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Cloning of the *Saccharomyces cerevisiae* Gene Whose Overexpression Overcomes the Effects of HM-1 Killer Toxin, Which Inhibits β-Glucan Synthesis

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A gene whose overexpression can endow *Saccharomyces cerevisiae* cells with resistance to HM-1 killer toxin was cloned from an *S. cerevisiae* genomic library. This gene, designated *HKR1* (*Hansenula mrakii* killer toxin-resistant gene *1*), contains a 5.4-kb open reading frame. The predicted amino acid sequence of the protein specified by *HKR1* indicates that the protein consists of 1,802 amino acids and is very rich in serine and threonine, which could serve as O-glycosylation sites. The protein also contains two hydrophobic domains at the N-terminal end and in the C-terminal half, which could function as a signal peptide and transmembrane domain, respectively. Hkr1p is found to contain an EF hand motif of the calcium-binding consensus sequence in the C-terminal cytoplasmic domain. Thus, Hkr1p is expected to be a calcium-binding, glycosylated type I membrane protein. Southern and Northern (RNA) analyses demonstrated that there is a single copy of the *HKR1* gene in the *S. cerevisiae* genome, and the transcriptional level of *HKR1* is extremely low. Gene disruption followed by tetrad analysis showed that *HKR1* is an essential gene. Overexpression of the truncated *HKR1* encoding the C-terminal half of Hkr1p made the cells more resistant to HM-1 killer toxin than the full-length *HKR1* did, demonstrating that the C-terminal half of Hkr1p is essential for overcoming the effect of HM-1 killer toxin. Furthermore, overexpression of *HKR1* increased the β-glucan content in the cell wall without affecting in vitro β-glucan synthase activity, suggesting that *HKR1* regulates β-glucan synthesis in vivo.

The yeast *Hansenula mrakii* secretes a protein with a small molecular mass (10.7 kDa) which kills *Saccharomyces cerevisiae* and other sensitive strains of yeasts. This protein, designated HM-1 killer toxin, consists of 88 amino acids, of which 10 amino acids are cysteine. HM-1 killer toxin is stable in a wide range of pHs (pH 2 to 11) and is heat labile but is sensitive to proteinases and reducing reagents (30). It has also been demonstrated that HM-1 toxin inhibited β-1,3-glucan synthesis in vivo when applied to yeast culture, whereas the synthesis of other cell wall components, such as chitin, mannan, and alkali-soluble glucan, was unaffected (31). In contrast to β-1,3-glucan synthesis, DNA, RNA, protein, and lipid syntheses were apparently not inhibited during the early period of toxin treatment (30). These results implied that HM-1 killer toxin specifically inhibits β-1,3-glucan synthesis and kills the targeted cells by an as yet unknown mechanism. Despite the strong cytocidal effect on *S. cerevisiae* cells which occurs at about 1 μg/ml, HM-1 toxin was weakly inhibitory to β-1,3-glucan synthase activity in the membrane fraction of *S. cerevisiae*; a higher concentration of HM-1 toxin (10 to 100 μg/ml) was needed to inhibit β-1,3-glucan synthase (30).

Concerning β-1,3-glucan synthesis, neither the enzyme(s) nor its gene has been isolated. The enzyme activity of β-1,3-glucan synthesis was detected in crude membrane fractions of *S. cerevisiae* cells and other fungal cells (14, 26), indicating that the enzyme exists in the membrane. It has also been demonstrated that ATP or GTP was required for enzyme activity (20, 27). Since its substrate (UDP-glucose) and activators (GTP and ATP) are available only in the cytoplasm, the active site of the enzyme would be in the cytoplasmic domain, and the nascent glucan may be transported to the cell wall, perhaps through a channel-like enzyme complex. In this context, Kang and Cabib (14) reported that glucan synthesis of *Hansenula anomala* and *Neurospora crassa* consisted of at least two components, a membrane-bound catalytic component and a cytosolic regulatory component, of which the latter could be solubilized by salt and detergent and may represent an affinity for GTP and ATP.

Beside β-1,3-glucan, there is another type of glucan polymer classified as β-1,6-glucan. β-1,6-Glucan accounts for only a small percentage of total β-glucan, while the ratio of β-1,3- and β-1,6-glucan varies among yeast species and under different growth conditions and stages (11). The synthesis of β-1,6-glucan apparently occurs on the long β-1,3-glucan chain via β-1,6-linkage of glucose, which gives rise to a cell wall glucan network (11). Although the mechanism underlying β-1,6-glucan synthesis and its regulation have not been fully understood, Bussey's group has succeeded in isolating various *S. cerevisiae* mutants defective in β-1,6-glucan synthesis. These mutants, designated *kre* mutants, were obtained by screening the *S. cerevisiae* cells that survived in the presence of K1 killer toxin, whose receptor has been identified as cell wall β-1,6-glucan (8, 12). The *kre* mutants which lack the ability to synthesize the normal level of β-1,6-glucan (and therefore acquired a K1 toxin-resistant phenotype) were used for cloning the genes involved in β-1,6-glucan synthesis. Genes whose introduction into *kre* mutants led to the normal level of β-1,6-glucan synthesis and to the loss of resistance to K1 killer toxin were elegantly cloned and characterized (4–7, 12, 19, 22).

In an attempt to clarify the mechanism of action of HM-1

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and to clone β-1,3-glucan synthesis-associated genes, we have used an expression cloning strategy with HM-1 killer toxin. Here we report the cloning of a gene whose overexpression gave rise to an HM-1 killer toxin-resistant phenotype in *S. cerevisiae*. A possible involvement of this gene product in β-1,3-glucan synthesis is discussed.

MATERIALS AND METHODS

Purification of HM-1 killer toxin. HM-1 killer toxin was purified from the culture medium of *H. mrakii* (IFO0895) by the method of Yamamoto et al. (31) with some modifications. A portion (1/100 volume) of the culture of *H. mrakii* grown overnight was inoculated in several liters of minimal medium containing 0.67% yeast nitrogen base (Difco) and 0.5% glucose. After 30 h, the culture of *H. mrakii* was centrifuged at 4,500 rpm for 10 min with a Kontron A6.9 rotor to remove cells. The supernatant was filtered through a cellulose acetate filter (pore size, 0.45 μm; Corning) to remove cell debris and aggregates. Filtered medium was concentrated by ultrafiltration with Filtron Omega Minisette (nominal molecular mass limit, 3 kDa; Filtron) and then with a YM2 filter (Amicon). HM-1 killer toxin was purified by Sephadex G-50 column chromatography. Several milliliters of the concentrated medium was applied to a Sephadex G-50 column and eluted with 50 mM NaH_2PO_4. Fractions containing HM-1 were combined, and HM-1 was further purified by high-pressure liquid chromatography. The partially purified toxin was loaded on an SP column (SP-2SW; 4.6 mm by 25 cm; Tosoh) and eluted with 25 mM sodium phosphate buffer (pH 5.8) with a linear gradient of 0 to 0.5 M NaCl. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and subsequent staining of the proteins with Coomassie brilliant blue revealed that the purified HM-1 was homogenous, and no visible contamination was detected.

The activity of HM-1 was determined on the basis of the growth inhibition of *S. cerevisiae* cells (strain A451) in synthetic medium containing glucose (SG) (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose) supplemented with required amino acids, 20 μg of uracil per ml, and 40 μg of adenine sulfate per ml. Part (1/100 volume) of a culture of *S. cerevisiae* cells grown overnight was inoculated into several milliliters of SG containing various concentrations of HM-1 killer toxin. After inoculation, cell growth was monitored by measuring A_{600} as a function of time.

Construction of a *S. cerevisiae* genomic DNA library. Wild-type *S. cerevisiae* (strain YNN295) genomic DNA purchased from Clontech was partially digested with *Sau*3AI and fractionated on a 0.5% agarose gel. The DNA fragments between 4 and 8 kb long were eluted electrophoretically from the gel (25) and purified with an Elutip-D column (Schleicher & Schuell). The vector used for constructing the library was YEp213, a derivative of YEp13, which carries a 2μm replication origin and *LEU2* gene as a genetic marker (1, 23). *S. cerevisiae* genomic DNA, partially digested with *Sau*3AI and ligated to the *Bam*HI cleavage site of YEp213, and the resulting plasmids were transfected to *Escherichia coli* DH5 competent cells. Plasmid DNA was extracted from the transformed *E. coli* cells through cesium chloride gradient centrifugation as described elsewhere (25).

DNA transfection and screening. *S. cerevisiae* cells (strain A451 *MATα can1 leu2 trp1 ura3 aro7*) were transfected with plasmid DNA by the lithium acetate method (13). For selecting the HM-1-resistant clones, cells were transfected with the genomic DNA library, and were seeded on SG agar plates that lacked leucine but contained 0.7 μg of HM-1 per ml. After incubation at 30°C for 3 to 4 days, plasmid DNA was recovered from the colonies growing on the HM-1-containing plates by lysing the cells with glass beads, extracting with phenol, and precipitating with ethanol (17). The insert DNA was excised by digesting the recovered plasmid DNA with various restriction endonucleases and subcloned in pUC18 and pUC19 vectors. DNA sequencing was performed with Sequenase version 2 kit (USB) and [α-^{35}S]dCTP (NEN).

For obtaining the full-length gene, *S. cerevisiae* genomic DNA was digested with *Bam*HI, and resulting DNA fragments between 5.5 and 8 kb long were purified, ligated at the *Bam*HI cleavage site of the pUC18 vector, transfected into *E. coli* DH5, and screened by colony hybridization with a radiolabelled 1-kb *Hin*dIII-*Bam*HI fragment of the cloned gene. Hybridization and washing of the filters were carried out under stringent conditions (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–1× Denhardt's solution–20 mM sodium phosphate buffer [pH 6.5]–0.1% SDS–50% formamide at 42°C for hybridization; 0.1× SSC–0.1% SDS at 60°C for washing) (25).

Southern and Northern (RNA) blotting. For the preparation of genomic DNA, *S. cerevisiae* cells were treated with Zymolyase 20T and the resulting spheroplasts were lysed by adding SDS to the cell suspension to give a final concentration of 0.1% (17). Cell debris was removed by centrifugation after the addition of potassium acetate. DNA was precipitated with 2-propanol, treated with pancreatic RNase, and digested with endonucleases as indicated. RNA was extracted from growing *S. cerevisiae* cells by lysing the cells with glass beads in the presence of SDS followed by phenol extraction and ethanol precipitation (25). Poly(A)$^+$ RNA was purified from total RNA with Oligotex-$(dT)_{30}$ as described previously (16). Endonuclease-digested DNA and poly(A)$^+$ RNA were fractionated by agarose gel electrophoresis, transferred to nylon membranes, hybridized with radiolabelled probes, and visualized by autoradiography. Radiolabelling of DNA probes was carried out by random-priming methods with [α-^{32}P]dCTP (25). Conditions for hybridization and washing of the filters were identical to those described for colony hybridization.

Overexpression of *HKR1*. In order to overexpress *HKR1*, a 5.9-kb *Tth*111I-*Hin*dIII fragment or a 2.6-kb *Hin*dIII-*Hin*dIII fragment which contains the full-length *HKR1* gene and part of the gene encoding the C-terminal part of Hkr1p, respectively, were ligated at the *Bgl*II cleavage site of pMT34-317, a derivative of pMT34 (28). These plasmids were designated pMT-*HKR* and pMT-*HKR*^{tr} (tr for truncated), respectively. In these plasmids, *HKR1* gene transcription was under control of the *GAL7* promoter. After transformation of *S. cerevisiae* A451 cells with these plasmid DNAs, uracil prototrophs were collected, analyzed, and used for the experiments. Induction of *HKR1* gene expression was carried out as described previously (28). Part (1/100 volume) of a culture of the cells grown overnight in glucose-containing medium was inoculated into synthetic medium containing 2% galactose, and the cells were further incubated at 30°C.

Disruption of the *HKR1* gene. The strains carrying null mutations of *HKR1* were generated by single-step gene disruption (24). A plasmid required for homologous recombination was constructed by replacing the *Kpn*I-*Xba*I region of *HKR1* with the *S. cerevisiae LEU2* gene. The chimeric *HKR1-LEU2* gene was then excised from the plasmid vector and used to transform a diploid *S. cerevisiae* strain (RAY3A-D **a**/α *ura3/ura3 leu2/leu2 his3/his3 trp1/trp1*). To confirm that the disrupted copy of *HKR1* had been integrated at the expected chromosomal locus in the diploid strain, genomic DNA was isolated from several leucine prototrophs and then digested

with *Eco*RV and *Hin*dIII followed by Southern blot analysis with a 0.9-kb *Xba*I-*Hin*dIII fragment of *HKR1* as a probe, which would give rise to a 5.7-kb normal *HKR1* allele and a 3.2-kb *HKR1-LEU2* chimeric allele.

Preparation of cell wall glucan. Cell wall polysaccharides were fractionated from *S. cerevisiae* A451 cells transformed with pMT34-317, pMT-*HKR*^{tr}, or pMT-*HKR* by the methods of Peat et al. (21) and Manners et al. (18) with some modifications. Lyophilized *S. cerevisiae* cells grown to late logarithmic phase in synthetic medium containing galactose and lacking uracil were autoclaved for 90 min at 120°C. The insoluble residues were collected by centrifugation and extracted four times with 1.0 N NaOH containing 0.5% $NaBH_4$ for 24 h at 30°C with gentle shaking. After centrifugation, the supernatant fractions containing alkali-soluble glucans were neutralized with acetic acid, dialyzed against H_2O, and lyophilized. The precipitates were also neutralized and extracted five times with 0.5 M acetic acid at 90°C for 90 min. The acid-insoluble glucans were centrifuged, dialyzed against H_2O, and lyophilized. The carbohydrate content of each fraction was estimated by the phenol-sulfuric acid method by using glucose as a standard (10).

RESULTS

Cloning of a gene with multicopy suppression activity of HM-1 killer toxin. In an attempt to clone *S. cerevisiae* genes involved in β-1,3-glucan synthesis, we employed an expression cloning approach using HM-1 killer toxin, which has been implicated as an inhibitor of β-1,3-glucan synthesis. Overproduction of the protein which might bind to toxin was anticipated to protect the cells from the lethal effect of HM-1 killer toxin. To this end, the minimal concentration of HM-1 required for its lethal effects on *S. cerevisiae* A451 was first examined. After serial dilutions of purified HM-1 toxin and inclusion in the *S. cerevisiae* proliferation assay, the threshold of the lethal concentration was estimated to be 0.5 μg/ml in agar plates. To ensure the growth arrest of the untransfected cells, a slightly higher concentration (0.7 μg/ml) of HM-1 was used for the screening. A genomic DNA library was constructed with a YEp213 vector which had a 2μm replication origin. The library contained partially *Sau*3AI-digested genomic DNA that was between 4 and 8 kb long. *S. cerevisiae* A451 cells were transformed with this library, and the transformants were then seeded on the leucine-depleted SG agar plates which contained the lethal dose (0.7 μg/ml) of the purified HM-1 killer toxin. Of 10^5 transformants, 10 colonies appeared after 4 days of incubation at 30°C. Cells from these colonies were grown in SG lacking leucine and were subjected to a secondary screening. Of 10 clones, 6 showed healthy growth, even in the presence of 0.7 μg of HM-1 killer toxin per ml. Plasmid DNA was extracted from cells derived from the six clones, and a restriction enzyme map of the insert DNA was determined. The size (7.3 kb) and restriction map of the insert DNA was found to be identical among the six clones, indicating that all of the HM-1-resistant clones contained the same genomic DNA fragment. Since the introduction of vector plasmid (YEp213) alone did not alter the sensitivity of the cells to the toxin, it was evident that this DNA fragment was essential for resistance to HM-1 killer toxin (Fig. 1A). Next, we have analyzed the essential region of the insert DNA fragment for the HM-1-resistant phenotype. Transformation of *S. cerevisiae* cells with DNA fragments which had been digested with various endonucleases revealed that the 2.6-kb *Hin*dIII-*Hin*dIII region located near the 5′ end of the 7.3-kb DNA fragment was sufficient to make the cells resistant to 0.7 μg of HM-1 per ml (Fig. 1A). A more detailed study indicated that the A451 cells harboring the 2.6-kb *Hin*dIII-*Hin*dIII fragment were resistant up to 10 μg of HM-1 killer toxin per ml (data not shown).

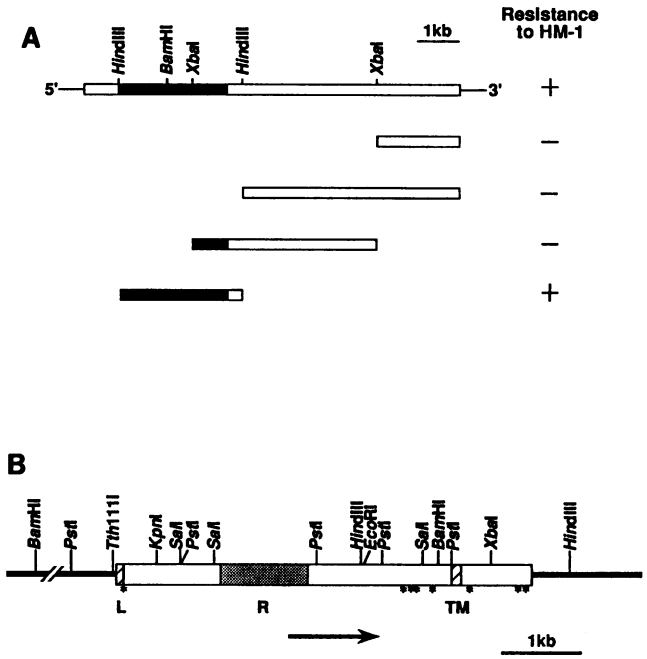

FIG. 1. Restriction map of *HKR1* and the essential region for resistance to HM-1. (A) Plasmid DNA was isolated from the HM-1-resistant cells, and the insert DNA was digested with various endonucleases. The resulting DNA fragments were subcloned into the YEp213 vector, transfected to A451 cells, and tested for the ability to overcome growth inhibition by 0.7 μg of HM-1 per ml. The solid box indicates an open reading frame found in this DNA fragment. The open box represents the coding region of *HKR1*. (B) Predicted structure of Hkr1p. R, repetitive sequence; L, possible leader sequence; TM, transmembrane domain. Asterisks indicate potential N glycosylation sites.

The sequencing of this 2.6-kb *Hin*dIII-*Hin*dIII fragment demonstrated that there was an open reading frame which was capable of coding for a protein with an approximate molecular mass of 73 kDa. We designated this gene *HKR1* (*Hansenula* killer toxin-resistant gene *1*). Despite its ability to rescue *S. cerevisiae* cells from 10 μg of HM-1 killer toxin per ml, the 2.6-kb *Hin*dIII-*Hin*dIII fragment seemed to be part of the gene, because the open reading frame could still continue to the 5′ end of the insert DNA. Furthermore, there was no typical promoter sequence, such as a TATA box in the 5′ sequence upstream from the first methionine codon. These observations strongly suggested that the 2.6-kb *Hin*dIII-*Hin*dIII fragment was part of the *HKR1* gene. To address this possibility, we carried out Northern blotting to determine the length of the endogenous mRNA for this gene. Cells transformed with YEp213 carrying the 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* expressed mRNA of the expected size at a high level (Fig. 2, lane 1), whereas mRNA of the same size was not detected in the cells transformed with the vector alone (Fig. 2). Instead, a larger (about 6-kb-long) mRNA was detected in the cells transformed with *HKR1* and with the vector alone. The level of expression of endogenous *HKR1* was very low; the 6-kb transcript was visible only when more than 10 μg of poly(A)$^+$ RNA was subjected to Northern blotting

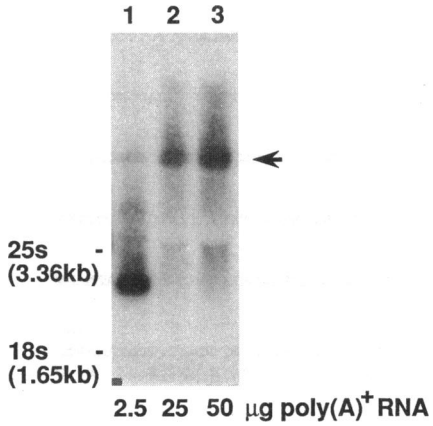

FIG. 2. Northern blot analysis of *HKR1* mRNA. The indicated amount of poly(A)$^+$ RNA was isolated from cells carrying the 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* in YEp213 (lane 1) or YEp213 alone (lanes 2 and 3), fractionated on an agarose gel, transferred to a nylon membrane, hybridized with ^{32}P-labelled probe, and visualized by autoradiography. The positions for 25S and 18S rRNA are indicated. The arrow indicates the position of the endogenous *HKR1* mRNA.

(Fig. 2, lane 2 and 3). From these results, we concluded that the 2.6-kb *Hin*dIII-*Hin*dIII fragment was part of the *HKR1* gene and that the full gene might be about 6 kb long. Transcription of the 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* might have started from promoter-like sequences in the YEp213 vector.

Southern blot analysis with genomic DNA revealed that there was a single copy of the *HKR1* gene in the genome and that there was a *Bam*HI site about 6.5 kb 5′ upstream from the *Bam*HI site located near the 5′ end of the cloned DNA fragment (Fig. 3). In order to clone the missing 5′ part of *HKR1*, *S. cerevisiae* genomic DNA was digested with *Bam*HI, and the resulting DNA fragments between 5.5 to 8 kb long

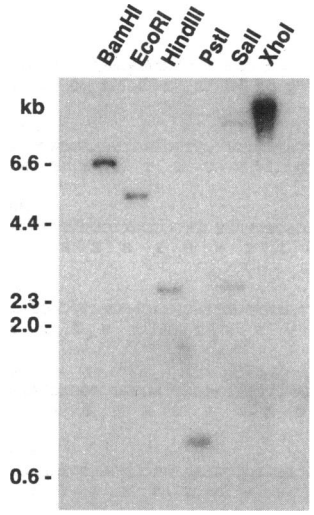

FIG. 3. Genomic Southern blot analysis of *HKR1*. Genomic DNA (5 μg) was digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, and *Xho*I, fractionated on an agarose gel, transferred to a nylon membrane, hybridized with ^{32}P-labelled probe, and visualized by autoradiography. The positions of size markers are indicated.

were purified from the gel, subcloned into pUC19 vector, and screened with radiolabelled *HKR1* probe. Of 10^4 clones, we obtained two clones which strongly hybridized to the probe. Restriction maps of these two clones were identical. Sequencing of the full-length *HKR1* gene demonstrated that there was a 5.4-kb open reading frame which could encode a protein with an approximate molecular mass of 189 kDa (1,802 amino acids) (Fig. 1B and 4).

The predicted amino acid sequence of the protein specified by *HKR1* (Hkr1p) showed two hydrophobic domains at the N terminus (21 amino acids) and in the C-terminal half (26 amino acids) (Fig. 1B, 4, and 5). According to Von Heijne's criteria (29), it was predicted that the N-terminal 21 amino acids could serve as a signal peptide. The hydrophobic domain in the C-terminal half could be a membrane-spanning domain, since this domain can form seven turns of α-helical structure. Another noteworthy feature of Hkr1p was that this protein was very rich in serine and threonine. Over 30% of the total amino acids were serine and threonine, which might serve as acceptors for O-linked glycosylation. In addition to the possible O-linked glycosylation, there were eight potential N-linked glycosylation sites (Fig. 1B). Repetitive sequences were found in the middle of the protein; there were 12 repeats of the 28 amino acids (Ser-Ala-Pro-Val-Ala-Val-Ser-Ser-Thr-Tyr-Thr-Ser-Ser-Pro-Ser-Ala-Pro-Ala-Ala-Ile-Ser-Ser-Thr-Tyr-Thr-Ser-Ser-Pro) (Fig. 4). Although there has been no gene or protein reported so far whose sequence was identical to that of *HKR1* or Hkr1p, *HKR1* had a significant sequence similarity to *MSB2*, a multicopy suppressor gene of *S. cerevisiae cdc24* (2, 3). Hkr1p shared 33% sequence identity and 45% sequence similarity with Msb2p throughout the proteins. Structural resemblance between Hkr1p and Msb2p was also observed; Msb2p, which consists of 1,306 amino acids, is very rich in serine and threonine and has a signal sequence at the N terminus, a hydrophobic transmembrane domain in the C-terminal half, and finally repetitive sequences in the middle of the protein (3).

Interestingly, Hkr1p had a sequence, Asp-Val-Asp-Glu-Asn-Gly-Asp-Ile-Arg-Leu-Tyr-Asp, starting at position 1645. This sequence strongly correlated with the EF hand motif of the calcium-binding site: Asp (or Asn)-Val-Asp (or Asn)-Glu-Asn (or Asp or Ser)-Gly-Asp-Ile (or Val)-Arg-Leu-Tyr-Asp (or Glu), in which the 1st Asp (or Asn), 3rd Asp (or Asn), 5th Asn (or Asp or Ser), 8th Ile (or Val), and 12th Asp (or Glu) are conserved (Fig. 6). This EF hand motif was originally identified in carp parvalbumin as the calcium-binding site (15) and has been found in several other calcium-binding proteins in a wide variety of organisms, including *S. cerevisiae* and higher eukaryotes.

Part of *HKR1* which could encode only a portion of the C-terminal half of Hkr1p is sufficient to overcome the effect of HM-1. As mentioned above, overexpression of the 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* which could encode part of the C-terminal half of Hkr1p was sufficient to evade the cytocidal effect of HM-1 (at least up to 10 μg/ml). We also overexpressed the full-length *HKR1* by using the *GAL7* promoter, because the entire *HKR1* gene could not be subcloned in YEp213 for unknown reasons. We succeeded in subcloning the full-length *HKR1* gene in pMT34-317, a vector carrying the *GAL7* promoter and the *URA3* gene as a selectable marker. Either the entire sequence or the 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* was inserted just downstream of the *GAL7* promoter (designated pMT-*HKR* and pMT-*HKR*^{tr}, respectively). The resulting plasmids were transfected into *S. cerevisiae* cells. Expression of *HKR1* was induced by culturing the Ura3$^+$ cells in the medium containing galactose instead of glucose. As

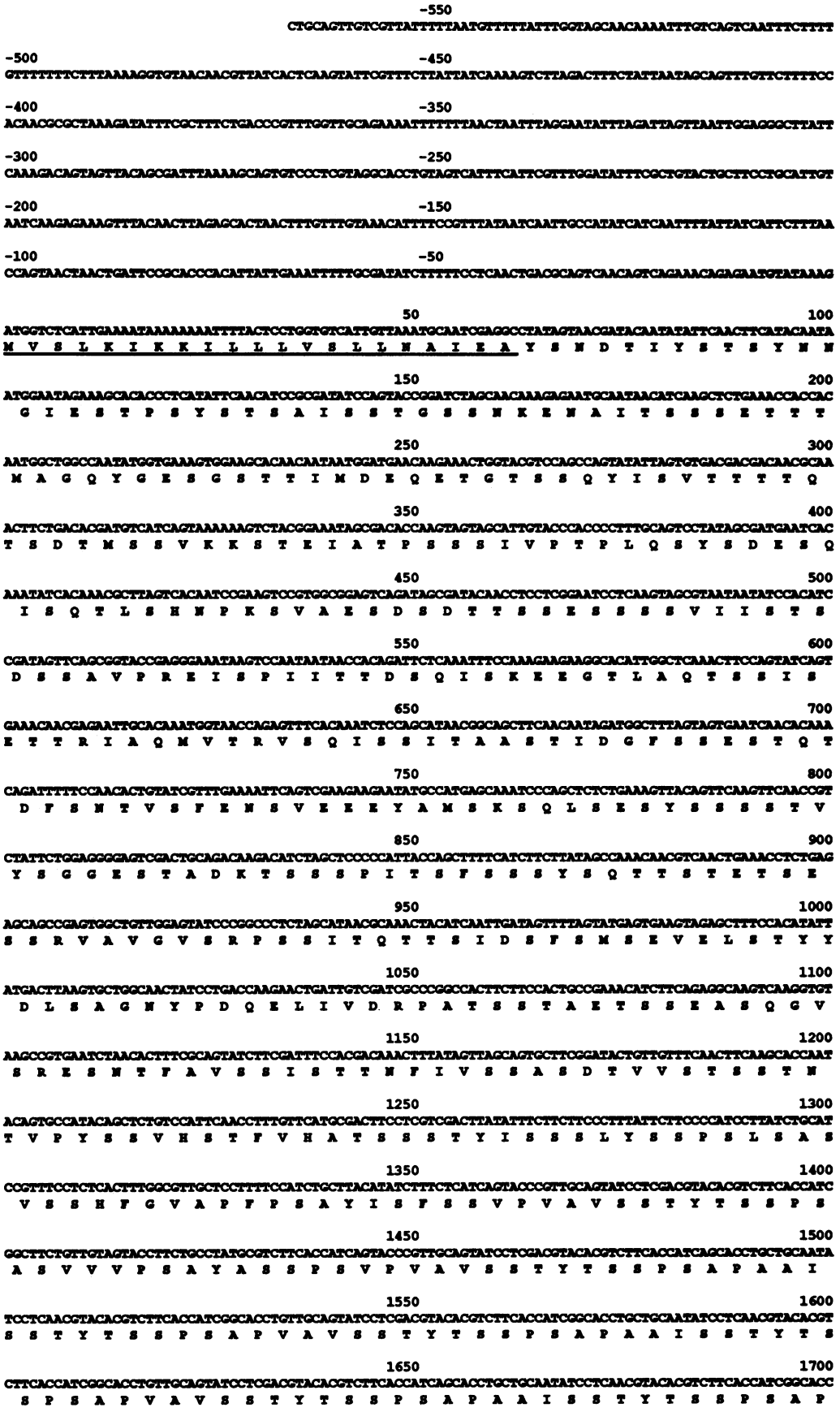

FIG. 4. Nucleotide sequence of *HKR1* and predicted amino acid sequence of Hkr1p. Possible leader sequence and transmembrane domain are indicated by underline and shaded box, respectively.

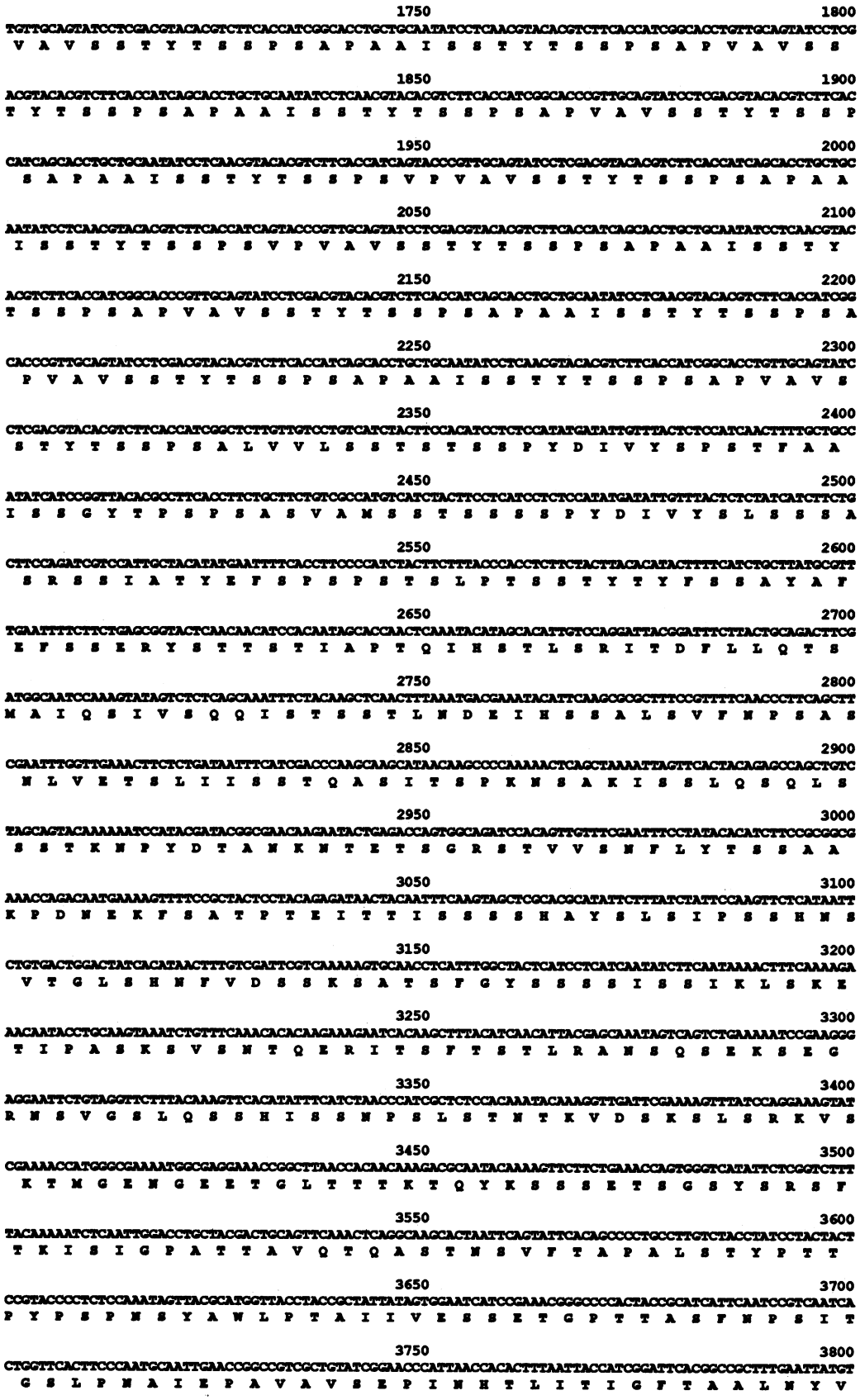

FIG. 4—*Continued.*

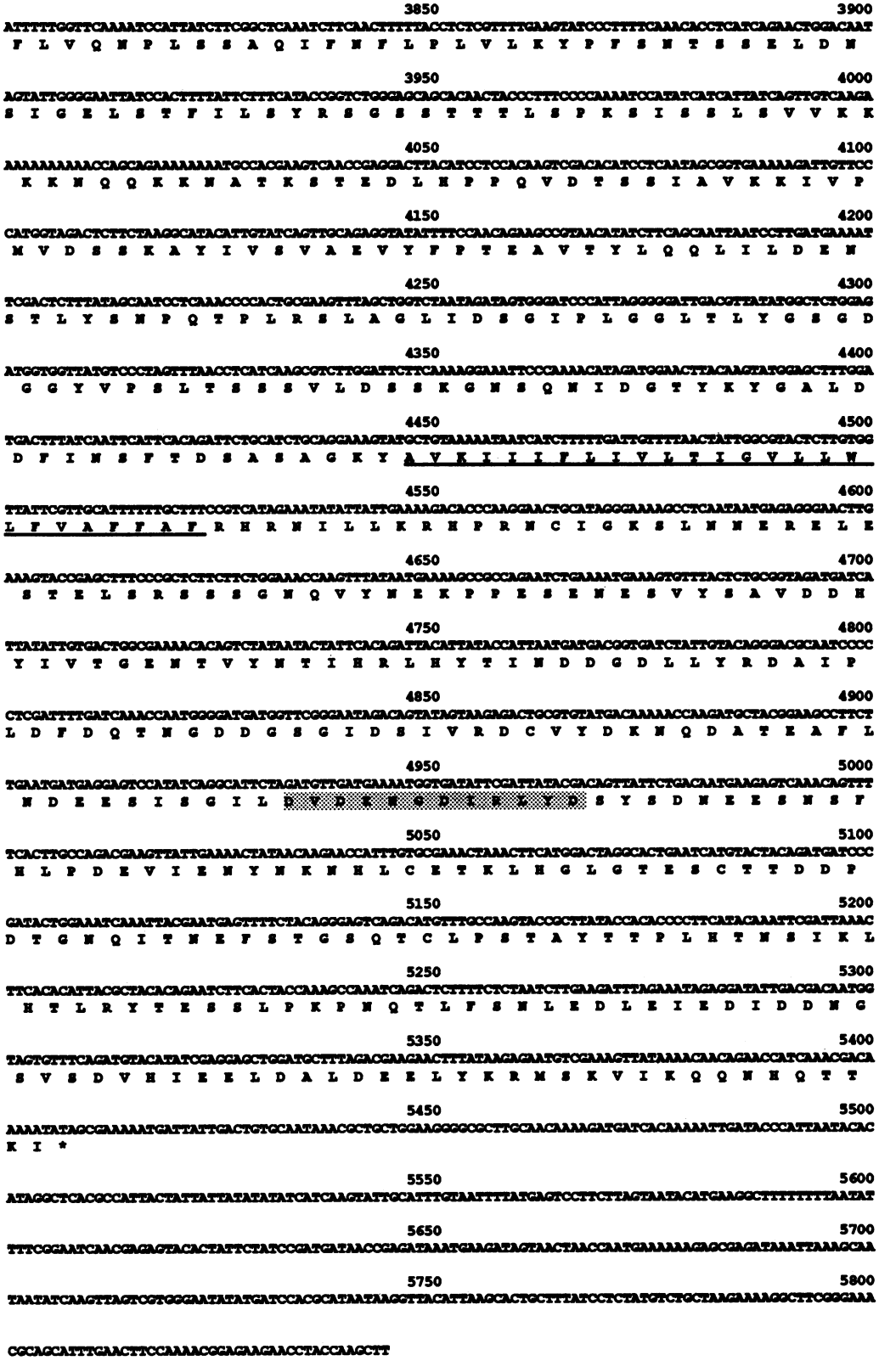

FIG. 4—*Continued.*

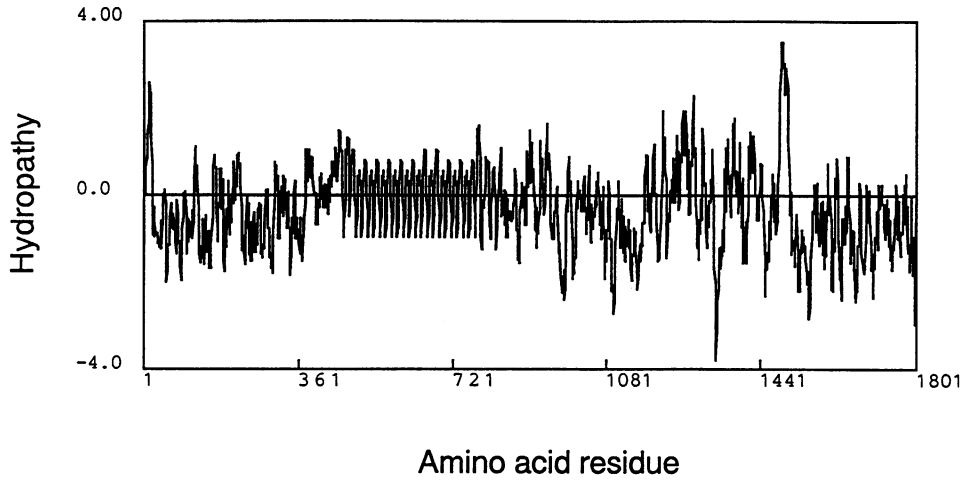

FIG. 5. Hydrophobic profile of Hkr1p. Hydropathy of Hkr1p was calculated by using Kyte and Doolittle parameters (16a) from the predicted amino acid sequence of the protein.

Consensus (EF-hand)		DXDXNGXIXXXD N N D V E S
Hkr1p	1645	DVDENGDIRLYD
Troponin C [human]	146	DKNNDGRIDYDE
Parvalbumin [human]	56	DKDKSGFIEEDE
Calmodulin [*P. hybrida*(plant)]	98	DKDQNGYISAAD
Calmodulin [*S. cerevisiae*]	99	DKNGDGLISAAE
Cdc31p [*S. cerevisiae*]	147	DLDGDGEINENE
H^+ pump [*S. cerevisiae*]	65	DSDNDGPVAAGE
Glucanase [*C. thermocellum*(bacterium)]	714	DVDGNGRINSTD

FIG. 6. Consensus sequence of the calcium-binding site (EF hand motif) in Hkr1p. The EF hand motif in several calcium-binding proteins and Hkr1p is shown. The amino acids indicated in boldface type are conserved, and X can be any amino acid. The number indicates the amino acid position of the first aspartic acid in this motif. *P. hybrida*, *Petunia hybrida*; *C. thermocellum*, *Clostridium thermocellum*.

shown in Fig. 7, culturing the cells in the galactose-containing medium resulted in more than 50-fold increases in the mRNA levels of both full-length and truncated *HKR1* compared with those of the cells cultured in glucose-containing medium. Then, the effect of HM-1 toxin on the *HKR1*-overexpressing cells was examined by monitoring the growth of these cells in the presence of HM-1. In galactose-containing medium, the cells harboring pMT-*HKR*^{tr} grew normally in the presence of 4 μg of HM-1 per ml (Fig. 8A). Overexpression of the full-length *HKR1* in galactose-containing medium delayed the onset of growth but resulted in normal growth of the cells in the presence of 2 μg of toxin per ml. Full-length *HKR1*, however, did not make the cells resistant to higher concentrations of the toxin; they grew poorly in the presence of 4 μg of HM-1 per ml

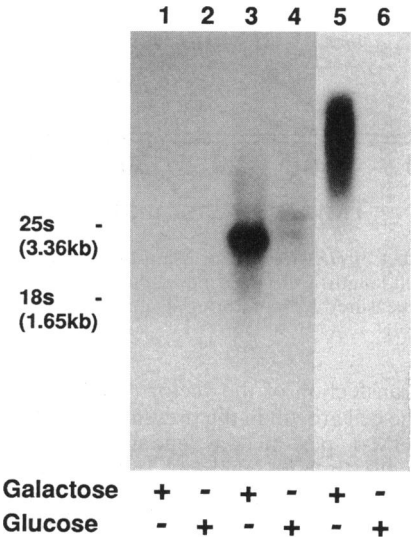

FIG. 7. Induction of *HKR1* gene expression by the *GAL7* promoter. Samples (2 μg) of poly(A)$^+$ RNA from cells carrying pMT34-317 (lanes 1 and 2), pMT-*HKR*^{tr} (lanes 3 and 4), or pMT-*HKR* (lane 5 and 6) which were cultured in galactose- or glucose-containing medium were fractionated on an agarose gel, transferred to a nylon membrane, hybridized with ^{32}P-labelled probe, and visualized by autoradiography. The positions of 25S and 18S rRNA are indicated.

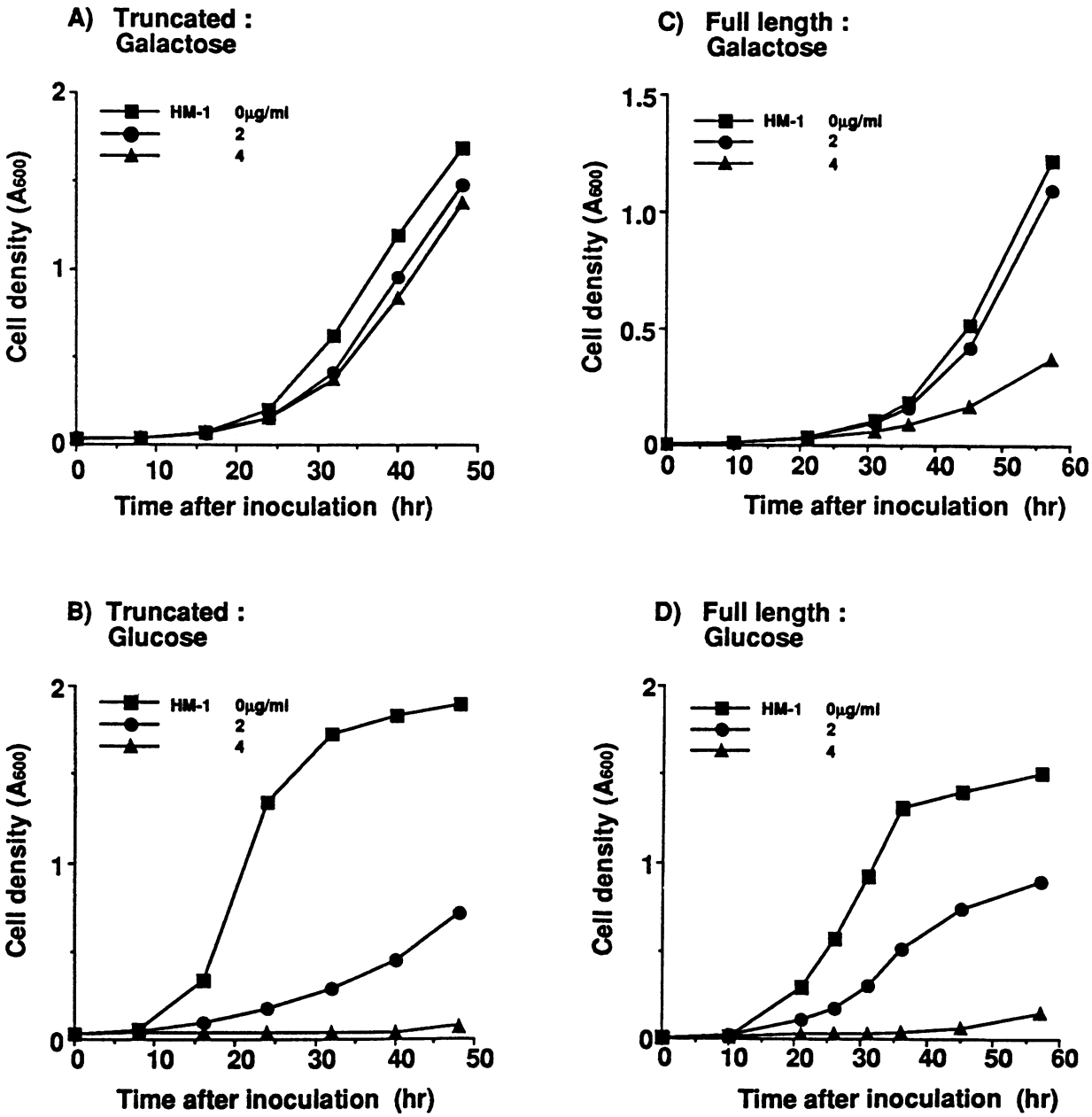

FIG. 8. Effect of *HKR1* overexpression on the growth of cells in the presence of HM-1. Cells transformed with pMT-*HKR*^{tr} (truncated) or pMT-*HKR* (full length) were cultured in glucose- or galactose-containing medium in the presence of the indicated amount of HM-1. Growth of the cells was measured by monitoring A_{600} at various times after inoculation.

(Fig. 8C). Transfection of the vector (pMT34-317) alone did not lead to the cell growth in the presence of HM-1 toxin at all; 0.7 μg of HM-1 per ml was enough to kill the vector-transfected cells in both glucose- and galactose-containing medium (data not shown). When cells were cultured in glucose-containing medium, only low levels of *HKR1* expression were detected, and under this condition, 2 μg of HM-1 per ml almost completely inhibited the growth of cells harboring pMT-*HKR* and pMT-*HKR*^{tr} (Fig. 8B and D). From these results, we concluded that *HKR1* causes the multicopy suppression of HM-1 killer toxin action in *S. cerevisiae* cells and that the C-terminal half of Hkr1p is essential for overcoming the killing effect of HM-1.

***HKR1* is an essential gene.** As described above, the *S. cerevisiae* genome contains a single copy of the *HKR1* gene (Fig. 3). This fact enabled us to examine whether *HKR1* is an essential gene. To address this question, we constructed a plasmid in which most of the coding region of *HKR1* (region between *Kpn*I and *Xba*I sites) was replaced with the *LEU2* gene (Fig. 9A). *S. cerevisiae* cells were transformed with this plasmid, and the *HKR1* disruptants were identified among Leu2$^+$ transformants by Southern blot hybridization with *HKR1* as a probe. Three independent disruptant clones were allowed to sporulate, and the viability of each spore was examined following tetrad dissection. We analyzed 20 asci in each three disruptant clones by tetrad dissection and found

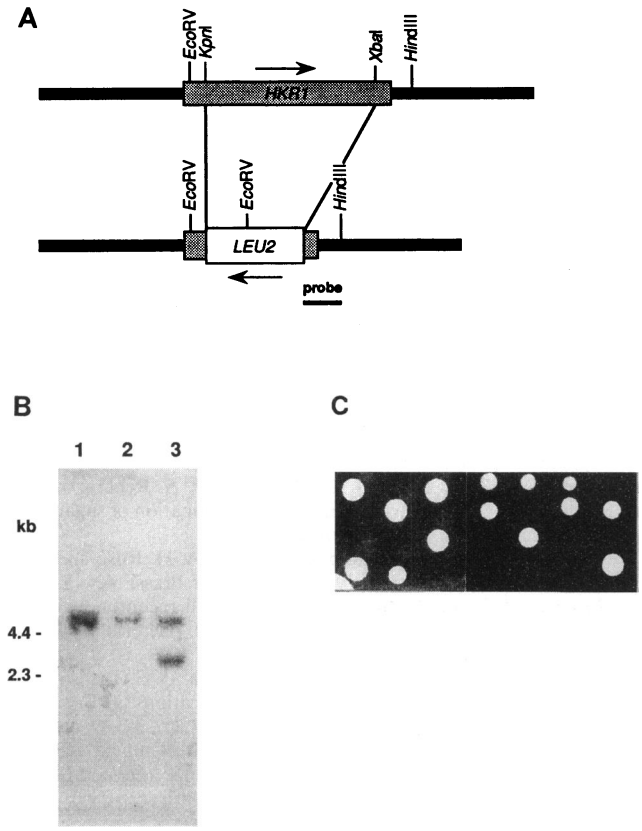

FIG. 9. Disruption of *HKR1* by homologous recombination and subsequent tetrad dissection. (A) The plasmid required for homologous recombination was constructed by replacing the *Kpn*I-*Xba*I region of *HKR1* with a *LEU2* cassette which was inserted in the direction opposite that of *HKR1*. Diploid RAY3A-D cells were transformed with the resulting *HKR1-LEU2* chimeric DNA, and several leucine prototrophs were allowed to sporulate. The probe used for the Southern blotting is indicated. (B) Southern blot analysis of DNA from parental RAY3A-D (lane 1), LEU2$^+$ transformants (lane 3), and haploid cells originated from a viable spore after tetrad dissection (lane 2). The positions of the size markers are indicated. The slower-migrating band corresponds to the normal *HKR1* allele, and the faster-migrating one is attributed to the disrupted allele. (C) Tetrad analysis of *HKR1* disruptants. RAY3A-D clones which were confirmed to contain the disrupted allele of *HKR1* were subjected to tetrad analysis. In most of the cases, only two of four spores were viable.

that only two of four spores were shown to be viable, even after 1 week of cultivation. Furthermore, cells that originated from viable spores were confirmed to contain only an intact allele of *HKR1* (Fig. 9B and C). All these results demonstrated that *HKR1* is an essential gene for the growth of *S. cerevisiae* cells.

Overexpression of *HKR1* increases the cell wall β-glucan content. Since HM-1 killer toxin specifically interferes with β-1,3-glucan synthesis of *S. cerevisiae* (30), it would be of interest to ask whether overexpression of *HKR1* affects β-1,3-glucan synthase activity. This was tested first by β-1,3-glucan synthase assay in vitro (9, 26) with membrane fractions prepared from the cells transformed with pMT-*HKR* or pMT-*HKR*^{tr}. Induction of *HKR1* expression by culturing the cells in the galactose-containing medium did not influence the in vitro β-1,3-glucan synthase activity (data not shown). Next, we examined whether *HKR1* is involved in β-glucan synthesis in

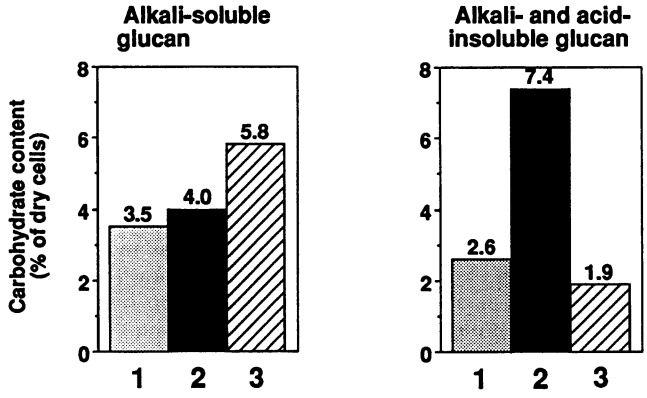

FIG. 10. Changes in β-glucan content by overexpression of *HKR1*. β-Glucan was fractionated from the cells transformed with pMT34-317 (bar 1), pMT-*HKR*^{tr} (bar 2), and pMT-*HKR* (bar 3), and carbohydorate contents in the alkali-soluble glucan fraction and in the alkali- and acid-insoluble glucan fractions were determined by the phenol-sulfuric acid method.

vivo. For this purpose, the β-glucan content of the cell wall was determined in cells overexpressing either the truncated or full-length *HKR1* gene. Comparison of the carbohydorate contents of *HKR1*-overexpressing cells and vector-transfected cells revealed that the overexpression of full-length *HKR1* resulted in about 50% increase in the alkali-soluble β-glucan content and that the alkali- and acid-insoluble β-glucan content was increased by about 2.5-fold by the overexpressed truncated *HKR1* (Fig. 10). Furthermore, most of the carbohydorates both in the alkali-soluble and in the alkali- and acid-insoluble glucan fractions were identified as β-1,3-glucan (not shown). These results demonstrated that the overexpression of either truncated or full-length *HKR1* increased the level of the cell wall β-1,3-glucan and suggest that *HKR1* is involved in β-1,3-glucan biosynthesis.

DISCUSSION

We have isolated a *S. cerevisiae* gene whose overexpression made *S. cerevisiae* cells resistant to HM-1 killer toxin. This gene, designated *HKR1*, encodes a high-molecular-weight protein (the calculated molecular mass is 189 kDa) that has the profile of a type I membrane protein. As mentioned in Results, *HKR1* shares a significant sequence similarity to *MSB2*, a multicopy suppressor of *cdc24* (3). Furthermore, both Hkr1p and Msb2p can code for high-molecular-weight proteins which are rich in serine and threonine and are structurally related; they have a signal sequence at the N terminus, possess a transmembrane domain in the C-terminal half, and contain amino acid repeats in the middle of the proteins (3). These results prompted us to examine whether *HKR1* could substitute for *MSB2* and rescue a *cdc24* mutant at the nonpermissive temperature. To address this question, we carried out a preliminary experiment in which pMT-*HKR* and pMT-*HKR*^{tr} were introduced into *S. cerevisiae* Y147 (*cdc24*) cells (2). *HKR1* mRNA was increased more than 50-fold in cells cultured in galactose-containing medium compared with in the cells cultured in glucose-containing medium, as shown in Fig. 7. This increased level of *HKR1* expression, however, did not support the growth of Y147 cells at 37°C, suggesting that *HKR1* cannot substitute for *MSB2*. *MSB2* has been reported to be a nonessential gene (3), while *HKR1* was shown to be essential for the

viability of *S. cerevisiae* cells. Taking these results together, we concluded that *HKR1* and *MSB2* are functionally distinct.

We found that overexpression of truncated *HKR1*, which could encode the C-terminal part of Hkr1p, was sufficient and much more efficient than the full-length gene in conferring resistance to HM-1 killer toxin. This difference may be caused mainly by the difference in the levels of expressed protein. Indeed, Western blotting (immunoblotting) with a specific antibody raised against the C-terminal portion of Hkr1p showed that the level of truncated Hkr1p was much higher than that of the full-length Hkr1p (data not shown).

It is not clear at present how the full-length or truncated Hkr1p could protect the cells from HM-1 toxin, and further experiments should be necessary to understand the molecular mechanism of HM-1 killer toxin action. Surprisingly, most, if not all, of the protein expressed from the truncated form of *HKR1* (2.6-kb *Hin*dIII-*Hin*dIII region) was found in the membrane fraction (data not shown). One possible interpretation is that Hkr1p contains a HM-1 toxin binding site in the C-terminal half of the protein and that overexpressed Hkr1p neutralizes the HM-1 killer toxin by binding to it.

On the other hand, Hkr1p contains an EF hand motif of the calcium-binding consensus sequence in the C-terminal cytoplasmic domain. The existence of a calcium-binding site in Hkr1p suggests that the changes in the intracellular calcium concentration may be associated with the cytocidal effect by HM-1 toxin. This finding also enables us to speculate that the overexpression of the full-length or truncated form of Hkr1p impairs the function of the endogenous Hkr1p at certain steps, e.g., binding to calcium ion and interaction with substrate.

As for the physiological function of Hkr1p, we demonstrated here that overexpression of *HKR1* increased the β-glucan content and that *HKR1* is involved in β-glucan biosynthesis. β-Glucan synthase activity in the cells, however, was unaffected by the overexpression of either truncated or full-length *HKR1*, suggesting that Hkr1p may be a regulatory factor of β-glucan synthesis. Interestingly, truncated and full-length *HKR1* affected the β-glucan content in different fractions. Since the level of truncated Hkr1p is much higher than that of full-length Hkr1p, the increase in the β-glucan content in the different fractions by the two forms of Hkr1p may also be the consequence of the difference in the protein levels.

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REFERENCES

1. **Andreadis, A., Y.-P. Hsu, G. B. Kohlhaw, and P. Schimmel.** 1982. Nucleotide sequence of yeast *LEU2* shows 5′ noncoding region has sequences cognate to leucine. Cell **31**:319–325.
2. **Bender, A., and J. R. Pringle.** 1989. Multicopy suppression of the *cdc24* budding defect in yeast by *CDC42* and three newly identified genes including the *ras*-related gene *RSR1*. Proc. Natl. Acad. Sci. USA **86**:9976–9980.
3. **Bender, A., and J. R. Pringle.** 1992. A Ser/Thr rich multicopy suppressor of a *cdc24* bud emergence defect. Yeast **8**:315–323.
4. **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**:273–283.
5. **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.
6. **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.
7. **Brown, J. L., Z. Kossaczka, B. Jiang, and H. Bussey.** 1993. A mutational analysis of killer toxin resistance in *Saccharomyces cerevisiae* identifies new genes in cell wall (1-6)-β-glucan synthesis. Genetics **133**:837–849.
8. **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*. J. Bacteriol. **140**:888–892.
9. **Cabib, E., and M. S. Kang.** 1987. Fungal 1,3-β-glucan synthase. Methods Enzymol. **138**:637–642.
10. **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.
11. **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.
12. **Hutchens, K., and H. Bussey.** 1983. Cell wall receptor for yeast killer toxin: involvement of (1-6)-β-D-glucan. J. Bacteriol. **154**:161–169.
13. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
14. **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.
15. **Kretsinger, R. H., and C. E. Nockolds.** 1973. Carp muscle calcium-binding protein. J. Biol. Chem. **248**:3313–3326.
16. **Kuribayashi-Ohta, K., S. Tamatsukuri, M. Hikata, C. Miyamoto, and Y. Furuichi.** 1993. Application of oligo(dT)$_{30}$-latex for rapid purification of poly(A)$^+$ mRNA and for hybrid subtraction with the in situ reverse transcribed cDNA. Biochim. Biophys. Acta **1156**:204–212.

16a. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.

17. **Lundblad, V.** 1989. *Saccharomyces cerevisiae*, p. 13.11.1–13.11.5. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Wiley Interscience, New York.
18. **Manners, D. J., A. J. Masson, and J. C. Patterson.** 1973. The structure of a β-(1,3)-D-glucan from yeast cell walls. Biochem. J. **135**:19–30.
19. **Meaden, P., K. Hill, J. Wagner, D. Slipetz, S. S. Somer, and H. Bussey.** 1990. The yeast *KRE5* gene encodes a probable endoplasmic reticulum protein required for (1-6)-β-D-glucan synthesis and normal cell growth. Mol. Cell. Biol. **10**:3013–3019.
20. **Notario, V., H. Kawai, and E. Cabib.** 1982. Interaction between yeast β-(1,3)-glucan synthetase and activating phosphorylated compounds. J. Biol. Chem. **257**:1902–1905.
21. **Peat, S., W. J. Whelan, and T. E. Edwards.** 1958. Polysaccharides of baker's yeast. Part II. Yeast glucan. J. Chem. Soc. **1958**:3862–3868.
22. **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.
23. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Rothstein, R.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101**:202–209.
25. **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.

26. **Shematek, E. M., J. A. Braatz, and E. Cabib.** 1980. Biosynthesis of the yeast cell wall. I. Preparation and property of β-(1,3)-glucan synthetase. J. Biol. Chem. **255**:888–894.
27. **Shematek, E. M., and E. Cabib.** 1980. Biosynthesis of the yeast cell wall. II. Regulation of β-(1,3)-glucan synthetase by ATP and GTP. J. Biol. Chem. **255**:895–902.
28. **Tajima, M., Y. Nogi, and T. Fukasawa.** 1986. Duplicate upstream activating sequences in the promoter region of the *Saccharomyces cerevisiae GAL7* gene. Mol. Cell. Biol. **6**:246–256.
29. **Von Heijne, G.** 1986. A new method for predicting signal sequence cleavage site. Nucleic Acids Res. **14**:4683–4690.
30. **Yamamoto, T., T. Hiratani, H. Hirata, M. Imai, and H. Yamaguchi.** 1986. Killer toxin from *Hansenula mrakii* selectively inhibits cell wall synthesis in a sensitive yeast. FEBS Lett. **197**:50–54.
31. **Yamamoto, T., M. Imai, K. Tachibana, and M. Mayumi.** 1986. Application of monoclonal antibodies to the isolation and characterization of a killer toxin secreted by *Hansenula mrakii*. FEBS Lett. **195**:253–257.