
Materials and Methods

Yeast Strains and Procedures

S484, S486 and S442 are isogenic strains of *S. cerevisiae* derived from S331 as previously described (Ridley et al., 1984). S484 has a genotype *MATa leu2 his3 can1 [HOK] [NEX], whereas S486 is similar but lacks [HOK] [NEX]. The genotype of S442 is *MATa lys2 cyh2 can1 mkn1 [HOK] [NEX]. The killer-resistant isolates were identified for mutants of S484 or S486. Once obtained, the resistant mutants were characterized through crosses with S442 followed by tetrad analysis. The strains presented in Table III result from spore progeny obtained from crosses of mutants with S442. Each strain in Table III is *MATa lys2 cyh2 can1 mkn1 [HOK] [NEX]; in addition, S706 and S731 are met 13.

TARGET, *MATa/MATa his3/his3 leu2/leu2 can1/can1*, is an isogenic diploid strain (Whiteley and Szostak, 1985). The strains 463-1A, 463-1B, 463-1C, and 463-1D presented in Table I were obtained as the spore progeny from a TA405 diploid made heterozygous for a krel disruption mutation (*KREI/krel*: *HIS3*). The genotype of strains 463-1A and 463-1B is *MATa leu2 his3 krel::H1S3*, whereas the genotype of strains 463-1C and 463-1D is *MATa leu2 his3 krel::H1S3*, and one of the other strains used throughout this work and their corresponding genotypes are as follows: 1) *MATa krel-1 ura3*; T158C/S14a *MATa/MATa his3-468-464 his3-2 AD52 [KIL-KI] (Bussey et al., 1979); 2) HAB150-1 *MATa/MATa krel::H1S3*: *HIS3 his3* [HIS3 leu2/LEU2 lys2/lys2]; 7B *MATa pelt his3 ura3*.

Growth conditions and media (YPEP, complete and Halvorson's) were as described previously by Bussey et al. (1982) and Wickner (1979). Standard techniques were employed for diploid construction and sporulation (Sherman et al., 1982). Transformation was performed using the lithium acetate technique of Ito et al. (1983).

\[(\rightarrow \beta)-\beta-\text{Glucan Quantification}\]

Yeast cells were grown as 5-10 ml cultures in YEPD or minimal media (if plasmid selection was required) until stationary phase. Cells were harvested, washed once with distilled water, and then extracted three times with 0.5 ml of 3% NaOH at 75°C (1 h per extraction). After alkali extraction, 0.5 ml of 3% NaOH at 75°C (1 h per extraction). After alkali extraction, $484, $486 and $442 are isogenic strains of *S. cerevisiae* as described previously by Bussey et al. (1982) and Wickner (1978). Standard techniques were employed for diploid construction and sporulation (Sherman et al., 1982). Transformation was performed using the lithium acetate technique of Ito et al. (1983).

Plasmids

Vector YCPSO and the yeast genomic library constructed by M. Rose were provided by B. Futcher (Cold Spring Harbor Laboratory). Plasmid, pFL44, was obtained from F. Lacroute (Centre Nationale de la Recherche Scientifique, Gif sur Yvette, France) and is a yeast-2-μm-based, multicopy, shuttle vector with URA3 and Ap+ markers, which contains the pUC19 polylinker. The plasmid pFL44 was used for subcloning DNA fragments of YCP30 KRE1. Bluescript + and Bluescript − vectors (Strategene Corp., La Jolla, CA) were used for various recombinant DNA constructions and for production of single-stranded DNA. The yeast expression vector, PYT100U, contains the krel origin of replication, also allowing the production of single-stranded DNA, and was provided by T. Vernet et al. (1987). Plasmid PBSK:HIS3 was recovered from a TA405 diploid made heterozygous for a krel disruption mutation (*KREI/krel*: *HIS3*). The genotype of strains 463-1A and 463-1B is *MATa leu2 his3 krel::H1S3*, whereas the genotype of strains 463-1C and 463-1D is *MATa leu2 his3 krel::H1S3*. The other strains used throughout this work and their corresponding genotypes are as follows: 1) *MATa krel-1 ura3*; T158C/S14a *MATa/MATa his3-468-464 his3-2 AD52 [KIL-KI] (Bussey et al., 1979); 2) HAB150-1 *MATa/MATa krel::H1S3*: *HIS3 his3* [HIS3 leu2/LEU2 lys2/lys2]; 7B *MATa pelt his3 ura3*.

Growth conditions and media (YPEP, complete and Halvorson's) were as described previously by Bussey et al. (1982) and Wickner (1979). Standard techniques were employed for diploid construction and sporulation (Sherman et al., 1982). Transformation was performed using the lithium acetate technique of Ito et al. (1983).

DNA Sequencing

Subclones of the KRE1 yeast genomic DNA were made in Bluescript vectors or in PVTT100U. Plasmids containing subclones were transformed into the bacterial strain, UT580, and single-stranded DNA was made using M13K07 helper phage (Vernet et al., 1987). Sequencing was by the dyeoxyme method (Sanger et al., 1977) and uracil protrophs were selected. Transformants were replica-plated to minimal media, Halvorson's 1x pH 4.7 agar, which had been seeded with 75 μl/ filter of a stationary culture of the diploid killer strain T158C/S14a and contained 0.002% of the vital stain methylene blue. After replica plating, the methylene blue plates were incubated at 18°C for 3-4 d; at the end of this period the Kre+ transformants had stained a dark blue color, whereas kre− colonies remained white. Individual cells were isolated from the stained blue colonies and these were later grown for plasmid recovery.

KRE1 Disruption

To create a krel::H1S3 disruption construct, a *HIS3* containing fragment was ligated into the Spe I and Kpn I sites, situated within the *KRE1* coding sequence (Fig. I) as described below. The KRE1 Bam HI-Pst I fragment was then introduced into an altered PUC19 plasmid, in which the Kpn I site of the polylinker had been removed, to create p411. Plasmid p411 was digested with Asp718 (an isoschizomer of Kpn I), made blunt ended with Klenow fragment, and then ligated with a nonphosphorylated Xho I linker (AM155CATCCGCGGAGG-3), to generate p458. The *HIS3* gene could be isolated from PBSK, *HIS3* as a Spe I-Xho I fragment and ligated into p558 also digested with Spe I and Xho I. The ligation product of this last reaction was called p463, digestion of p463 with Neo I and Sph I, which cut within the KREI portion of the insert but not the *HIS3* portion, allowed disruption of the KREI locus upon transformation.

Mapping KRE1

A Southern blot of chromosomes separated by pulse-field electrophoresis (Carle and Olson, 1985) was probed with KREI DNA. The KREI sequence hybridized to chromosome XIV (data not shown). Tetrads analysis provided the following linkage for KREI: the krel-pha2 map distance is 8 cM (1) parental ditypes [PD], 0 nonparental ditypes [NPD], and 8 tetratypes [TT]). The krel-pha2 map distance is 8 cM (1PD, 1 NPD, and 56 TT). The krel-pha2 map distance is 8 cM (1PD, 1 NPD, and 56 TT). Seven tetrads examined where krel was recombinant with pha2; five tetrads were also recombinant for krel with met2 and pet2, suggesting the order krel pha2 met2 pet2. The map distances were calculated according to Mortimer and Schuld (1985).

Electron Microscopy

The conditions presented below represent a modified version of the proce-
Cells were grown in minimal media supplemented with 1X Halvorson's salts to stationary phase, and harvested and washed with distilled water. Cell pellets were fixed in a solution containing 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 70 minutes. After fixation, cell pellets were rinsed in buffer, then postfixed for 1 h in 0.1% OsO4 in 0.1 M sodium phosphate buffer (pH 7.2) and then rinsed again. Cell pellets were subsequently dehydrated through a graded ethanol series, infiltrated and embedded in Spurr's epoxy resin (Spurr, 1969). Gold- and silver-stained sections were mounted on formvar-coated grids and sections were subsequently dehydrated through a graded ethanol series, infiltrated and embedded in Spurr's epoxy resin (Spun', 1969). Gold- and silver-stained sections were mounted on formvar-coated grids and sections were then stained with 2% aqueous uranyl acetate followed by Reynold's lead citrate (Reynold, 1963). Sections were viewed on a Philips EM410 electron microscope at an operating voltage of 80 kV.

**pVT::KRE1**

To create the pVT::KRE1 insert, blunt-end restriction sites were introduced into subclones of the KRE1 locus and the resultant constructs ressembled to form an uninterrupted open reading frame. Single-stranded DNA was prepared from p486 and in combination with oligo IB (5'-CAATCAA-AAAACCGGGAAATAGCATC-3'), an Smal restriction site was introduced three nucleotides before the ATG of the KRE1 open reading frame, resulting in plasmid p567. Plasmid p567 was then digested with Sma I and religated so that most of the 5'-untranslated region of KRE1 was removed and the introduced Smal site was situated next to a Bam HI site of the Bluescript polyclinker (p585). Single-stranded DNA was also prepared from p492 and used in combination with oligo 3B (5'-GTTCTTATAAAGGCCTAITrTTATTC-3') to insert a Stu I restriction site just after the KRE1 open reading frame in p567. The 0.4-kb Spe I-Hind III fragment of p563 was isolated and ligated into Spe I, Hind II digested p580. Plasmid p580 was digested with Stu I and religated by Hinc II and the resultant 0.8-kb fragment was purified. This fragment was ligated into p585 after digestion with Xho I and Hinc II to give p596. Plasmid p607 was made when p596 was digested with Stu I and Eco RV, and the vector fragment (containing KRE1) religated. This procedure situated the Hind III site of the Bluescript polyclinker just after the KRE1 open reading frame. The KRE1 containing 1-kb (Bam HI-Hind III) fragment of p607 was purified and ligated into Bam HI and Hind III digested pVT100U, to generate pVT::KRE1.

**pVT::Δ24::KRE1**

Single-stranded DNA from p486 was used for oligonucleotide specific mutagenesis with oligo 6B (5'-GCTCCGCGTGTTAACTACGCGCT-3') to form p647. Oligo 6B directs the introduction of a Hpa I site just before Met 25 of Krelp. The plasmid p647 was digested with Hpa I and Smal 1, and religated, so as to remove the KRE1 leader encoding DNA (p676). This process also situates the 5' end of the leader deleted KRE1 fragment next to a Bam HI restriction site in the Bluescript polyclinker. The Sat I-Hinc II (0.25 kb) fragment of p676 was ligated into the plasmid p607, which had also been digested with Sat I and Hinc II, creating p688. The leader deleted KRE1 construct of p688 was isolated via Bam HI and Hind III digestion, then ligated into similarly digested pVT100U to generate pVT::Δ24::KRE1.

**pVT::KRE1/SP and pVT::Δ24::KRE1/SP**

Plasmids p607 and p688 were digested with Sma I and Hind III, and the vector containing fragment isolated for each digestion. These fragments were ligated with the complementary oligonucleotide 15B (5'-GACTCGCGACTCGTAACTAGTGCGATCT-3') and 16B (5'-AGCTTTACAGGCGCGAGGCGCT-3'), to create KRE1-substance P epitope fusion constructs p715 and p718. The oligos (15B and 16B) basepair to form a small segment of DNA encoding the peptide DQQFGLML followed by a stop codon, the last six amino acids are part of the neuroepitope, substance P. Plasmids p715 and p718 were digested with Bam HI and Hind III and the KRE1-substance P fusion fragments were introduced into pVT100U to create pVT::KRE1/SP and pVT::Δ24::KRE1/SP, respectively.

**pVT::α20::KRE1**

Single-stranded DNA was prepared from pVT::KRE1 and oligonucleotide 6B (see above) was used for directed mutagenesis to insert a Hpa I site just before the codon encoding Met 25 of Krelp. The newly created plasmid was designated p758. The 0.9-kb Hpa I-Hind III (KRE1 fragment without the leader) was isolated and ligated into Hinc II-Hind III digested p339 vector fragment to give p751. A segment of DNA with the prepro-α factor leader spliced in frame with a deleted KRE1 gene fragment can be removed from p771 as a 1.0-kb Bgl II-Hind III fragment. Ligation of this Bgl II-Hind III fragment into Bam HI-Hind III digested pVT100U generated pVT::α20::KRE1.

**Seeded Plate Assay for Killer Resistance**

Yeast strains were grown to stationary phase in liquid media (under plasmid-selective conditions if necessary) and 30 µl of this culture was used to inoculate 10 ml of minimal media, 1% agar. 1X Halvorson's, pH 4.7. Con. 7% (7 µl of 1,000× concentrated media from S14a/T158C; Bussey et al., 1983) was introduced onto the solidified agar and the plates incubated at 18°C overnight, followed by a 30-C incubation for 24 h.

**Western Analysis of Substance P Hybrid Proteins**

Approximately 1 × 10^7 transformed yeast cells (grown in minimal media under plasmid selection) were harvested in log phase and the cellular contents prepared for electrophoresis as described by Segev et al. (1988). Electrophoretic transfer blots were analyzed with anti-substance P antibody (NCI/34 HL; Accurate Chemical & Scientific, Westbury, NY) as described by Musro and Pelham (1984), in combination with an alkaline phosphatase immunoblot detection kit (Bio-Rad Laboratories, Richmond, CA).

**Large-Scale (1→6)-β-Glucan Preparation**

Yeast (1→6)-β-glucan was isolated from a 2-liter culture of wildtype cells (strain 7B) or a 5-liter culture of krel mutant cells (strain 3), each grown to stationary phase in YEPD, 1X Halvorson's salts. The cells were harvested, (strain 3, cells were split into two samples each treated as given below) washed with distilled water, and stored at -70°C. Mannoprotein and alkali soluble glucan was removed via five 100-ml extractions with 3% NaOH, each for 1 h at 70°C. After alkali extraction the cell walls were neutralized (with phosphate buffer, pH 6.8), and digested with 33 µg of Zymolase 100,000 in 10 mM sodium phosphate buffer pH 6.8 (with a 40-min final volume containing 0.1% sodium azide) for 16 h at 37°C. After this digestion, insoluble material was removed by centrifugation (12,000 rpm) and the supernatant treated with 20 µm amylase (10 mg/ml, Boehringer Mannheim Canada Ltd., Dorval, Quebec) for 2 h at room temperature. After amylase treatment the glucan containing solution was extracted twice with 5-ml portions of phenol. Several 10-ml ether extractions removed residual phenol. The aqueous phase was collected and dialyzed against distilled water in Spectra/por tubing with a pore size of 6,000-8,000 D (Spectrum Medical Industries, Inc.) for 5 h, then freeze-dried. The freeze-dried material was solubilized in 5 ml of distilled water and further dialyzed in Spectra/por tubing with a 2000 D pore size for 30 h before a second freeze drying. The water-soluble material, which remains after this procedure was used for structural analysis. 2 liter of a culture of wild-type cells yielded 40–50 mg of (1→6)-β-glucan and 5 liter of a culture of krel mutant cells produced an equivalent amount.

**[^1]C Nuclear Magnetic Resonance (NMR) Spectroscopy**

[^1]C NMR spectra were obtained using 10-mm-diam tubes, with 40 mg of glucan dissolved in 3 ml D2O. Data were collected under conditions of proton decoupling, using a Bruker spectrometer (model WH 400; Bruker Instruments, Billerica, MA) operated in the Fourier-transform mode, at 100.62 MHz, with a sweep width of 6493.5 Hz and an acquisition time of 0.631 s. The pulse angle was 73° and the pulse interval was 4.0 s, during which the decoupler was gated off. The probe temperature was maintained at 19°C. Each spectrum was recorded several times, from independent glucan samples, with ~10,000 scans. The reference for the chemical shift values was external Dioxane at 67.4 ppm.

**Gel Filtration Chromatography**

A Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) column of dimensions 110.0 x 10 cm was used at a flow rate of 16 ml/h. The eluant was 0.1 M NaOH and 0.4-ml fractions were collected. Calibration of the column was carried out using dextran blue (Pharmacia Fine Chemicals) to indicate the void volume and several dextrins of known molecular weights (Sigma Chemical Co., St. Louis, MO; Fig. 6). Determination of the carbohydrate content of each fraction was carried out by the phenol-sulfuric acid method (Dubois et al., 1956).

1. Abbreviations used in this paper: NMR, nuclear magnetic resonance.
positions of restriction sites inserted using site specific mutagenesis (see Materials and Methods). Various restriction sites used for recom-

Figure 1. The binant DNA constructs are designated above the DNA sequence. These sequence data are available from EMBL/Genbank/DDBI under

The Journal of Cell Biology, Volume 110, 1990 1836

Isolation of Killer-resistant Mutants

To isolate mutants resistant to K1 killer toxin, 1 × 10^7 cells of S46 or S484 were mixed with 2 × 10^6 of a nonreverting, homozygous leu2 K1+
diploid strain and plated on complete media lacking leucine, pH 4.7. After 8 d, colonies of resistant S46 and S484 could be seen above a lawn of ini-

Isolation of the KREI Gene

To identify the KREI gene product and initiate a study of its function, we isolated the wild-type KREI locus. The krel-1 ura3 yeast strain 11A was transformed with a yeast genomic
library in the URA3-containing centromeric vector YCp50, and uracil prototrophs were selected (Rose et al., 1987). Transformants were screened for a killer-sensitive phenotype (Kre+) as described in Materials and Methods. Two independent Kre+ transformants were obtained and found to be unstable for both the Kre+ and Ura+ phenotypes when grown under nonselective conditions in YEPD. A unique plasmid was isolated from each of these transformants that could complement the krel-1 mutation. One plasmid, YCp50: KRE1, contained a 6.5-kb insert of yeast genomic DNA and restriction endonuclease mapping revealed that this DNA fragment was contained within a larger (11 kb) insert, of the other complementing plasmid. Genetic analysis showed that the complementing fragment contained the KRE1 locus (see below).

**Nucleotide Sequence of KRE1**

Subcloning of the insert of plasmid YCp50:KRE1 determined that a 3.9-kb Bam HI-Pst I restriction fragment could complement the kre-, phenotype of strain 11A. However, subclones on either side of an internal Kpn I site failed to complement, suggesting that the Kpn I site is located within the KRE1 functional region. Further subcloning experiments localized the complementing activity to a 1.5-kb Nhe I-Nsi I fragment, the DNA sequence of this fragment (Fig. 1) was determined using the dideoxy nucleotide method of Sanger et al. (1977). This sequence contains a single extended open reading frame that spans the Kpn I site. This open reading frame would encode a protein of 313 amino acids with a molecular weight of 32,000 (Fig. 1).

The protein, Krelp, displays a striking abundance of threonine (25%) and serine (15%) residues. The amino terminus of Krelp is hydrophobic and resembles the signal sequences of secreted proteins. There are two potential signal cleavage sites (von Heijne, 1984) found after amino acid residues 23 and 27 (Fig. 1). The last 21 amino acid residues of Krelp also form a hydrophobic sequence. No sites for N-linked glycosylation were observed, however, the abundance of serine and threonine residues may provide sites for O-linked glycosylation (Tanner and Lehle, 1987). Krelp contains an internal repeat of 15 amino acids. Comparison of both the KRE1 nucleotide sequence, and the deduced primary amino acid sequence with those from available data bases, has not revealed any sequences with significant similarities to KRE1.

**Disruption of KRE1**

A null mutation of the KRE1 locus was generated by the one step gene disruption procedure using HIS3 as a selective marker (Rothstein, 1983). The krel::HIS3 disruption construct is described in Materials and Methods. The diploid TA405, homozygous for a his3 mutation, was transformed with a restriction fragment of the cloned DNA containing a disruption of the KRE1 coding region. His+ transformants were sporulated and subjected to tetrads analysis. Several independent transformants gave rise to two His+ kre- segre- gants and two His- kre+ segregants (18 out of 18 tetrads analyzed). The killer-resistant segregants consistently formed slightly smaller colonies upon spor germination when compared with the killer sensitive segregants, but individual cells were of normal size and morphology as judged by light microscopy. The structure of the integrated krel::HIS3 deletion replacement was confirmed by Southern analysis of the chromosomal DNA from disrupted haploids (data not shown). The diploid HAB150-1 (krel-3/krel::HIS3) was sporulated for tetrad analysis, 22 of 23 tetrads were parental dihybrid for killer resistance and 1 was a tetraploidy. These results show that the cloned sequence is tightly linked to the KRE1 locus and that KRE1 is nonessential for both mitotic growth and meiotic spore formation. In further experiments, we have determined the location of KRE1 on the yeast genetic map (see Materials and Methods). Closest linkage was with the PHA2 locus (required for phenylalalanine biosynthesis), analysis of recombinants between krel and pha2 suggests that krel is the distal most known marker on the left arm of chromosome XIV.

Cell wall (1→6)-β-glucan can be isolated from the alkali insoluble glucan fraction following acid extraction or treatment with an endo-(1→3)-β-glucanase (Manners et al., 1973b). Yeast strains with a mutant krel-1 allele were found to display an ~40% reduced level of the (1→6)-β-glucan fraction when isolated by either protocol (Hutchins and Bussey, 1983), however, the yield was greater with the glucanase method. To avoid any subfractionation that may occur upon incomplete acid extraction, (1→6)-β-glucan was isolated using the endoglucanase technique (see Materials and Methods). Analysis of the (1→6)-β-glucan levels of the spore progeny for the krel::HIS3 disruption mutation, demonstrated that the level of this glucan was reduced in progeny with a disrupted allele and the reduction was ~40% of wild-type levels (Table I). This finding suggests that the mutation that defines the krel-1 allele leads to a null phenotype. Consistent with this idea, krel-1 mutant yeast strains display a complete killer resistant phenotype, which appears similar to the phenotype of krel::HIS3 mutant strains. However, small in-frame insertions or deletions of the KRE1 coding sequence can lead to a partial resistant phenotype (see Fig. 3, below).

**Electron Microscopy of krel Mutant Cell Walls**

The krel::HIS3 mutant yeast cells were examined by EM and compared with wild-type cells. Under the conditions used, wild-type cells were found to have a finely delineated dark-staining outer layer. This layer was missing from mutant cell walls and the outer surface appeared rough in texture (Fig. 2). The mutant cell wall material also stained more intensely, especially in the outer half of the wall. These struc-

| Yeast strain | Allele at KRE1 locus | (1→6)-β-Glucan μ/mg dry wt |
|--------------|----------------------|---------------------------|
| 463-1A       | krel::HIS3           | 24.9 ± 3.5                |
| 463-1B       | krel::HIS3           | 19.3 ± 2.6                |
| 463-1C       | KRE1+                | 34.2 ± 3.2                |
| 463-1D       | KREI+                | 34.5 ± 0.6                |
| 7B           | KREI+                | 27.0 ± 0.8                |
| 3            | krel::HIS3           | 17.2 ± 2.2                |

The levels of (1→6)-β-glucan were analyzed for the spore progeny of a tetrad, from the isogenic diploid TA405 made heterozygous for a krel::HIS3 disruption mutation, (KRE1/krel::HIS3). Strains 463-1A and 463-1B display a reduced level of (1→6)-β-glucan and carry the krel::HIS3 mutation. Disruption of the KRE1 locus of the haploid strain, 7B (gle1, ura3, his3), resulted in strain 3 (gle1, ura3, his3, krel::HIS3). Error represents 1 SD.
et al., 1987). Upon transformation of a phenotype and led to \( (\mathbf{1} \rightarrow \mathbf{6}) \)-\( \beta \)-glucan levels equivalent to those induced by YCp50:KRE1 (Table II). Transformation of deleted construct (pVT:A24/KRE1) did not complement the resulting plasmid, pVT: KRE1, fully complemented the nucleotides of the open reading frame (predicted to encode an increased amount of \( (\mathbf{1} \rightarrow \mathbf{6}) \)-\( \beta \)-glucan.\\n
The KRE1 Gene Encodes a Product with a Functional Signal Peptide

Restriction endonuclease sites were introduced three nucleotides before, and immediately after, the \( \text{ADH} \)/promoter and terminator (Vernet et al., 1987). Upon transformation of a \( krel-1 \) mutant, the resultant plasmid, pVT:KRE1, fully complemented the \( kre- \) phenotype and led to \( (\mathbf{1} \rightarrow \mathbf{6}) \)-\( \beta \)-glucan levels equivalent to those induced by YCp50:KRE1 (Table II). Transformation of a wild-type \( (Kre{+}) \) strain with pVT:KRE1 did not lead to an increased amount of \( (\mathbf{1} \rightarrow \mathbf{6}) \)-\( \beta \)-glucan.\\n
To determine whether the \( KRE1 \) sequence encoded a functional signal peptide, a deletion was made of the first 72 nucleotides of the open reading frame (predicted to encode 24 NH\(_2\)-terminal amino acids of Krelp, Fig. 1). The resultant construct was introduced into pVT100U, positioning Met 25 of Krelp next to the \( ADHI \) promoter. When transformed into yeast cells mutant at the \( KRE1 \) locus, the leader-deleted construct (pVT:Δ24/KRE1) did not complement the \( kre- \) phenotype. However, if the leader deleted portion of the \( KRE1 \) sequence was replaced with a segment of DNA which encodes the first 20 amino acids of the alpha factor precursor (Kurjan and Hershowitz, 1982) (pVT:α20/KRE1), a \( Kre+ \) phenotype was observed (Fig. 3). Another hybrid gene was constructed that replaced the DNA segment of \( KRE1 \) encoding the last 59 amino acids of Krelp, with a sequence that codes for a six-amino acid portion of the neuropeptide substance P. This construct was introduced into pVT100U (pVT:KRE1/SP) and allowed partial complementation of the \( krel \) mutant strain (Fig. 3). The substitution P portion provides an epitope that can be detected by an mAb (Munro and Pelham, 1984). Yeast strain 11A was transformed with the \( ADHI \) expression vector carrying hybrid constructs both with and without (pVT:Δ24/KRE1/SP), the Krelp signal peptide. Electrophoretic transfer blot analysis of total protein isolated from transformed yeast cells showed that the leader allowed a 50-kD modification of the Krel-substance P hybrid protein (Fig. 4). This sizing is approximate because extended electrophoresis of the modified polypeptide resulted in smearing of the immunoreactive band. Similar analysis of concentrated yeast culture media revealed that only the modified hybrid protein was exported (data not shown). The leader-deleted Krel-substance P hybrid protein has a predicted molecular weight of \( \sim 25,000 \), while the apparent size as determined by SDS-PAGE was found to be 30 kD (Fig. 4). A similar discrepancy has been observed for other serine- and threonine-rich proteins, suggesting that it is associated with a high content of hydroxy-aminos (Early et al., 1988). The observed modification of the Krel-substance P hybrid protein is probably the result of O-linked mannose addition. Evidence to support this conjecture comes from immunoprecipitation experiments, using other fusion constructs, where the modification was found to be endoglucosaminidase H resistant (data not shown).

\[\text{Structural Analysis of } (\mathbf{1} \rightarrow \mathbf{6}) \beta\text{-Glucan from a krel Mutant}\]

To facilitate \([^{13}\text{C}]\text{NMR analysis of the } (\mathbf{1} \rightarrow \mathbf{6}) \beta\text{-glucan fraction isolated from a } KRE1 \text{ disruption strain (mutant glucan), a large-scale procedure for the purification of } \sim 50 \text{ mg of Zymolyase-resistant glucan was designed (see Materials and Methods). The yeast strain } 7B \text{ (his3 ura3 glc1) used for wild-type glucan purification carried the } glc1 \text{ mutation to minimize glycogen contamination (Tkacz, 1984); disruption of the } KRE1 \text{ locus in this strain created a } krel \text{ null mutant, (strain 3), with a reduced amount of } (\mathbf{1} \rightarrow \mathbf{6}) \beta\text{-glucan (Table I).}\\n
The proton decoupled \([^{13}\text{C}]\text{NMR spectrum of glucan purified from the wild-type strain (7B), is presented in Fig. 5A. The data for this spectrum were obtained under conditions where the signal area reflects relative amounts of the constituent carbon atom(s) (Shimamura, 1989). The wild-

\[\text{Table II. Plasmid-dependent Maturation of Cell Wall } (\mathbf{1} \rightarrow \mathbf{6}) \beta\text{-Glucan}\]

| Yeast strain (allele at } KRE1 \text{ locus) | Transformation plasmid | \( \text{\(\mu\)g/mg dry wt} \) |
|--------------------------------------------|------------------------|-----------------|
| 11A (krel-1)                               | YCp50                  | 15.7 ± 1.4      |
| 11A (krel-1)                               | YCp50:KRE1             | 41.4 ± 4.7      |
| 11A (krel-1)                               | pVT100U                | 17.5 ± 1.1      |
| 11A (krel-1)                               | pVT:KRE1               | 42.6 ± 4.1      |
| 7B (KRE1)                                  | pVT100U                | 38.9 ± 3.9      |
| 7B (KRE1)                                  | pVT:KRE1               | 45.2 ± 6.4      |

Yeast \((\mathbf{1} \rightarrow \mathbf{6}) \beta\text{-glucan levels were analyzed for various 11A (krel-1, ura3) transformants. Plasmid YCp50:KRE1 contains a yeast genomic insert that complements the } krel-1 \text{ mutation ligated into the centromeric (single copy) vector YCp50. Plasmid pVT:KRE1 contains the } KRE1 \text{ open reading frame ligated into the } 2 \mu \text{ derived (multicyclic) expression vector pVT100U. Transcription of the } KRE1 \text{ from pVT:KRE1 occurs via the } ADHI \text{ promoter. Yeast } (\mathbf{1} \rightarrow \mathbf{6}) \beta\text{-glucan levels were also analyzed for transformants of strain 7B (ura3, his3, glc1). Error represents 1 SD.}
Figure 3. Leader-dependent function of the KREI gene product. Inserts of various pVT:100U-derived vectors are designated by the plasmid name and drawn schematically, indicating structural features as described in the text. The amino acid sequence of the Krelp leader is compared with the sequence of the prepro-α factor leader. The signal cleavage site of the prepro-α factor leader and a site predicted for Krelp are indicated. Examples of the seeded plate assay used to assess complementation of the killer-resistant phenotype of transformants of strain IIA (krel-1, ura3) are also shown (see Materials and Methods). Plasmids pVT:KREI and pVT:α20/KREI, completely complement the kre- phenotype. Plasmid pVT:KREI/SP can only partially complement and pVT:Δ24/KREI does not complement the kre- phenotype.

Type glucan showed predominant signals at 103.8, 76.4, 75.7, 73.8, 70.3, and 69.6 ppm (Fig. 5 A; C-1, C-3, C-5, C-2, C-4, and C-6 linked, respectively). These chemical shifts are characteristic of linear (1→6)-β-glucan (Gopal et al., 1984; Bassieux et al., 1977; Saito et al., 1977). Several minor signals can be ascribed to the presence of linear (1→3)-linked, branched (1→3, 1→6)-linked and terminal β-glucopyranosyl residues in the polymer. For example, the signal with a chemical shift of 61.5 ppm is the result of residues unsubstituted at C-6 (Fig. 5 A; C-6), as found for terminal β-glucopyranosyl residues or those which have a linear (1→3)-linked structure, other assignments are presented in Fig. 5. Integration analysis suggests that 82% of the residues are O-substituted at C-6 position.

Gel filtration chromatography of mutant and wild-type glucans over a Sepharose CL-6B column demonstrated that
Figure 4. Western blot analysis of the products of Krel-substance P fusion constructs was carried out as described in Materials and Methods. Plasmids pVT:KREI/SP and pVT:Δ24/KREI/SP contain similar DNA inserts, encoding Krel-substance P fusion proteins (with the substance P epitope replacing the last 59 amino acids of Grep), except that the insert of pVT:Δ24/KREI/SP is deleted for DNA encoding the predicted leader of Krelp. Strain 11A transformed with pVT:KREI/SP (see Fig. 3) produces an immunoreactive band that migrates with a molecular mass of 80 kD (lane 3). Strain 11A transformed with pVT:Δ24/KREI/SP leads to an immunoreactive band which migrates with a predicted molecular mass of 30 kD (lane 2). Strain 11A transformed with the expression vector pWTi00U provided a control that did not give rise to an immunoreactive band (lane 1).

The mutant glucan had a smaller average degree of polymerization than the wild type (Fig. 6). The wild-type glucan displayed a range of different-sized material, with an average predicted molecular mass of 40 kD. The mutant glucan displayed a range of material with smaller predicted molecular masses and an average of 20 kD. These results suggest that the average degree of polymerization of the wild-type glucan was ~200, whereas that of the mutant glucan was 100.

**Killer-resistant Mutants Identify a Group of Genes Required for Cell Wall (1→6)-β-Glucan Production**

The observation that *krel::HIS3* mutant yeast strains produced an altered form of cell wall (1→6)-β-glucan with a smaller average polymer size, suggests that *KREI* could be required for the stepwise synthesis of the mature polymer. Additional genes required for the production of cell wall (1→6)-β-glucan were identified through further selection of mutants resistant to killer toxin (see Materials and Methods). 44 resistant mutants were characterized by performing genetic analysis in an isogenic background. Six complementation groups were defined by recessive mutations, each of which segregated as a defect in a single gene. Two of the complementation groups were found to be equivalent to *KREI* and *KRE2* described by Al-Aidroos and Bussey (1978). The other complementation groups designated *KRE4*, *KRE5*, *KRE6*, and *KRE8* are novel. Segregation analysis indicated that the *krel*, 2, and 5 mutations identify three separate loci, and that *KRE5* and *KRE6* are not allelic. The mutants that define the *KRE4* and *KRE8* complementation groups proved to be only weakly resistant, and will not be considered further here.

Mutants in three complementation groups were found to have reduced levels of cell wall (1→6)-β-glucan (Table III). The level in strain S708 that harbors the *krel-3* allele was reduced 40% in agreement with the previous observations for mutants containing *krel-1* or a *krel::HIS3* disruption mutation. Strains S726 and S731 carrying mutations at the *KRE5* and *KRE6* loci respectively showed a significant reduction in (1→6)-β-glucan and demonstrated a slow growth phenotype which cosegregated with the killer resistance when compared with wild-type strains or the *krel* mutant, S708. The level of (1→6)-β-glucan was not altered in the *krel* strain S706 (Table III).

Double mutants were constructed for the strains that showed a reduced level of cell wall (1→6)-β-glucan. The level of (1→6)-β-glucan in double mutants of *krel* with *kre5* or *kre6* was not significantly lower than that found in *kre5* or *kre6* single mutants. This result suggests that mutations at both the *kre5* and *kre6* loci lead to killer toxin resistance because they are epistatic to *KREI*. In contrast, *kre5* and *kre6* mutants showed a reduced level of cell wall (1→6)-β-glucan (Table IV). The level in strain S708 that harbors the *krel-3* allele was reduced 40% in agreement with the previous observations for mutants containing *krel-1* or a *krel::HIS3* disruption mutation. Strains S726 and S731 carrying mutations at the *KRE5* and *KRE6* loci respectively showed a significant reduction in (1→6)-β-glucan and demonstrated a slow growth phenotype which cosegregated with the killer resistance when compared with wild-type strains or the *krel* mutant, S708. The level of (1→6)-β-glucan was not altered in the *krel* strain S706 (Table III).

**Figure 5.** (A) [13C]NMR spectrum of (1→6)-β-glucan purified from a wild type (*Kre +*) yeast strain. The predominant signals (A, C-1, C-3, C-5, C-2, C-4, and C-6 linked) have chemical shifts that are characteristic of linear (1→6)-β-glucan as described in the text. Presently, there are insufficient reference data to assign identities to each of the minor signals; however, some can be assigned as presented below. The signal at 85 ppm (A, C-4; C-3 linked) corresponds to O-substituted at C-3 found in branched and linear (1→3)-linked residues (Yoshioka et al., 1985). The signal at 68.8 ppm can be assigned to C-4 of residues O-substituted at C-3 and the signal at 61.5 ppm results from residues unsubstituted at C-6, the latter are found in linear (1→3)-linked and terminal β-glucopyranosyl residues (Brunet et al., 1988). Some of the expected minor signals coincide with a major signal. For example, the signal with a chemical shift of 69.6 ppm is the result of residues O-substituted at C-6 (Fig. 5A; C-6 linked), as found for those which have a linear (1→6)-linked or branched structure. Assignment of the signal at 69.6 and 61.5 ppm as the result of a CH2 group was confirmed by a DEPT NMR pulse sequence (Doddrell et al., 1982). The area of the assigned minor signals was similar predicting that the relative proportion of branched and terminal β-glucopyranosyl residues was approximately equal, as expected. (B) [13C]NMR spectrum of the (1→6)-β-glucan fraction purified from a *krel* mutant.
double mutants displayed a further reduction in the cell wall (1->6)-β-glucan level (reduced by ~80% over wild type), and are associated with a severe growth impairment.

Discussion

We have cloned the KRE1 gene from S. cerevisiae and shown that a disruption of the KRE1 locus results in an ~40% reduction of cell wall (1->6)-β-glucan. Haploid yeast strains with a disrupted krel allele grow somewhat more slowly than wild type and were found to have an unusual cell wall ultrastructure. Yeast cell wall (1->6)-β-glucan is a highly branched glucosyl polymer composed mostly of linear (1->6)-linked residues as well as some linear (1->3)-linked residues. Branching occurs through triply linked (1->3, 1->6)-β-glucopyranosyl residues. Structural analysis of the (1->6)-β-glucan, which remains in a krel mutant (mutant glucan) when compared with the glucan purified from isogenic wild type cells, showed that each glucan was composed of a similar set of linked residues. However, the mutant glucan contained fewer (1->6)-linked residues, which were incorporated into a polymer of smaller average size. It is possible that the KRE1 gene product is required for the addition of extended chains, composed predominantly of linear (1->6)-β-glucan, onto a highly branched acceptor glucan.

We favor this interpretation because krel mutants are completely resistant to the K1 killer toxin of S. cerevisiae. The killer toxin displays a lectin-like affinity for linear (1->6)-β-glucan chains and unlike the cell walls of wild type yeast strains, krel mutant cell walls lack a component with similar toxin affinity (Bussey et al., 1979).

A potential pathway of gene products necessary for yeast (1->6)-β-glucan biosynthesis is implicated by the finding that other mutants are resistant to killer toxin. Mutations at either the KRE5 or KRE6 loci result in killer resistance and a reduced amount of cell wall (1->6)-β-glucan. This reduction is not affected by a krel mutant allele, suggesting that mutations at the KRE5 or KRE6 loci are epistatic to KRE1. Mechanistically it seems reasonable that the KRE5 and KRE6 gene products could be required for the production of an acceptor glucan, which is defined by the (1->6)-β-glucan fraction that remains in a krel mutant (Fig. 7). This interpretation implies that the mutant krel-1 or krel-6 alleles lead to the production of an altered acceptor glucan, which cannot be extended in a KREI-dependent fashion and therefore result in killer toxin resistance. Recent experiments have shown that disruption of the KRE5 locus leads to a yeast strain which is not impaired for (1->3)-β-glucan biosynthesis, but has an extremely slow growth rate, and appears to lack cell wall (1->6)-β-glucan (Meaden, P., unpublished results). The lack of (1->6)-β-glucan in yeast strains carrying a krel5 null mutation further indicates that mutations at the KRE5 locus are epistatic to KRE1.

The KRE1 gene product (Krelp) has a functional aminoterminal signal sequence that directs the protein into the yeast secretory pathway, where it is extensively modified probably through the addition of O-linked mannose residues. Yeast mating-type agglutinin proteins (Lasky and Ballou, 1988; Watzele et al., 1988) and a large proportion of the bulk cell wall protein (Frevert and Ballou, 1985) are serine/threonine-rich and O-glycosylated. Therefore by analogy, Krelp may also be localized at the yeast cell surface. In support of this idea, fusion constructs which place a leader-deleted KRE1 fragment next to the carboxy terminus of the PH05 open reading frame (Meyhack et al., 1982), lead to a fusion protein that partially complements a krel mutant and directs acid phosphatase activity to the cell surface (data not shown). The 21 carboxy-terminal amino acid residues of Krelp form a hydrophobic sequence, which may serve as a membrane spanning domain or provide a signal for attachment of a glycosyl-phosphatidylinositol membrane anchor (Conzelmann et al., 1988).

The appearance of krel mutant cells, as examined using EM, revealed that the outer portion of the wall was abnormal. Particularly noticeable was the lack of a finely delinated dark staining region, thought to be a surface layer of mannoprotein (Zlotnik et al., 1984). This alteration, although possibly enhanced by the fixation procedure, may have functional significance, as krel mutants are more sensitive to...
zymolyase treatment than wild-type cells (data not shown), and over secrete proteins normally found in the growth medium (Bussey et al., 1983). It is likely that wild-type cells release a certain portion of wall-localized proteins into the growth medium and this process is exaggerated in krel mutant cells. However, krel mutants do not show significant reduction in total wall mannoprotein (Hutchins, 1982), indicating that the bulk of the mannoprotein is efficiently targeted within the wall (Valentin et al., 1987) or periplasmic space. In addition, the krel mutant cell walls were found to stain more intensely, especially in the outer half of the wall, leading to a bipartite appearance. This may suggest that a krel mutant is partially defective in the assembly of the outer wall, which could be the region of (1→6)-β-glucan localization (Cabib et al., 1982).

Efficient in vitro synthesis of chitin and linear (1→3)-β-glucan has been observed with membrane preparations and UDP-charged substrates (Kang and Cabib, 1986; Cabib et al., 1982), but an analogous system for yeast (1→6)-β-glucan or branched (1→3)-β-glucan synthesis has not yet been achieved. Although it is not known if Krelp functions directly in (1→6)-β-glucan biosynthesis, that Krelp is targeted to the yeast secretory pathway and potentially localized on the cell surface supports this possibility. In accord with this idea, kinetic experiments after hyphal cell wall biosynthesis of Schizosaccharomyces pombe suggested that cell wall deposited (1→3)-β-glucan could be subsequently modified by attachment of (1→6)-β-linked branches (Sietsma et al., 1985). Multiple copies of the KREI gene under the control of the ADH1 promoter did not lead to the overproduction of (1→6)-β-glucan, but this does not rule out the possibility of a glucan synthase or transferase function. For instance, the gene products required for the synthesis of an acceptor glucan could be rate limiting for (1→6)-β-glucan biosynthesis (Glazebrook and Walker, 1989). Indirect mechanisms may lead to the observed phenotypes of krel mutants. For example, each of the krel mutants could be required for preservation rather than synthesis of yeast (1→6)-β-glucan. Krelp could then function as an inhibitor of a putative cell wall glucanase, with an activity towards linear (1→6)-β-glucan, resulting in partial degradation of the polymer. Several glucanase activities have been reported to occur in S. cerevisiae, but their functions are unknown (Kuranda and Robbins, 1987).

Cell wall (1→6)-β-glucan has been reported to occur among species from taxonomically diverse genera of yeasts including Candida albicans (Manners et al., 1974). C. albicans is of particular interest because of its dimorphic nature and pathogenicity. Glucan accounts for 50–70% of the C. albicans cell wall and appears to function as the main structural component of both the yeast and mycelial forms (Fleet, 1985). As was observed for S. cerevisiae most of the cell wall glucan was isolated from whole cells as an alkali insoluble fraction which was found to contain two glucan subclasses. One glucan subclass closely resembled the S. cerevisiae (1→6)-β-glucan and while the other was found to contain relatively more (1→3)-linked glucopyranosyl residues, both types of glucan appear to be highly branched and composed predominantly of (1→6)-linked residues (Gopal et al., 1984). We have recently isolated a DNA fragment from the C. albicans genome capable of complementing the krel-- phenotype of an S. cerevisiae krel mutant. It is likely that C. albicans homologues of the S. cerevisiae KRE genes described here, have a similar function in the production or assembly of the C. albicans cell wall. However, the greater abundance of (1→6)-linked residues in the total cell wall glucan of C. albicans may imply that KRE homologues are associated with additional structural or morphological roles in this fungus. Parity because of the functional similarity of gene products required for most eukaryotic cellular processes, it has been difficult to devise specific antifungal antibiotics. Identification of the synthetic machinery for components, like fungal cell wall β-glucans, that are absent in mammalian cells, should reveal proteins that are excellent potential targets for specific antifungal inhibitors.

We are grateful to Anne-Marie Sdcu and Josephine Wagner for providing superb assistance with many of the experiments presented here. We also appreciate the assistance provided by Marc Laroche, Seona Hanson, and Amy Glossbach with the genetic analysis. We thank Susan Welch and Vivian MacKay for help with the KREI-Substance P fusion studies, Elizabeth Mangeney for electron microscopy expertise, and Erica Boone for preparation of the manuscript. Antony Cooper, Ola Dmochowska, Kathryn Hill, Linda Hougan, Philip Meaden, Thierry Vernet, Malcolm Whiteway, and Hong Zhu are also thanked for thoughtful reading of the manuscript, providing experimental insights, and helpful discussions.

This work was supported by an Operating Grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada. C. Boone was an NSERC and Canadian Pacific scholar.

Received for publication 28 August 1989 and in revised form 21 December 1989.

References

Al-Aidroos, K., and H. Bussey. 1978. Chromosomal mutants of Saccharomyces cerevisiae affecting the cell wall binding site for killer factor. Can. J. Microbiol. 24:228–237.

Aloni, Y., D. P. Delmer, and M. Benziman. 1982. Achievement of high rates of in vitro synthesis of 1,4-β-D-glucan: activation by cooperative interaction of the Acetobacter xylinum enzyme system with GTP, polyethylene glycol, and a protein factor. Proc. Natl. Acad. Sci. USA. 79:6448–6452.

Badin, J., C. Jackson, and M. Schubert. 1953. Improved method for determination of plasma polysaccharides with tryptophan. Proc. Soc. Exp. Biol. Med. 84:288–291.

Balton, C. E. 1982. Yeast cell wall and cell surface. In The Molecular Biology of the Yeast Saccharomyces cerevisiae. Metabolism and Gene Expression. J. N. Strathern, E. W. Jones, and J. R. Broach, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 335–357.

Bassieux, D., D. Gagnaire, and M. Vignon. 1977. l~tude par R. M. N. -~3C et -~13C du (l-*6)-β-glucane et des oligosaccharides lin~aires et cycliques correspondants. Carbohydr. Res. 56:19–33.

Bruneteau, M., I. Fabre, J. Perret, and G. Michel. 1988. Antitumor active β-D-glucans from Phythophora parasitica. Carbohydr. Res. 175:137–143.

Bulawa, C. E., M. L. Slater, E. Cabib, J. Au-Young, A. Shubrati, W. L. Adair, and P. W. Robbins. 1986. The S. cerevisiae structural gene for chitin synthase is not required for chitin synthesis in vivo. Cell. 46:213–225.
Bussey, H., D. Saville, K. Hutchins, and R. G. E. Palfree. 1979. Binding of yeast killer toxin to a cell wall receptor on sensitive *Saccharomyces cerevisiae*. Curr. Genet. 1:409-417.

Bussey, H., W. Sacks, D. Galley, and D. Saville. 1982. Yeast killer plasmid mutations affecting toxin secretion and activity and toxin immunity function. *Mol. Cell. Biol.* 2:346-354.

Bussey, H., G. O. Szniterski, and D. Saville. 1983. Protein secretion in yeast: two chromosomal mutants that oversecrete killer toxin in *Saccharomyces cerevisiae*. *Curr. Genet.* 7:449-456.

Cabib, E., W. R. Roberts, and B. Bowers. 1982. Synthesis of the yeast cell wall by *Saccharomyces cerevisiae*. *Curr. Genet.* 7:2233-2240.

Davis, R. W., M. Thomas, J. Cameron, T. St. John, S. Scherer, and R. A. Sherman. 1982. Yeast killer plasmid mutations affecting toxin secretion and activity and toxin immunity function. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:657-680.

Dmochowska, A., D. Dignard, D. Henning, D. Thomas, and H. Bussey. 1987. Yeast KEX1 gene encodes a putative protease with a carboxypeptidase B-like function involved in killer toxin and a-factor precursor processing. *Cell* 50:573-584.

Doddrell, D. M., D. T. Pegg, and M. R. Bendall. 1982. Distortionless enhancement of NMR signals by polarization transfer. *J. Magn. Reson.* 48:323-327.

Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28:350-356.

Fleet, G. H., and H. J. Phaff. 1981. Fusarium-glucans-structure and metabolism. In *Encyclopedia of Plant Physiology*. New series. Vol. 13b. Plant Carbohydrates. (R. K. Ting, editor. Elsevier Science Pub. Amsterdam, New York, Oxford. 287-295.

Fleet, G. H., and D. J. Manners. 1976. Isolation and composition of an alkali-soluble glucan from the cell walls of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* 94:180-192.

Frevert, J., and C. E. Ballou. 1985. *Saccharomyces cerevisiae* structural cell wall mannoprotein. *Biochemistry.* 24:53-759.

Gleazerbrook, J., and G. C. Walker. 1989. A novel exopolysaccharide can function in place of the calcitonin-binding exopolysaccharide in nodulation of *Medicago truncatula*. *J. Biol. Chem.* 264:561-672.

Gopal, P. K., M. G. Shepard, and P. A. Sullivan. 1984. Analysis of wall glucans from yeast, hyphal and germ-tube forming cells of *Candida albicans*. *J. Gen. Microbiol.* 130:3295-3301.

Hutchins, K. T. 1982. Yeast cell wall receptor for killer toxin. MSc. thesis. Department of Biology, McGill University, Montreal, Canada. 46-50.

Ito, H., Y. Fukuda, M. Murata, and A. Kimura. 1983. Transformations of interest phenotype and its regulation. *Proc. Natl. Acad. Sci. USA.* 80:3770-3774.

Kuranda, M. J., and P. W. Robbins. 1987. Cloning and heterologous expression of the *Saccharomyces cerevisiae* ARD1 gene which encodes a putative protease with a carboxypeptidase B-like activity. *Cell* 52:915-924.

Lasky, R. D., and C. E. Ballou. 1982. Cell-cell recognition in yeast: isolation of intact α-aggutinin from *Saccharomyces kluveri*. *Proc. Natl. Acad. Sci. USA.* 85:5806-5812.

Manners, D. J., A. J. Masson, and J. C. Patterson. 1973a. The structure of α-(1→6)-d-glucan from yeast cell walls. *Biochem. J.* 153:19-30.

Manners, D. J., A. J. Masson, and J. C. Patterson. 1973b. The structure of a β-(1→6)-d-glucan from yeast cell walls. *Biochem. J.* 135:31-36.

Manners, D. J., A. J. Masson, and J. C. Patterson. 1974. The heterogeneity of glucans preparations from the walls of various yeast. *J. Gen. Microbiol.* 94:571-583.

Mortimer, R. K., and D. Schild. 1985. Genetic map of *Saccharomyces cerevisiae*, edition 9. *Microbiol. Rev.* 49:181-212.

Meyhak, B., W. Bajuja, H. Rudolph, and A. Hinnen. 1982. Two yeast acid phosphatase structural genes. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:657-680.

Munro, S., and H. R. B. Pelham. 1984. Use of peptide tagging to detect proteins expressed from cloned genes: deletion mapping functional domains of *Drosophila* hsp70. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3087-3093.

Redmond, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208-212.

Rudikoff, S., P. S. Sonmer, and R. W. Wickner. 1984. Superkiller mutations in *Saccharomyces cerevisiae* suppresses exclusion of M2 double-stranded RNA by L-A-MN and confer cold sensitivity in the presence of M and L-A-HN. *Mol. Cell. Biol.* 4:761-770.

Rosen, M. D., F. Novick, J. H. Thomas, D. Botstein, and G. R. Fink. 1987. *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene (Amst.).* 60:237-243.

Rothstein, R. J. 1983. One step gene disruption in yeast. *Methods Enzymol.* 101:202-211.