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Molecular Cloning, Chromosomal Integration and Expression of the Homoserine Kinase Gene *THR1* of *Saccharomyces cerevisiae*

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트레오닌 생합성에 관여하는 효모유전자 *THR1*의 클로닝, 염색체통합 및 발현

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ABSTRACT: The yeast gene *THR1* encodes the homoserine kinase (EC 2.7.1.39: HKase) which catalyses the first step of the threonine specific arm at the end of the common pathway for methionine and threonine biosynthesis. A recombinant plasmid pMC3 (12.6 kilobase pairs, vector YCp50) has been cloned into *E. coli* HB101 from a yeast genomic library through its complementing activity of a *thr1* mutation in a yeast recipient strain M39-1D. When subcloned into pMC32 (8.6 kbp, vector YRp7) and pMC35 (8.3 kbp, vector YIp5), the HindIII fragment (2.7 kbp) of pMC3 insert was positive in the *thr1* complementing activity in both yeast and *E. coli* auxotrophic strains. The linearized pMC35 was introduced into the original recipient yeast strain and the mitotically stable chromosomal integrant was identified among the transformants. Through the tetrad analysis, the integration site of the pMC35 was localized to the region of *THR1* structural gene at an expected genetic distance of approximately 11.1 cM from the *ARG4* locus on the right arm of the yeast chromosome VIII. When episomically introduced into the auxotrophic cells and cultured in Thr omission liquid medium, the cloned gene overexpressed the HKase in the order of thirteen to fifteenfold, as compared with a wildtype. HKase levels are repressed by addition of threonine at the amount of 300 mg/l and 1,190 mg/l for pMC32 and pMC3, respectively. Data from genetic analysis and HKase response thus support that the cloned HindIII yeast DNA fragment contains the yeast *thr1* structural gene, along with necessary regulatory components for control of its proper expression.

KEY WORDS□Chromosomal integration, Molecular cloning, Overexpression, *Saccharomyces cerevisiae*, Tetrad analysis, Yeast homoserine kinase gene *THR1*

INTRODUCTION

A common pathway is shared for biosynthesis of the amino acids methionine and threonine from aspartate to homoserine in the yeast, *Saccharomyces cerevisiae*. The intermediate homoserine is produced after three enzymetically catalyzed steps, after which the pathway diverses into two branches; one to threonine, and the other to methionine. Homoserine kinase (EC 2.7.1.39; HKase) phosphorylates homoserine, yielding O-phosphohomoserine which is catalyzed in turn to produce threonine. The gene *THR1* was assigned to the HKase through enzyme assay by de Robichon-Szulmajster *et al.* (1973). Regulation mechanism of the genes on the threonine specific arm is not clearly understood as reviewed by Jones and Fink (1982), and investigation of coregulation of those genes requires first characterization of each gene at the molecular level.

The four genes encoding enzymes for the threonine biosynthesis have been isolated and se-

quenced (*HOM3*: Rafalski and Falco, 1988; *HOM2*: Thomas and Surdin-Kerjan, 1989; *THR1*: Schultes *et al.*, 1990; *THR4*: Aas and Rognes, 1990). We also independently isolated *HOM3* (Choi and Lea, 1988 and Choi *et al.*, 1990), *THR4* (Lea and Lea, 1987) and *THR1* (this work). Farther upstream sequences than what Schultes *et al.* isolated (−201 bp) may be necessary for identification of possible regulatory sequence(s) in detail. In an attempt to molecularly characterize the yeast *THR1* gene , especially for upstream sequences beyond −201, we also isolated a 2.7 kilobase pair (kbp) fragment of yeast genomic DNA which is capable of complementing a *thr1* mutation in yeast and also corresponding *E. coli* gene *thrB1000*. We will happen to confirm or disconfirm Schultes' sequence data, keeping also in mind that our genomic library might have been originated from different source yeast strain. Here we report the isolation procedure and genetic and biochemical evidence that the isolated DNA segment contains the yeast *THR1* structural gene, as well as necessary regulatory region(s) for control of its proper expression.

MATERIALS AND METHODS

Microbial strains and plasmids

The yeast *Saccharomyces cerevisiae* and *Escherichia coli* strains and plasmids used in this work are listed in Table 1. The recipient yeast strain M39-1D was so constructed that it possesses markers of a target gene *thr1* for cloning and also markers *trp1* and *ura3* for most commonly used yeast-*E. coli* shuttle vectors. In a serial crosses (M24, M33, M37, M39, and M441), sporulation, asci dissection, spore germination and marker screening were performed following the procedure of Sherman *et al.* (1986). The target gene *thr1* was introduced into M39-1D from the strain X3127-27D and genetic background for high transformation efficiency from the strains M1-2B, RH218 and YNN281. For the linkage analysis, the component strains (M441-1C, M441-15B, M441-20B) in the crosses with the integrative transformant strain M39-1D::pMC35 were selected among the germinating spores obtained from a cross (M441) between M33-1D and X3127-27D. The *E. coli* strain Hfr3000YA73 harbors a mutant allele *thrB1000* for the bacterial HKase structural gene, which is homologous to the yeast *THR1* gene (Schultes *et al.*, 1990). Vectors and recombinant plasmids were maintained, amplified and extracted, using the *E. coli* strain HB101.

The vectors and yeast genomic libraries were kindly gifted by Drs. F. LaCroute (Strasborg, Cedex, France), R. Davis (Stanford, Ca, USA), and A. Wright (Imperial College, London, UK). The yeast genomic library was a pool of recombinant plasmids, in each of which partial Sau3A segments of the yeast chromosomal sequence are inserted into the BamHI site of a centromeric shuttle vector YCp50 (Parent *et al.*, 1985).

Enzymes and fine chemicals

Enzymes and fine chemicals were purchased from KOSCO Biotechnology Lab., Seoul, South Korea and Korean representatives of New England BioLabs (Beverly, Mass, USA) and Promega Biotech (Madison, Wi, USA), or directly from Sigma Chemical Co. (St. Louis, Mo, USA), and used as recommended by the supplier.

Culture media and growth conditions

Various yeast and *E. coli* culture media were prepared according to Sherman *et al.* (1986) and Sambrook *et al.* (1989), respectively. Yeast cells were cultured at 30℃ and those of *E. coli* at 36℃. Cell growth in liquid culture was monitored with a spectrophotometer at A_{600} or with a Klett-Summerson colorimeter at A_{500}-A_{570}.

Preparation and manipulation of DNA

Plasmid DNA was extracted from *E. coli* in large scale and purified, according to the procedure of Sambrook *et al.* (1989). For rapid screening of plasmidDNA, the method for a 5 m*l* culture was employed, as described by Tschumper and Carbon (1980), Birnboim and Doly (1979) or Guerry *et al.* (1973). Plasmid DNA from yeast cells was prepared following the procedure of Nasmyth and Reed (1980) for a large 100 m*l* culture, and of Hoffman and Winston (1987) or of Ward (1990) for a small 1.5 m*l* culture. DNA was manipulated through the procedure of restriction enzyme digestion, agarose gel electroporesis, electroelution and ligation, as described by Sambrook *et al.* (1989). Errington's one-step method was also employed for isolating DNA fragments from agarose gels (1990).

Table 1. *Microbial strains and plasmids used in this study.*

Strain/Plasmid	Relevant genotype, Marker or Phenotype		Source[1], Reference
S. cerevisiae			
X3127-27D	*MAT@ ade1 ade5 arg4 leu1 leu2 lys7 thr1 trp1*		YGSC
M1-2B	*MAT@ trp1 ura3*		YGSC
RH218	*MATa trp1 CUP1 gal1 mal SUC2*		YGSC
YNN281	*MATa ade2 his3 lys2 trp1 ura3*		YGSC
M24-12B	*MAT@ thr1 trp1*		this study
M33-1C	*MAT@ thr1 trp1 ura3*		this study
M33-1D	*MATa thr1 trp1 ura3*		this study
M37-3B	*MAT@ thr1 trp1 ura3*		this study
M39-1D	*MAT@ thr1 trp1 ura3*		this study
M39-1D/pMC3[2]	*MAT@ thr1/THR1 trp1 ura3/URA3*		this study
M39-1D/pMC32[2]	*MAT@ thr1/THR1 trp1 ura3/URA3*		this study
M39-1D::pMC35[3]	*MAT@ thr1::THR1 trp1 ura3::URA3*		this study
M441-1C	*MATa arg4 thr1 trp1 ura3*		this study
M441-15B	*MATa arg4 thr1 trp1 ura3*		this study
M441-20B	*MATa arg4 thr1 trp1 ura3*		this study
E. coli			
Hfr3000YA73	*thrB1000 thi-1 relA1 spoT1*		EGSC
HB101	*hsdR hsdM recA13 supE44 lacZ24 leuB6 proA2 thi-1 Smr*		ATCC
Plasmid			
YCp50	*ARS1 CEN4 URA3*	Am^r TC^r[4]	Rose *et al.* (1987)
YIp5	*URA3*	Am^r TC^r	Struhl *et al.* (1979)
YRp7	*ARS1 TRP1*	Am^r Tc^r	Hinnen *et al.* (1978)
pMC1	*ARS1 CEN4 URA3*	Am^r	this study
pMC2	*ARS1 CEN4 URA3*	Am^r	this study
pMC3	*THR1 ARS1 CEN4 URA3*	Am^r	this study
pMC4	*ARS1 CEN4 URA3*	Am^r	this study
pMC31	*THR1 ARS1 TRP1*	Am^r Tc^r	this study
pMC32	*THR1 ARS1 TRP1*	Am^r Tc^r	this study
pMC33	*THR1 ARS1 TRP1*	Am^r Tc^r	this study
pMC34	*ARS1 CEN4 URA3*	Am^r Tc^r	this study
pMC35	*THR1 URA3*	Am^r Tc^r	this study

[1]YGSC: Yeast Genetic Stock Center, University of California Berkeley, Berkeley, California, USA. EGSC: *E. coli* Genetic Stock Center, Department of Microbiology, Yale University School of Medicine, 310 New Haven CT 06510, USA. ATCC: American Type Culture Collection, 12301 Parklawan Drive, Rockville, MD 20852, USA.
[2]Yeast strains of episomal transformation with pMC3 and pMC32 into M39-1D, respectively.
[3]A yeast strain of integrative transformation with pMC35 into M39-1D.
[4]Am^r: ampicillin resistant, Tc^r: tetracycline resistant.

Yeast and *E. coli* transformation

The whole cell method of Ito *et al.* (1983) or of Bruschi *et al.* (1987) was used for yeast transformation. *E. coli* cells were transformed, following the standard procedure (Sambrook *et al.*, 1989). For the integrative transformation with the cloned yeast DNA, the technique and the procedure were carried out as described previously (Hinnen *et al.*, 1978 and Orr-Weaver *et al.*, 1981).

Genetic analysis with the yeast tetrads

The standard procedure of Sherman *et al.* (1986) was followed for mating, diploid selection, tetrad dissection, and marker scoring for each tetrad. Ascus wall was partially digested with zymolyase-100T (Kirin Brewery Co., Tokyo, Japan) and each ascus was dissected under a microscope attached with a mechanical micromanipulator (Lawrence Precision Machine, Pioneer, Ca, USA). For linkage analysis, marker segregation data were taken only from the tetrad where all four spores

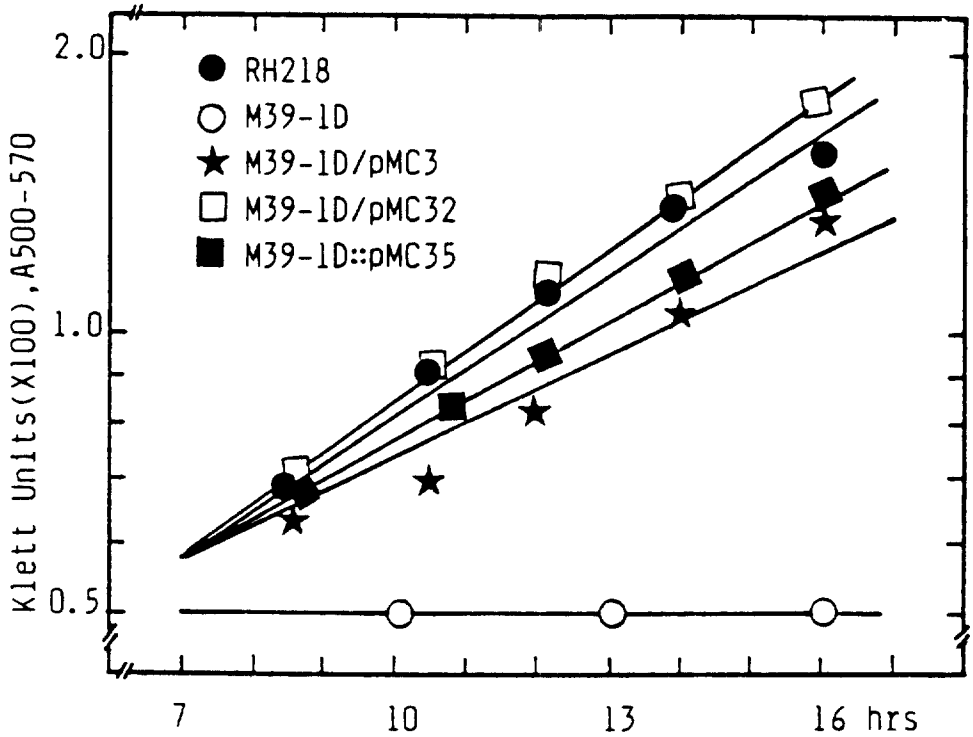

Fig. 1. *Cell growth of yeast strains in synthetic complete liquid medium without threonine at 30°C.* RH218, wild type (*THR1*)-threonine prototroph; M39-1D, recipient mutant strain (*thr1*) for transforming DNAs-threonine auxotroph; M39-1D/pMC3 and M39-1D/pMC32, yeast episomal transformants of M39-1D harboring recombinant plasmids pMC3 and pMC32, respectively; M39-1D::pMC35, yeast integrative transformant of M39-1D. Cell turbidity was measured with a Klett-Summerson colorimeter.

were germinated and segregated in a regular 2:2 ratio.

Enzyme purification and assay

HKase was purified from the acelluar extracts of exponentially growing yeast cells, as described by Natalini *et al.* (1979). HKase activity was quantitatively measured from the rate of coupled phosphoenolpyruvate- and NAD-linked oxidation of homoserine, according to the procedure of Flavin (1962), which procedure was based on a coupling of the ADP formed by the reaction of HKase (primary enzyme) to pyruvate kinase and lactate dehydrogenase (two auxiliary enzymes). One unit of enzyme activity was calibrated from A_{339} of NAD^+, representing the amount of enzyme that catalyses liberation of micromole of substrate phosphorylated per minute per milligram of protein, which concentration was measured by the method of Wittenberg and Kornberg (1983).

RESULTS AND DISCUSSION

Isolation and subcloning of a yeast DNA segment with the *thr1* complementing activity

The yeast recipient strain M39-1D cells were transformed with a yeast genomic library constructed in YCp50. A total number of 1,230 Ura^+ transformants were picked and transferred onto threonine omission (Thr^-) plates. Two Thr^+ transformants were selected, from each of which plasmid DNA was extracted to be amplified in *E. coli* cells. Ampicillin resistant (Am^r) transformants were screened for harboring plasmids, among which 4 plasmids were selected: pMC1 (14.9 kbp), pMC2 (15.0), pMC3 (12.6), and pMC4 (12.7). When these plasmids were reintroduced into the original recipient yeast strain M39-1D, the *thr1* complementing activity was stably maintained only with pMC3, which was chosen

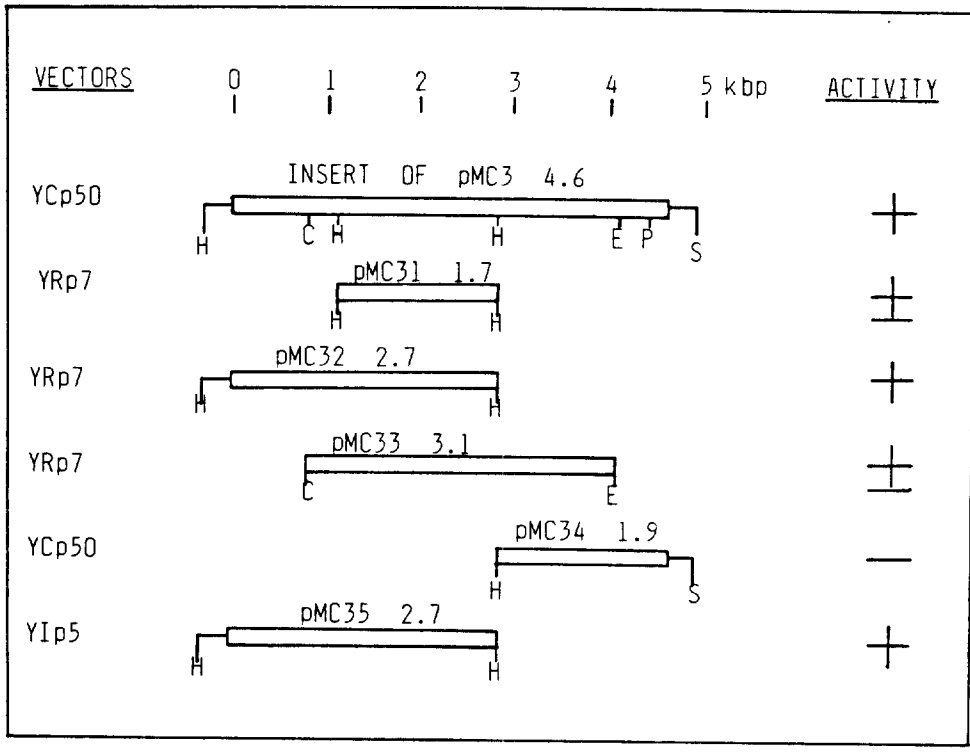

Fig. 2. *Localization of the THR1 gene and complementation analysis of the original clone and its subclones. Fragments of the pMC3 insert were subcloned into the vectors YCp50, YRp7 and YIp5 and tested for complementing activity of the thr1 mutation by transformation of the yeast M39-1D strain.*
C, ClaI; E, EcoRI; H, HindIII; P, PstI; S, SalI. Single line, pBR322; double line, yeast genomic sequence. +, complementing activity is positive; ±, episomal maintenance of plasmids is unstable; −, complementing activity is negative.

for further characterization and subcloning. Cosegregation of Ura^+ and Thr^+ was unstable in yeast cells transformed with pMC4, where only 10% of transformed cells maintained the characteristic cosegregation. The *thr1* complementing activity of pMC3 was further defined by comparison of cell growth in Thr^- liquid medium among yeast strains of the original recipient strain M39-1D, two episomal transformants M39-1D/pMC3 and M39-1D/pMC32, an integrative transformant M39-1D::pMC35, and a wild-type RH218 (Fig. 1). A partial restriction map was obtained through analysis of restriction fregments on the insert of pMC3 as shown in Fig. 2. In order to delimit the *thr1* complementing sequence, 1.7 kbp HindIII fregment was inserted into YRp7 to give rise to a subclone pMC31, 2.7 kbp HindIII fragment to pMC32, and 3.1 kbp ClaI-HindIII fragment to pMC33. After removal of 2.7 kbp HindIII fragment, the remaining portion of pMC3 was reclosed to yield pMC34. When reintroduced into the original recipient yeast strain M39-1D, complementing activity of pMC31 and pMC33 was leaky and unstable, while that of pMC34 was negative (Fig. 2). However, the subclone pMC32 completely rescued the *thr1* mutation, as tested by the cell growth of an episomal transformant yeast strain M39-1D/pMC32 (Fig. 1). Therefore, complementing sequence was primarily delimited within the 2.7 kbp insert of the original pMC3 from the genomic library. The 2.7 kbp fragment was in turn transferred into YIp5 to obtain an integrating plasmid pMC35, which complemented the *thr1* mutation in an integrative transformant yeast strain M39-1D::pMC35. The cell growth of M39-1D::pMC35 (Fig. 2) was also comparable to that of a wildtype RH218 and other episomal transformants M39-1D/pMC3 and M39-

Table 2. *Linkage of the integrated THR1-URA3 genes to ARG4 on the yeast chromosome VIII.*

M39-1D::pMC35 crossed with	No. of asci analyzed	Gene pair	Ascus type[1] PD	NPD	T	Genetic[2] distance
M441-1C	27	*thr1-arg4*	21	0	6	11.1cM
		ura3-arg4	21	0	6	11.1
		thr1-ura3	27	0	0	0.0
M441-15B	18	*thr1-arg4*	15	0	3	8.3
		ura3-arg4	15	0	3	8.3
		thr1-ura3	18	0	0	0.0
M441-20B	12	*thr1-arg4*	9	0	3	12.5
		ura3-arg4	9	0	3	12.5
		thr1-ura3	12	0	0	0.0

[1] The three tetrad classes are represented by PD (parental ditype), NPD (nonparental ditype) and T (tetratype).
[2] Genetic distances were calculated with the formula of Perkins (1949).

1D/pMC32. These results were summarized and illustrated in Fig. 1 and 2.

The enzyme HKase is encoded in *E. coli* by the gene *thrB*, which is equivalent to *THR1* of yeast. A mutant allele *thrB1000* of *E. coli* is a deletion of *thrB*, which is harbored in a threonine auxotrophic *E. coli* strain, Hfr3000YA73. The subclone pMC32 transformed successfully Hfr3000YA73 to a threonine prototroph, indicating that pMC32 contains a functional yeast HKase structural gene which can complement the bacterial *thrB* mutation. This observation is in accordance with the result of Schultes *et al.* (1990) that the *S. cerevisiae* homoserine kinase is homologous to prokaryotic homoserine kinases, which is in turn provides an additional evidence that the cloned DNA segment contains a structural gene for yeast HKase gene *THR1*.

Chromosomal integration of the cloned DNA

S. cerevisiae is one of the most useful microbial system in which the classical Mendelian genetic techniques and principles can be applied to molecular genetic enquiries for gene cloning and manipulation, mainly because of its rather unique biological features (Mortimer and Hawthorne, 1969). A critical genetic method has been established in order to prove that a cloned DNA does not represent a suppressor but contains a structural gene. Plasmid-borne DNA is first integrated additively by homologous recombination (Hinnen *et al.*, 1978; Struhl *et al.*, 1979) and then the integration site is localized by genetic linkage analysis (Baroni *et al.*, 1986; Kim *et al.*, 1988). The integrative plasmid pMC35 was partially digested with HindIII to obtain linear pMC35 molecules cleaved only once within the insert. This linearization should increase the frequency of homologous chromosomal integration and also target the integrating DNA to a specific chromosomal location from which the integratively transforming DNA was derived (Orr-Weaver *et al.*, 1981). The linear molecules of pMC35 were introduced into the original yeast recipient strain M39-1D. One hundred and eighty seven Ura^+ Thr^+ transformants were obtained, out of which 10 were initially screened for mitotically stable integrative transformants with expected phenotype of MAT@ Arg^+ Thr^+ Ura^+ Trp^-. One of them was arbitrarily chosen as an integrant yeast strain and designated as M39-1D::pMC35 for a subsequent genetic linkage test through classic tetrad analysis.

Genetic localization of the integration site

The structural gene of yeast *THR1* is genetically linked proximal to *ARG4* on the right arm of the chromosome VIII, within variation of genetic distance of approximately 10.0 cM (Mortimer *et al.*, 1989), 13.7 cM (Shultes *et al.*, 1990) and 15.6 cM (Fogel *et al.*, 1979). In order to obtain strains with allelic linkage relationship of recessive coupling state between two linked loci of *thr1* and *arg4*, M33-1C was crossed with X3127-27D. Resulting tetrads were manipulated and screened for spores with desirable genotypes. Three counterpart component strains were thus constructed and designated as M441-1C, M441-15B, and M441-20B (Table 1). The three strains were in turn crossed with the integrant yeast strain M39-1D::pMC35.

Table 3. *Homoserine kinase activity in yeast strains transformed with the plasmids pMC3 and pMC32.*

Strain[1/]	Medium	KHase activity[2/]	
		Unit/mg protein	Ratio to WT
RH218, WT	Complete without Threonine	9.5	1.0
M39-1D/pMC3	Complete without Threonine	145.1	15.3
M39-1D/pMC3	Complete with Thr, 300 mg/*l*	11.1	1.2
M39-1D/pMC3	Complete with Thr, 1190 mg/*l*	0.1	0.0
M39-1D/pMC32	Complete without Threonine	126.4	13.3
M39-1D/pMC32	Complete with Thr, 300 mg/*l*	2.1	0.2

[1/] RH218 was used as a wild type strain for the *THR1* gene. The recipient M39-1D did not grow in minimal medium (GN) and in threonine omission medium (Thr^{-1}).
[2/] One unit of enzyme activity was calibrated from A_{339} of NAD^+, representing the amount of enzyme that catalyses liberation of 1 micromole of substrate phoshphorylated per mg protein per minute.

which allelic linkage relationship was in dominant coupling state between loci of *THR1* and *ARG4*, for the purpose of providing genetic evidence that the cloned DNA segment was in fact originated from the structural gene of *THR1*.

The result of tetrad analysis is presented in Table 2. Segregation data were taken from three separate crosses. Out of a total number of 57 asci analyzed, only 12 asci contained recombinant type spores in tetratype, while no ascus of 4-strand double crossover type or nonparental ditype was observed. As expected, two genes of *THR1* and *URA3* linked on the plasmid pMC35 indeed cosegregated each other in all three crosses, which clearly indicates that Thr^- phenotype of the recipient M39-1D has not been converted or suppressed by any other possible mechanism but truly transformed by integration and expression of the newly introduced DNA. Linkage data further localize the plasmid-borne *thr1* complementing DNA fragment to a position about 8.3-12.5 cM from *ARG4* locus, which falls well in the range of previous genetic data 10.0-15.6 cM, indicating that the cloned DNA has been integrated into the predicted chromosomal locus from which it was originally derived. Therefore, genetic evidence is provided that the gene we have isolated is corresponding to the structural component of yeast homoserine kinase gene *THR1*.

Expression of the cloned *THR1*: HKase activity

In order to confirm through biochemical evidence that the cloned 2.7 kbp DNA fragment contains that *THR1* structural gene, HKase activity was measured in M39-1D yeast cells, which were transformed episomically with an original isolate pMC3 and its subclone pMC32, and then compared with that of wildtype. When cultured in Thr^- liquid medium (complete synthetic medium without threonine), the enzyme HKase was overexpressed 15 and 13 folds in cells transformed with pMC3 and pMC32, respectively. At the concentration of threonine 300 mg/*l* yeast cells with pMC3 responsed with about the same level of HKase activity as compared with the wildtype cells, however, expression of *THR1* gene was inhibited to negligible level in cells with pMC32. Inhibition level of threonine against HKase expression was much higher (1,190 mg/*l*) for the cells haboring pMC3 (Table 3). At the moment, only speculative explanations are possible on this differences in inhibition level of threonine to *THR1* expression between pMC3 and pMC32, which may be related to differences in copy number or insert size between pMC3 (vector YCp50, insert 4.6 kbp) and pMC32 (vector YRp7, insert 2.7 kbp). In conclusion, data support that the cloned DNA fragment contains sequences which are biochemically active for HKase expression, and also that includes necessary regulatory region(s) for control of its proper expresion.

적 요

효모의 *THR1* 유전자는 아미노산 트레오닌 생합성 경로상의 네번째 단계인 homoserine을 O-phosphohomoserine으로 전환시켜 주는 효소 homoserine kinase (HKase)의 구조유전자이다. 트레오닌 생합성에 관여하는 유전자들의

발현조절기작을 연구하기 위한 첫 단계 연구로서, 영양요구성의 상보현상을 이용하여 재조합 플라스미드 pMC3(12.6 kbp, vector YCp50)상으로 *THR1* 유전자를 1차 단리하였다. pMC3 삽입부위내의 2.7 kbp HindIII 절편을 2차 클로닝하여 pMC32(8.6 kbp, vector YRp7)와 pMC35 (8.3 kbp, vector YIp5)를 얻은 후, 이들을 세포내로 재도입시켜 본 결과 효모의 *thr1*과 대장균의 *thrB* mutation을 모두 상보하였다. pMC35로 형질전환시킨 효모세포 중에서 염색체내로 안정하게 통합된 균주를 선별하고, 사분체연관분석법을 통하여 pMC35가 통합된 부위는 예상했던 대로 효모 제6번염색체 우완상의 *ARG4*로부터 약 11.1 cM의 유전적 거리에 위치하는 *THR1* 구조유전자 좌위임을 확인하였다. 또한 *THR1*의 발현산물인 HKase의 활성을 측정하여본 결과, pMC3과 pMC32로 형질전환된 효모 균주들은 트레오닌 결핍 배지에서 각각 15배와 13배로 초과발현 되었으며, 발현억제 수준은 각각 트레오닌 1,190 mg/*l*와 300 mg/*l*이었다.

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