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Cloning and characteristics of a positive regulatory gene, *THI2* (*PHO6*), of thiamin biosynthesis in *Saccharomyces cerevisiae*

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A *thi2(pho6)* mutant of *Saccharomyces cerevisiae*, defective in the expression of structural genes for thiamin-repressible acid phosphatase and enzymes involved in thiamin biosynthesis, was found to retain sufficient thiamin transport activity. The transport activity was repressed by thiamin in growth medium. We isolated from a *S. cerevisiae* genomic library two hybrid plasmids, pTSR1 and pTSR2, containing 10.2- and 12.0-kilobase (kb) DNA fragments, respectively, which complement the *thi2(pho6)* mutation of *S. cerevisiae*. This gene was localized on a 6.0-kb *Cla*I–*Cla*I fragment in the subclone pTSR3. Complementation of the enzyme activities for thiamin metabolism in the *thi2(pho6)* mutant transformed by some plasmids with the *THI2(PHO6)* gene was also examined.

Cloning; Positive regulatory gene; Thiamin metabolism; *Saccharomyces cerevisiae*

1. INTRODUCTION

The thiamin-repressible acid phosphatase (T-rAPase) in the periplasmic space of *Saccharomyces cerevisiae* is coded by the *PHO3* gene [1] and requires two additional complementary genes, *PHO6* and *PHO7*, for its synthesis [2]. In a previous paper [3], we indicated that a *pho6* mutant was auxotrophic for thiamin and the *PHO6* gene was involved in the regulation of the synthesis not only of T-rAPase but also of enzymes synthesizing thiamin monophosphate from 2-methyl-4-amino-5-hydroxymethyl-pyrimidine (hydroxymethylpyrimidine) and 4-methyl-5-β-hydroxyethylthiazole (hydroxyethylthiazole) in *S. cerevisiae* [4–7]. Our previous study also showed that the activities of these enzymes are coordinately repressed by exogenous thiamin and that these activities were decreased in correlation with the increase in the intracellular thiamin pyrophosphate level in yeast cells [3]. Recently, we isolated the *thi80* mutant which has a partial deficiency of thiamin pyrophosphokinase (EC 2.7.6.2). The mutant had the constitutive phenotype of thiamin transport, T-rAPase, and enzymes involved in thiamin synthesis from hydroxymethylpyrimidine and hydroxyethylthiazole [8], suggesting that thiamin pyrophosphate is a negative effector of the regulatory mechanism in the thiamin metabolism of *S. cerevisiae*. These results indicated that the coordinated expression of T-rAPase and enzymes involved in the thiamin biosynthesis in *S. cerevisiae* is regulated positively by *PHO6*, whereas the expression of thiamin transport, T-rAPase and these enzymes for thiamin synthesis is controlled negatively by the intracellular thiamin pyrophosphate. Since *PHO6* was demonstrated to be closely related to thiamin metabolism, we redesignated this gene as *THI2*.

In this paper, we report that the *thi2(pho6)* mutant defective in a regulatory gene for enzymes involved in thiamin metabolism described above retains sufficient thiamin transport activity, and we have also isolated two hybrid plasmids, pTSR1 and pTSR2, containing a 10.2- and 12-kb DNA fragment, respectively, from a *S. cerevisiae* genomic library which complements the *thi2(pho6)* mutation of *S. cerevisiae*. Furthermore, this gene was localized on a 6.0-kb *Cla*I–*Cla*I fragment in the subclone pTSR3. These results show that gene expression for T-rAPase and thiamin-synthesizing enzymes in *S. cerevisiae* is subject to the positive regulatory gene, *THI2(PHO6)*, whereas yeast thiamin transport system, which is also repressed by thiamin, is not governed by this regulatory gene.

2. MATERIALS AND METHODS

2.1. *Strains and media*

The *thi2(pho6)* mutant, O58-M5(*MATathi2(pho6-1)*gal4), isolated from a haploid strain of *S. cerevisiae*, H42 (*MATagal4*) [1], was kindly supplied by Y. Oshima (Department of Fermentation Technology, Osaka University, Osaka, Japan). The other yeast strains, IFO 10483 (*MATαleu2-3,112gal2*) and KYC272-7C (*MATαthi2(pho6-7)leu2-3,112 ura3 trp1*), were constructed during this study. *Escherichia coli* strains used were DH5α and MV1184. Yeast strains were cultured at 30°C in YPD medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose) or in a defined medium containing 0.67% yeast-nitrogen base (Difco Laboratories) supplemented with essential amino acids or in

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Wickerham's synthetic medium supplemented with essential amino acids with or without thiamin [9]. *E. coli* cells were cultured in LB medium (0.5% yeast extract, 1% Bacto-Peptone, 1% NaCl, 0.2% Glucose) at 37°C. When necessary, the medium was supplemented with ampicillin (20 μg/ml).

2.2. *Transport and enzyme assays*

The transport of thiamin was determined by the method described previously [10]. T-rAPase activity with *p*-nitrophenyl phosphate as a substrate was determined from the amount of *p*-nitrophenol produced as described earlier [11].

Yeast cells were harvested, washed once with cold water, then suspended in 0.05 M potassium phosphate buffer (pH 7.5) containing 1 mM 2-mercaptoethanol and 1 mM EDTA. The cell suspensions were sonicated, and after centrifugation at 28,000 × *g* for 30 min the supernatant was used as a crude extract. Overall thiamin-synthesizing enzyme activity from hydroxymethylpyrimidine and hydroxyethylthiazole and the activity of thiamin phosphate pyrophosphorylase were assayed as previously described [3].

2.3. *Transformation and DNA preparations*

K272-7c (*thi2(pho6)* mutant) was transformed after lithium acetate treatment as described by Ito et al. [12]. Bacterial transformations were performed as described by Maniatis et al. [13]. Plasmid DNA from yeast cells was isolated as described by Sherman et al. [14] with some modification. Bacterial plasmids were prepared by the alkaline lysis method of Maniatis et al. [13].

2.4. *Cloning and subcloning of* THI2(PHO6) *gene*

A recipient strain, K272-7c, was transformed with a gene library by *S. cerevisiae* YCp 50 "CEN BANK" A, obtained from the American Type Culture Collection (Rockville, MD). Transformants were screened for the ability to grow without uracil and thiamin on Wickerham's synthetic medium. After 3 days of incubation at 30°C, colonies were selected for further analysis. Two plasmids, pTSR1 and pTSR2, that complemented mutations in *THI2(PHO6)* were obtained. Plasmids were constructed by subcloning into a multicloning vector pRS316.

2.5. *Plasmids, enzymes and chemicals*

Plasmid YCp50 [15] was obtained from the American Type Culture Collection (Rockville, MD) and plasmid pRS316 was provided by Philip Hieter (John Hopkins University, School of Medicine, Baltimore, MD). Restriction enzymes were purchased from Toyobo Co. (Osaka, Japan), Nippon Gene (Toyama, Japan) and Takara Shuzo (Kyoto, Japan). [^{14}C]Thiamin ([*thiazole*-2-[^{14}C]thiamin hydrochloride, 24.3 Ci/mol) was purchased from Amersham International (Buckinghamshire, UK). All other chemicals were purchased from commercial suppliers.

3. RESULTS AND DISCUSSION

3.1. *Thiamin transport activity in* thi2(pho6) *mutant strain*

The *thi2(pho6)* mutant defective in a regulatory gene for the synthesis of T-rAPase [2] was auxotrophic for thiamin, and the activities of enzymes involved in thiamin synthesis were markedly low in the crude extract from the *thi2(pho6)* mutant [3]. However, as shown in Fig. 1, thiamin transport activity in the mutant cultured in minimal medium with thiamin (~2 × 10^{-8} M) was retained, in a manner similar to that of the wild-type strain, H42. When the thiamin transport activity of the wild-type strain and the *thi2(pho6)* mutant in minimal medium supplemented with various concentrations of thiamin was determined, the activities of both strains decreased with increasing concentration of thiamin. This suggests that there is a negative regulatory factor for the thiamin transport system in the *thi2(pho6)* mutant as well as the wild-type strain that functions to control the transport activity depending on the intracellular thiamin pyrophosphate level [8]. Furthermore, the transport activity in the mutant showed a weak constitutive phenotype compared with that in the parent strain (Fig. 1). Since it was found that the *thi2(pho6)* mutant had reduced thiamin pyrophosphokinase (0.69 nmol/mg of protein/30 min) compared with that of the wild-type strain (10.2 nmol/mg of protein/30 min), the lower level of thiamin pyrophosphokinase in the mutant cells may result in the weak constitutivity for thiamin transport activity. These results indicate that the gene expression of T-rAPase and enzymes involved in thiamin synthesis is controlled positively by the *THI2(PHO6)* gene which is not involved in the expres-

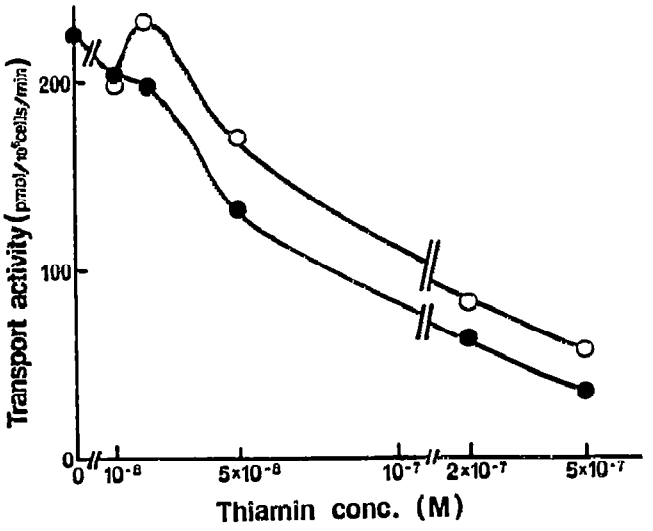

Fig. 1. Effect of thiamin added to the growth medium on activities of thiamin transport in the wild-type strain (H42) and the *thi2(pho6)* mutant (O58-M5). Wild-type strain, ●; *thi2(pho6)* mutant, ○.

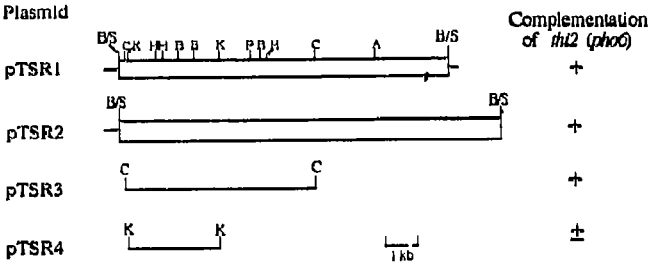

Fig. 2. Restriction map of a *S. cerevisiae* DNA insert in pTSR1 and subcloning of the DNA fragments with the ability to complement the *thi2(pho6)* mutation. The broken line represents a deleted fragment. Complementation of *thi2(pho6)*: +, complements; ±, weakly complements. Restriction enzyme cleavage sites: A, *Apa*I; B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*II; S, *Sau*3AI.

sion of the thiamin transport. Thus, the thiamin transport system in *S. cerevisiae* appeared to be regulated by a positive regulatory gene other than this gene.

3.2. *Cloning of yeast DNA gene fragment carrying the* thi2(pho6) *gene*

The *THI2(PHO6)* gene was identified on a recombinant DNA plasmid by its functional complementation of a *thi2(pho6)* mutation in K272-7c (*thi2,ura3*). The *S. cerevisiae* genomic DNA library was used to transform K272-7c. After alkaline cation treatment [12], the transformed cells were plated on agar containing Wickerham's synthetic medium without thiamin and uracil. The plates were incubated at 30°C for 3 days, and Thi^+ and Ura^+ colonies were isolated. DNA prepared from these transformants was used to transform *E. coli* DH5α to Amp^+. Two plasmids obtained from the Amp^+ transformant conferred the thiamin prototrophic phenotype on yeast strain K272-7c (*thi2,ura3*) in minimum medium without thiamin and uracil. As shown in Fig. 2, these plasmids, pTSR1 and pTSR2, had insertions of the 10.2- and 12.0-kb DNA fragment, respectively, in YCp50. Several deletion fragments were constructed into the multicloning vector pRS316 from the cloned 10.2-kb fragment in pTSR1 (Fig. 2). Plasmid pTSR3, constructed by subcloning a 6.0-kb *Cla*I–*Cla*I fragment into a pRS316, complemented the *thi2* mutation, and plasmid pTSR4, bearing the 3.5-kb *Kpn*I–*Kpn*I fragment into pRS316, weakly complemented the *thi2* mutation. This result suggests that either of the *Kpn*I sites are located within a region necessary for restoring the Thi2 function. The 6.0-kb *Cla*I–*Cla*I DNA fragment derived from plasmid pTSR3 against yeast chromosomes that had been resolved by pulsed-field electrophoresis hybridized only to one chromosome which was identified, by comparison with a standard, as chromosome II (data not shown).

3.3. *Expression of the activities of T-rAPase and enzymes involved in thiamin synthesis of the* thi2 *mutant transformed by* TH12(PHO6) *gene*

The effect of some plasmids bearing the *THI2* gene on thiamin metabolism was examined by measuring the activities of T-rAPase and enzymes involved in thiamin synthesis. Transformants of the *thi2(pho6)* mutant, K272-7c, harboring the low-copy numbered vector plasmid YCp50, pRS316 and four plasmid (pTSR1, pTSR2, pTSR3 and pTSR4) were cultured in the absence of thiamin and assayed for T-rAPase activity (Table I). The T-rAPase activity in these transformants harboring the *THI2(PHO6)* gene was less active than the wild-type strain and its activity in the recipient cells transformed with the control plasmid, YCp50 and pRS316, were as markedly low as that of the *thi2(pho6)* mutant, O58-M5 [2]. Furthermore, the activity in these transformants with pTSR1, pTSR2 and pTSR3 was repressed by thiamin (2×10^{-7} M) in the minimal medium in the same manner as that of the wild-type strain. These results suggest that the DNA fragments inserted in pTSR1, pTSR2 and pTSR3 contain a full length promotor region. The T-rAPase in a transformant cell with pTSR4, which weakly complemented the thiamin auxotroph, was appreciably low. The overall-enzyme activity for thiamin synthesis from hydroxymethylpyrimidine and hydroxyethylthiazole and the activity of an enzyme involved in the formation of thiamin monophosphate (thiamin phosphate pyrophosphorylase) in the crude extract of these transformants which cultured in thiamin-deficient Wickerham's synthetic medium were also assayed (Table I). In the crude extract of the cells transformed with pTSR1, pTSR2 and pTSR3, the activities of overall enzyme reaction for thiamin synthesis and thiamin phosphate pyrophosphorylase were the same as those of the wild-type strain. These activities in the extracts of the recipient cells

Table I

Activities of T-rAPase and thiamin-synthesizing enzymes in the *thi2(pho6)* mutant strain transformed by various plasmid-borne DNA fragments

Strain	Plasmid	T-rAPase[a] (nmol/10^6 cells/5 min)		Enzyme activity (nmol/mg of protein/30 min)[a]	
		None	0.2 μM[b]	Overall reaction[c]	Thiamin phosphate pyrophosphorylase
IFO 10483	None	20.5	0.83	0.49	11.1
K272-7c (*thi2* mutant)	YCp50[d] (vector)	0.4	–	0.01	0.2
	pTSR1	6.6	0.35	0.23	9.8
	pTSR2	7.1	0.78	0.28	8.6
	pRS316[d] (vector)	0	–	0	0.3
	pTSR3	7.2	0.71	0.42	12.7
	pTSR4	1.9	–	–	–

[a]Each value is the mean of two experiments.
[b]Thiamin (0.2 μM) was added to the growth medium.
[c]Overall thiamin-synthesizing enzyme activity from hydroxymethylpyrimidine and hydroxyethylthiazole.
[d]Strains harboring control vector were cultured in Wickerham's synthetic medium supplemented with 10^{-8} M thiamin.

transformed with the control plasmids, YCp50 and pRS316, were also as markedly low as those of the *thi2*(*pho6*) mutant, O58-M5 [2].

The system regulating the synthesis of phosphatases (*PHO* system) in *S. cerevisiae* has been investigated extensively by Oshima and his co-workers. The gene expression of phosphatase is controlled in response to a simple effector, inorganic phosphate, and two positive regulatory genes, *PHO2* and *PHO4*, are indispensable for the transcriptional control of the structural genes of repressible acid phosphatase encoded by *PHO5* in *S. cerevisiae* [16]. In yeast thiamin metabolism the positive regulatory gene, *THI2*(*PHO6*), is indispensable for the transcriptional control of the structural genes of T-rAPase and enzymes involved in thiamin synthesis from hydroxymethylpyrimidine and hydroxyethylthiazole. This regulatory system of thiamin metabolism appears of interest to further investigate in connection with the *PHO* regulatory system in *S. cerevisiae*.

Studies are currently underway to determine the nucleotide sequence of the *THI2*(*PHO6*) gene, after which investigation at the molecular level will be required to elucidate the mechanism of the regulation of thiamin metabolism in *S. cerevisiae*.

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