Cloning and Nucleotide Sequence of the *KHR* Killer Gene of *Saccharomyces cerevisiae*

Kuniyasu GOTO, Yasuhiro IWATUKI, Kazuyosi KITANO,
Takaji OBATA and Shodo HARA

National Research Institute of Brewing, 2–6–30 Takinogawa,
Kita-ku, Tokyo 114, Japan

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The *KHR* gene cloned from a genomic library was on 4.7-kbp DNA fragment and was inserted into YCpG11 vector (KHR-YCp) and YE_p vector (KHP-YE_p). Transformants with KHR-YE_p could secrete 3–4 times as much killer toxin into the media as the donor strain. The complete nucleotide sequence of the *KHR* gene was analyzed. It was found that the *KHR* gene consisted of 888 bp. It was suggested that this protein was processed before being secreted into the media, because its molecular mass presumed from the nucleotide sequence was larger than that of the mature killer toxin.

Some yeast strains secrete into the culture media factors, called killer toxin, which kill sensitive strains of yeast. Previous reports suggested that the use of K1 type killer yeasts in sake making¹⁾ and K2 type killer yeasts in wine making²⁾ would improve their qualities.

Recently, Kitano *et al.*³⁾ described new two killer types encoded on the chromosomal DNA of *S. cerevisiae*. One of them was *KHS* (Killer of Heat Sensitive) mapped on the right arm of chromosome V and the other *KHR* (Killer of Heat Resistant) on the left arm of chromosome IX. These killer activities were weaker than those of known killer strains. The isolation and characterization of *KHR* killer toxin was described in our previous paper.⁴⁾ The mature killer toxin was a single protein having a molecular mass of about 20 kDa.

To study the function and the secretion system of *KHR* killer toxin in comparison with other killer toxin, the elucidation of its primary structure is necessary. We report here the molecular cloning and the nucleotide sequence of the *KHR* gene.

Materials and Methods

Strains, plasmids, and media. *S. cerevisiae* No. 18p⁻ (a

KHR KHS) was used as the DNA donor to construct a gene library, *S. cerevisiae* No. 111 (α *KHR khs*) as the standard *KHR* strain, *S. cerevisiae* DBY746 (α *leu2-3 leu2-112 trp1 ura3 his3 khr khs*) as a recipient strain for transformation. *E. coli* JA221, and MV1184 were used as hosts, plasmid YCpG11,⁵⁾ as a vector for the construction of a gene library, and YE_p24,⁶⁾ pUC118, pUC119, and M13-KO7⁷⁾ as vectors for DNA sequencing and DNA manipulation.

LB supplemented with 100 μ g/ml ampicillin and 40 μ g/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) was used for selection of *E. coli* transformants.

YNBD medium consisted of 2% dextrose, 0.67% yeast nitrogen base without amino acids, 0.04% each of L-methionine and L-leucine, and 0.008% L-histidine. YNBD media supplemented with 0.008% L-tryptophan and uracil (YNBD+) were used for selection of yeast transformants with YE_p24 and YCpG11 derivative vectors respectively. The medium consisting of YNBD+ in 50 mM citrate-phosphate buffer (pH 6.0) and 0.003% methylene blue were used for the killer assay. For agar plates, 2% agarose was added to each medium.

Construction of a YCpG11 library

Genomic DNA of *S. cerevisiae* No. 18p⁻ was prepared by the method of Cryer *et al.*⁸⁾ partially digested with *Sau*3A, and separated by agarose-gel electrophoresis. Four to ten kbp DNA fragments were collected and directly inserted into YCpG11 *Bam*HI site.

PFGE and Southern hybridization. Electrophoresis was done at 12°C on a LKB 2015 Pulsaphor hexagonal system with 0.5 $\times$ TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA) containing 1.5% agarose at 300 V, for 40 hr

with a switching interval of 100 sec. Gels were stained with ethidium bromide for 10 min at a concentration of 0.5 μ g/ml in 0.5 $\times$ TBE, and destained overnight in 0.5 $\times$ TBE.

Southern transfer and hybridization were done by the method of Maniatis *et al.*⁹⁾ A 1.9-kbp *Bam*HI–*Pst*I fragment prepared from KHR-YCp was used as the *KHR* probe. The probe was labeled with [α -³²P] dCTP by nick-translation.

Yeast transformation. Transformation was done by the method of Ito *et al.*¹⁰⁾

Assay for killer activity. The cells for the assay were grown on killer assay plates for 2 days at 25°C, and then *C. glabrata* IFO0622 was sprayed over the plates. After incubation at 20°C for 2 days, the fungicidal zone around each colony was observed. Killer activity was measured as described before.⁴⁾

Nucleotide sequencing analysis. Genomic DNA was subcloned using plasmids pUC118, pUC119, and *E. coli* MV1184. The nucleotide sequence was analyzed by the dideoxy method of Messing¹¹⁾ and by the fluorescent-labeled DNA sequence method¹²⁾ using an automated DNA sequencing system (Applied Biosystems Model 370A). The nucleotide sequence and amino acid sequence were analyzed with the GENETYX Program (SDC Co., Ltd., Japan).

Results

Cloning of the *KHR* gene

About 5,000 clones (Amp^r and Tet^s) were selected from *E. coli* transformants with the YCpG11 vector containing *S. cerevisiae* No. 18 ρ^- yeast DNA fragments partially digested with *Sau*3A. These recombinant plasmids were amplified in *E. coli* JA221, purified, and stored as the gene banks. From about 9,000 non-tryptophan-requiring yeast clones, one clone was selected by killer activity at pH 6.0. This clone had also the same characteristics as the *KHR* killer toxin, for example, the optimum pH, temperature stability, and antimicrobial spectrum⁴⁾ like those produced by *S. cerevisiae* No. 111.

The plasmid was prepared from this killer yeast clone and was designated KHR-YCp. The inserted DNA fragment was about 4.7 kbp.

The KHR-YCp DNA was digested with several restriction enzymes to make a restric-

Fig. 1. The Structure of Plasmid KHR-YCp and Subcloning Fragments.

A: The 4.7-kb *Sau*3A fragment containing the entire *KHR* gene was inserted into YCpG11 (11.1 kbp).

B: Subcloning fragments were as indicated by solid lines. These fragments were inserted into YEp24 vector and examined their killer activities. Their *KHR* killer activities are positive (+) or negative (–).

Abbreviations: B, *Bam*HI; E, *Eco*RI; N, *Nco*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; B/Sau, the ligation site of *Bam*HI and *Sau*3A; X, *Xho*I.

tion map (Fig. 1). By the physical mapping (Fig. 2), this fragment was located on chromosome IX, which was the same as the genetic mapping.³⁾

Construction of a plasmid carrying an internal region of *KHR*

In the preliminary experiments, the biologically functional *KHR* gene was found on a part of the *Bam*HI–*Sal*I fragment (about 3.7 kbp) in KHR-YCp.

The DNA was recovered from *Bam*HI–*Sal*I digestion of KHR-YCp and then inserted into plasmid vectors pUC118 and 119 and YEp24 (Fig. 3). The resultant pUC (KHR-pUC) and YEP (KHR-YEP) were used for sequencing, subcloning, and testing of the killer activity respectively.

The *E. coli* transformants with KHR-pUC and KHR-YEP did not express the killer activity.

The growth of the yeast transformant with KHR-YEP was inhibited at the optimum con-

Fig. 2. Physical Mapping of *KHR* Gene in *S. cerevisiae* No. 18p⁻.

A: lane 1, the gel was stained with ethidium bromide; lane 2, the 1.9 kbp fragment containing the *KHR* gene was used as the probe.

B: The open and solid boxes indicated the insert DNA. The 1.9 kbp fragment treated by *Pst*I and *Bam*HI (the solid box) was used as the probe.

Abbreviations: P, *Pst*I; B, *Bam*HI.

Fig. 3. The Strategies for Constructions of *KHR*(BS)-pUC119 and *KHR*-YEp.

conditions for producing *KHR* toxin, but the cell growth was not inhibited below pH 5.0 or by shaking culture, under those conditions the *KHR* toxin was inactivated. Those phenomena were the same as observed in the *KHR* multiploid strains.⁴⁾

Fig. 4. The Structure of the *S. cerevisiae* Genome Surrounding *KHR* Gene and the Strategy for Its DNA Sequencing.

▨, presumed signal sequence; ▨, homologous sequence to δ -sequence; ○, Asn-X-Thr/Ser presumed glycosylation site; △, presumed recognizing site with *KEX2* proteinase; ⇄, length and direction in sequencing; ⇄, 5'→3' direction of the ORFs'.

Abbreviations: E, *Eco*RI; Pv, *Pvu*II; Ev, *Eco*RV; Ps, *Pst*I; M, *Mlu*I; N, *Nco*I; X, *Xho*I; B, *Bam*HI; S, *Sal*I.

Subcloning of the *KHR* gene and their expression

In subcloning, we selected yeast transformants with *KHR*-YEp derivatives by the complementation of *ura3* on the pH 4.5 agar plate and the extent of the fungicidal zone formed on the killer assay plate.

Figure 1 shows the relationship between

transformants with YEp carrying *KHR* gene fragments and their killer activities. The functional *KHR* gene was found on the *EcoRI*–*XhoI* fragment (about 1.7 kbp).

The nucleotide sequence around the *KHR* gene

The inserted DNA of *BamHI*–*EcoRI* fragment was sequenced as described in Materials

and Methods. Figure 4 shows the sequencing strategy and summarizes the open reading frame and other information on *BamHI*–*EcoRI* fragment DNA.

In this fragment, two open reading frames existed. The sequence around the *XhoI* site (about 350 nucleotides) had more than 85% homology to the δ sequence that was a part of

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*1
aat. cca. att. act. aac. att. cgc. aac. aca. ttc. atc. aat. gtt. taa. tat. tta. aac. ggc. tat. ata
*61
ttt. cgc. ggt. agg. aca. atg. tac. taa. aac. atg. aaa. ggc. cac. gga. aaa. aaa. aat. cga. tac. aaa
*121
taa. gca. cac. atc. ata. gtc. ATG. GGC. CAC. TTA. GCG. ATC. CTT. TTC. AGT. ATT. ATC. GCT. GTA. TTG
Met-Gly-His-Leu-Ala-Ile-Leu-Phe-Ser-Ile-Ile-Ala-Val-Leu
*181
AAT. ATA. GCT↓ACA. GCT↓GTT. GCA↓TCG. AGC. GAC. AGC. ATT. TAC. CTT. AAG. GGA. CAC. AGA. GTA. GGA
Asn-Ile-Ala-Thr-Ala-Val-Ala-Ser-Ser-Asp-Ser-Ile-Tyr-Leu-Lys-Gly-His-Arg-Val-Gly
*241
CAG. GAT. ATC. GAC. AGT. CTA. TAC. AGA. GTG. TAC. GAT. AAT. GGT. ACT. ATG. TAC. CCT. GTC. ACT. TTC
Gln-Asp-Ile-Asp-Ser-Leu-Tyr-Arg-Val-Tyr-Asp-Asn*Gly-Thr-Met-Tyr-Pro-Val-Thr-Phe
*301
AAT. GAG. TGG. TTA. AAT. GAT. CTA. ACT. GGG. ATG. AAT. GAC. TTG. GCA. ACA. AAT. AAC. GCG. ACA. ATA
Asn-Glu-Trp-Leu-Asn-Asp-Leu-Thr-Gly-Met-Asn-Asp-Leu-Ala-Thr-Asn-Asn*Ala-Thr-Ile
*361
TTA. AAA. CGT. GAC. AGT. AGC. GAT. GTT. TCT. TGC. GTT. ACT. GAA. ACA. TGC. CAA. TAC. GTG. GAT. TAC
Leu-Lys+Arg-Asp-Ser-Ser-Asp-Val-Ser-Cys-Val-Thr-Glu-Thr-Cys-Gln-Tyr-Val-Asp-Tyr
*421
CAC. GTG. GAT. GAC. GAA. GGA. GTT. ATA. ACT. ATA. GAC. ATA. TCT. ACG. TAT. CGT. ATC. CCT. GTC. GAA
His-Val-Asp-Asp-Glu-Gly-Val-Ile-Thr-Ile-Asp-Ile-Ser-Thr-Tyr-Arg-Ile-Pro-Val-Glu
*481
TGG. GAT. AGT. GGT. TCT. GCA. GGC. AAC. GCA. TCA. TAT. GGA. GTC. TCA. AAG. CGT. GAT. ACA. AAA. TAT
Trp-Asp-Ser-Gly-Ser-Ala-Gly-Asn*Ala-Ser-Tyr-Gly-Val-Ser-Lys+Arg-Asp-Thr-Lys-Tyr
*541
GAG. ACA. TTC. TGT. AAG. AAG. AAA. ATA. TGC. GGT. ATA. AAC. GTT. TCC. GGT. TTC. TGT. AAC. GCG. TAT
Glu-Thr-Phe-Cys-Lys+Lys+Lys-Ile-Cys-Gly-Ile-Asn*Val-Ser-Gly-Phe-Cys-Asn-Ala-Tyr
*601
GAC. TTC. GCC. GTT. CAT. GCT. TTC. GAC. TTC. GGT. GGG. AGT. GTC. TAC. AAC. CCT. GTT. AGT. GGT. ATC
Asp-Phe-Ala-Val-His-Ala-Phe-Asp-Phe-Gly-Gly-Ser-Val-Tyr-Asn-Pro-Val-Ser-Gly-Ile
*661
ACT. GAT. AGG. ATT. AAA. GAA. GCT. ACG. AAG. AGG. GAT. AAG. ACT. GAA. TGT. CTA. GGT. TAC. GAA. CTA
Thr-Asp-Arg-Ile-Lys-Glu-Ala-Thr-Lys+Arg-Asp-Lys-Thr-Glu-Cys-Leu-Gly-Tyr-Glu-Leu
*721
GAT. CAT. GTG. AGA. ATA. GAT. CCT. GCT. GTT. GAT. TGG. TCC. ATA. TCT. ATT. TCT. ACT. TGG. AAA. CAA
Asp-His-Val-Arg-Ile-Asp-Pro-Ala-Val-Asp-Trp-Ser-Ile-Ser-Ile-Ser-Thr-Trp-Lys-Gln
*781
GGG. TCG. GCC. AAT. TGT. GAC. ACA. CAG. GCA. TCT. GCT. GAC. AGC. TTG. AAA. TGT. GCT. GCC. CAA. AAA
Gly-Ser-Ala-Asn-Cys-Asp-Thr-Gln-Ala-Ser-Ala-Asp-Ser-Leu-Lys-Cys-Ala-Ala-Gln-Lys
*841
GCA. CTT. GAA. AGT. GAA. CAC. AAT. CAC. CAA. AAA. ACA. GCT. TTC. TGT. ATT. CAC. CTA. GAT. AAT. GGT
Ala-Leu-Glu-Ser-Glu-His-Asn-His-Gln-Lys-Thr-Ala-Phe-Cys-Ile-His-Leu-Asp-Asn-Gly
*901
GGA. TCA. TTT. AAC. TTA. GAC. ATT. AGG. CTA. ATA. TCT. GAG. CTT. TCA. TTT. TCG. AAA. TAT. AAC. CCA
Gly-Ser-Phe-Asn-Leu-Asp-Ile-Arg-Leu-Ile-Ser-Glu-Leu-Ser-Phe-Ser-Lys-Tyr-Asn-Pro
*961
TGG. GCT. CTT. CCA. TGT. CCG. AAG. TAC. AAA. GGC. TCC. AAT. TCT. TGG. CAA. GTT. GTG. AGC. GAC. TGT
Trp-Ala-Leu-Pro-Cys-Pro-Lys-Tyr-Lys-Gly-Ser-Asn-Ser-Trp-Gln-Val-Val-Ser-Asp-Cys
*1021
TTT. CAA. TAA. att. aaa. caa. att. caa. gct. cta. tta. cat. tat. caa. tct. gtt. gga. ata. aaa. tta
Phe-Gln-Stop
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Fig. 5. Nucleotide Sequence and Deduced Amino Acid Sequence of the *KHR* Gene of *S. cerevisiae*.

Protein-coding sequence are given in uppercase letters. The TATA box is overlined; the presumed signal peptide is underlined (presumed cleavage site is indicated by an arrow); * indicates Asn-X-Thr/Ser site; + indicates presumed cleavage recognizing site with *KEX2* proteinase.

transposon yeast (*Ty1*).

The nucleotide and amino acid sequence of the KHR gene

In the *Eco*RI-*Xho*I fragment, a sole ORF existed.

Figure 5 shows the nucleotide sequence and the predicted amino acid sequence of the *KHR* gene. This ORF consisted of 888 bp, with no intron. A typical eucaryotic promoter region was found in the *KHR* gene. A TATATA sequence, which seems to function as a TATA box, was found about 80 bp upstream of this ORF.

The total amino acid sequence of *KHR* deduced from its nucleotide sequence *KHR* protein was supposed to consist of 296 amino acids. As shown in Fig. 5, a presumed signal peptide consisting of 17–21 amino acids was found at the N-terminal side, and a 4 glycosylated amino acid sequence, Asn-X-Thr/Ser, was found at the N-terminal side. The calculated molecular mass of this coding protein was 32,781 Da.

Discussion

Here we reported the molecular cloning and the nucleotide sequence of the genomic *KHR*

gene of *S. cerevisiae*. The *KHR* gene is contained within the 1.7-kbp *Eco*RI-*Xho*I fragment (Figs. 1 and 4). By the physical mapping of *KHR* gene, it was found to be located on chromosome IX as was already reported by the genomic mapping.

The nucleotide sequence of the genomic *KHR* gene was analyzed and it was found that the *KHR* gene consisted of 888 bp (a molecular mass of about 33 kDa). This gene and presumed amino acid sequence had no homology to previously reported yeast killer genes or killer toxins, and this gene was unique as far as we searched in the EMBL and NBRF-PDB data bases.

The molecular mass of mature killer toxin was about 20 kDa.⁴⁾ The gap between these molecular masses indicated that the processing step existed during maturation of killer toxin. Remarkable findings were that the 21 very hydrophobic amino acids that were supposed to make a signal sequence existed in the N-terminal site region, and 4 competent sites for glycosylation were located at the N-terminal side, and 5 cleavage sites that might be cut by *KEX2* proteinase existed in the *KHR* gene.

The AT-rich region, which seems to function as a TATA box, was found upstream of the *KHR* gene. Downstream from this gene, a

Table I. COMPARISON OF CODON USAGE BETWEEN *KHR* TOXIN AND K1 KILLER TOXIN

<i>KHR</i>	K1		<i>KHR</i>	K1		<i>KHR</i>	K1		<i>KHR</i>	K1
Phe	UUU 3 9		Ser	UCU 8 2		Tyr	UAU 5 8		Cys	UGU 8 3
	UUC 8 2			UCC 3 2			UAC 9 3			UGC 3 3
Leu	UUA 4 7			UCA 4 6		non	UAA 1 0		non	UGA 0 0
	UUG 3 3			UCG 3 4			UAG 0 1		Trp	UGG 6 9
	CUU 5 3	Pro		CCU 4 5		His	CAU 2 7		Arg	CGU 3 6
	CUC 0 2			CCC 0 0			CAC 6 4			CGC 0 0
	CUA 6 6			CCA 2 5		Gln	CAA 6 4			CGA 0 0
	CUG 0 4			CCG 1 2			CAG 2 3			CGG 0 0
Ile	AUU 6 8	Thr		ACU 8 3		Asn	AAU 10 11		Ser	AGU 7 8
	AUC 5 7			ACC 0 7			AAC 7 8			AGC 5 2
	AUA 10 5			ACA 8 10		Lys	AAA 10 3		Arg	AGA 3 2
Met	AUG 3 6			ACG 2 5			AAG 7 10			AGG 3 1
Val	GUU 9 4	Ala		GCU 10 5		Asp	GAU 14 11		Gly	GGU 8 11
	GUC 4 5			GCC 3 8			GAC 12 10			GGC 3 8
	GUA 2 8			GCA 6 8		Glu	GAA 8 8			GGA 5 3
	GUG 5 8			GCG 3 4			GAG 3 6			GGG 3 1

Total amino acid 296 317

sequence homologous to the δ sequence was found, but no sequence homologous to the *Ty* gene containing δ was found.

The homologies of nucleotide and amino acid sequences between the KHR and K1 type¹⁴⁾ killer toxin were not found. Table I shows the comparison of their codon usage and amino acid composition. In the K1 type killer preprotoxin gene, for example, such rare codons as CUC and CUG for Leucine¹⁴⁾ were used, but these codons were not used in the KHR gene, and more common codons were used in it.

Of the KHR killer toxin, the hydrophobic amino acid residues were a little fewer than those of K1 killer toxin. The K1 killer subunit which is the domain of killer activity has two hydrophobic regions and their function is an ionophore, but the KHR toxin did not have a clear hydrophobic region. These data suggest that the mechanism of the KHR toxin killing sensitive strains was different from that of K1 type killer. We are studying this killer's physiological function now.

The KHR-YEp plasmid had some useful characteristics. For example, the killer activity serves as a positive marker for *S. cerevisiae*. It has the two *PvuII* sites, one of which is near the end of the signal peptide and the other is within the plasmid YEp24. By digestion with *PvuII*, the domain of KHR killer activity was cut out, and the KHR promoter and its almost entire signal sequence remained intact on the YEp vector. The resultant linear DNA can be used as a secretion vector. The KHR-YEp vector had a sole *MluI* site in the domain of KHR toxin activity. By insertion of other DNA fragment into this site, the killer activity was lost, and transformants with this vector

can be selected from those with KHR-YEp by growth under the optimum KHR toxin producing condition.

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