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Molecular Cloning and Restriction Analysis of Aspartokinase Gene (*HOM3*) in the Yeast, *Saccharomyces cerevisiae*

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아스파테이트족 아미노산 대사에 관여하는 효모유전자(*HOM3*)의 클로닝 및 구조분석

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ABSTRACT: The yeast gene *HOM3* encodes aspartokinase, which catalyses the first step (aspartate to and from beta-aspartyl phosphate) of common pathway to threonine and methionine. The yeast *HOM3* gene expression is known to be regulated by threonine and methionine specific control, and also by general control of amino acid biosynthesis. Isolation and characterization of the *HOM3* gene are essential for the molecular genetic study on its regulation of expression. A recombinant plasmid pSC3 (15.5kb, vector YCp50) has been cloned into *E. coli* HB101 from yeast genomic library through their complementing activity of *HOM3* mutation in a yeast recipient strain M34-24B. Organization of the plasmid was characterized by delineation of restriction cleavage sites in the insert fragment.

KEY WORDS □ ***Saccharomyces cerevisiae*, Aspartokinase gene(*HOM3*), Molecular cloning, Restriction map.**

Yeast aspartokinase (ATP: L-aspartate 4-p -transferase, EC 2.7.2.4.) catalyses the interconversion of aspartate and beta-aspartylphosphate, which is the first step in the three reactions leading aspartate to homoserine on a common pathway for threonine and methionine biosynthesis in the yeast, *Saccharomyces cerevisiae*. The structural gene (*HOM*3) for aspartokinase is located on the right arm of chromosome V (De Robichon-Szulmajster *et al.*, 1966 and 1973). Regulation of *HOM*3 gene expression is under threonine and methinone specific control and also under general control or coregulation of amino acid biosynthesis in yeast. The level of aspartokinase is decreased 4-7 fold by addition of threonine or homoserine to the medium and is also repressed threefold by lysine. Starvation for threonine, as effected by starvation of a threonine auxotroph or by addition of a threonyl-tRNA synthetase inhibitor, borrelidin, causes derepression of aspartokinase synthesis. A weak repression of aspartokinase is caused by addition of methionine to the medium. Synthesis of aspartokinase is derepressed by starvation of a methionine auxotroph for methionine (De Robichon *et al.*, 1973; Jones and Fink, 1982; Cherest *et al.*, 1969 and 1971).

Biochemical study on regulatory aspect of threonine and methionine biosynthesis through the enzyme activity and protein quantity does not provide exact information on fine levels of regulation; transcriptional, translational or enzymatic. In order to study the regulation of *HOM*3 expression, especially in relation to coregulation of the genes on the common and the branch pathways to threonine and methionine, we have cloned the *HOM*3 gene by compementation of *hom*3 muta-

tion through yeast transformation using a genomic library constructed in a centromeric plasmid vector YCp50 (8.0kb).

MATERIALS AND METHODS

Strains

E. coli strain HB101 (ATCC, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, USA) was exclusively used for maintenance, amplification and isolation of recombinant plasmids and vectors. The relevant genotypes and sources of yeast, *Saccharomyces cerevisiae* strains for the present work are shown in Table 1. Yeast strain RH218 was used as a wild type (*HOM3*) control of the mutant type (*hom3*) for yeast aspartokinase gene activity. The recipient strain M34-24B for initial yeast transformation was constructed according to the methods of Sherman *et al.* (1982) for mating, sporulation, micro-dissection of zygotes and screening for desired marker genes. Diploids were screened on selective agar plates by nutritional complementation activity in a diploid state. When markers do not complement each other in a diploid, mating mixture was streaked on a standard rich medium (YPD) to obtain single colonies. The larger colonies in size were selected as diploids and further confirmed by sporulation. Yeast ascus was partially digested with zymolyase-100T (Kirin Brewery Co., Tokyo, Japan) before being dissected under a microscope with the mechanical micromanipulator (Lawrence Precision Machine, 1416 W. Winton Ave, Hayward, California, USA). Four progeny spores from each ascus were germinated on YPD plates and screened for strains with the desired genetic markers.

Plasmids and enzymes

Drs. F. LaCroute (Strasbourg, Cedex, France), R. Davis (Stanford, Ca, USA), and A. Wright (Imperial College, London, UK) kindly provided the yeast-*E. coli* shuttle vectors and yeast genomic libraries. The yeast genomic library consisted of pooled recombinant plasmids, in each of which a partial Sau3A fragment of the yeast chromosomal sequence is inserted into the BamHI site of YCp50 (Parent *et al.*, 1985). Enzyme and fine chemicals were purchased from KOSCO Biotechnology Lab., Seoul, Korea and Korean representatives of Sigma Chemical Co. (St. Louis, Mo, USA), New England BioLabs (Beverly, Mass, USA) and Promega Biotech (Madison, Wi, USA).

Media and growth conditions

LB medium for *E. coli* culture was prepared according to Maniatis *et al.* (1982). Solid media contained 1.5% agar (Difco Laboratories, Detroit, MI, USA). Ampicillin (200mg/liter) and tetracycline (15mg/liter) were added to both liquid and solid media, when necessary. Minimal (GN), standard rich (YPD), synthetic complete(COM), selective omission, and selective addition media were prepared according to Sherman *et al.* (1982). Yeast cells were grown in incubators, shaking water baths or on a rotary spinner at 30°C. Cell growth in liquid culture was monitored with a spectrophotometer at A600 or Klett-Summerson colorimeter (A500-A570); 100 Klett units is equivalent to approximately 3×10^7 cells/m*l* for the budding yeast. Homoserine auxotrophic cells were selected on homoserine omission plates; COM medium without methionine and threonine. The original *hom3* marker from YGSC was identified and confirmed by complementation test in a diploid from a cross between strains with one of each marker of *hom3*, *hom2* and *hom6*.

Preparation of DNA

Plasmid DNA from yeast transformants was prepared following procedures by Nasmyth and Reed (1980) and from *E. coli* by Tschumper and Carbon (1980). For rapid analysis, the alkaline extraction procedure of Birnboim and Doly (1979) was also used as a small scale preparation. In the small preparations, the cleared lysate of *E. coli* cells was precipitated with ethanol prior to enzyme digestion and agarose gel electrophoresis. For genomic library and for back-transformation into the original recipient yeast strain, plasmid DNA was purified by CsCl ultracentrifugation.

Yeast and *E. coli* transformation

Yeast cells were transformed by the methods of Hinnen *et al.* (1978) with modifications as described by Tschumper and Carbon (1980). The

whole cell transformation method by Ito *et al.* (1983) and by Bruschi *et al.* (1987) were also adapted to back-transformation of isolated sequences into original recipient M34-24B. Transformation of *E. coli* HB101 was as described by Tschumper and Carbon (1980).

RESULTS AND DISCUSSION

Construction of a yeast recipient strain

The yeast strain K382-19D was crossed with RH218 (Mating serial number M13, Table 1). K382-19D had the target marker gene for cloning *hom*3 and one of a commonly used vector marker *ura*3. RH218 contained the vector marker *trp*1 and highly transformable genetic elements. The resulting diploid cells were sporulated, the asci were dissected, and the germinating spores were scored to select a strain M13-17A which possessed the desired genetic markers (*trp*1 and *ura*3 for yeast-*E. coli* shuttle vector selection and *hom*3 as the target gene for isolation). The M13-17A had been backcrossed to RH218 again to improve transformation efficiency of the progeny segregant M22-36, which unfortunately lost one of vector marker *trp*1. M22-36 was mated with YNN 281, which is highly transformogenic and also contains *trp*1 marker. M32-12B was selected as a result of marker test of germinating progeny spores. Strains with desirable markers were selected among germinating spores only from asci in which all markers segregated in normal 2:2 mendelian ratios to secure that the selected markers are chromosomal. M32-12B was crossed with LA196 -504D (M34), which is characterized with high sporulation rate in diploid condition. The final recipient strain M34-24B had been selected from the germinating progeny spores of M34 asci. The M34-24B contained all the desired genetic markers and was observed to be highly transformable. It also demonstrated high sporulation rate in diploids.

Table 1. *List of yeast strains.*

Mating serial No.	Designation	Relevant genotype	Source[a]
M13	RH218	MATa trpl	YGSC
	K382-19D	MAT@ his7 hom3 tyrl ura3	YGSC
M22	RH218	MATa trpl	YGSC
	M13-7A	MAT@ hom3 trpl ura3	
M32	YNN281	MATa his3 lys2 trpl ura3	YGSC
	M22-36	MAT@ his7 hom3 ura3	
M34	LA196-504D	MATa	YGSC
	M32-12B	MAT@ hom3 trpl ura3	
	M34-24B	MAT@ hom3 trpl ura3	

[a]YGSC: Yeast Genetic Stock Center, University of California, Berkeley, California 94720, USA. All other strains are from this work.

Isolation of a recombinant plasmid containing yeast DNA sequence with the *hom*3 complementing activity

The genomic library, constructed in a centromeric vector YCp50, was used to transform spheroplasted cells of the yeast recipient strain M34-24B. In yeast, simultaneous (double) selection from the regeneration plates is usually unsuccessful for a target gene and a vector marker. DNA-treated cells were first spread on uracil-omission plates to select cells with the vector marker *URA*3. Then, approximately 3×10^3 Ura+ cells were picked with tooth-picks and replicated onto homoserine-omission plates to select cells which harbor plasmids with the target gene *HOM*3 sequence, as identified by its complementing activity of the *hom*3 mutation in the recipient M34-24B. Four homoserine prototrophic yeast transformants were obtained from the initial transformation. When cultured in liquid rich medium YPD, curing rate of yeast transformants was about 1.4% per generation, which fell within the normal range (0.5-5%) of curing rate for the yeast centromeric plasmids. The cured cells lost concomitantly the Ura+ and Hom+ phenotypes, which indicated that *URA*3 and *HOM*3 genes were on the same recombinant plasmid. It is expected if the origin of the marker genes was the genomic library used in the original yeast trans-

formation. Crude yeast plasmid DNA was prepared from one of the transformants KC32, and used to transform *E. coli* HB101, while selecting for ampicillin resistant transformants. Two *E. coli* transformants were obtained and replicated onto tetracycline plates to confirm that they harbor recombinant plasmids with inserts at the BamHI site of pBR322. The plasmids from the two *E. coli* transformants were identified to be the same through PstI digestion of plasmid DNA from 5-m*l* cultures, and designated as pSC3. The pSC3 was amplified in *E. coli* HB101, extracted on a large scale, and purified by CsCl ultracentrifugation. The purified plasmid was used for back-transformation into the original recipient yeast strain M34-24B. The recombinant plasmid pSC3 transformed successfully the homoserine auxotrophic M34-24B to prototrophic M34-24B/pSC3, confirming the yeast *hom3* complementing activity of the sequence contained in the insert of pSC3. Cell growth of the back-transformant M34-24B/pSC3 was compared with that of wild type RH218 in the homoserine omission liquid cultures and demonstrated an equivalent growth to that of RH218 (Fig. 1). The original *hom3* mutation was proved to be a mutation of aspartokinase structural gene (De Robichon *et al.*, 1966 and 1973). Therefore it is least likely that the isolated sequence would represent any other genetic element than a structural gene itself. However, integration test of the pSC3 insert, Northern analysis, and direct enzyme assay would provide additional proof that the isolated sequence contains the structural gene (*HOM3*) for the yeast aspartokinase.

Characterization of the pSC3 insert through restriction mapping

Fragments from double digestion were analyzed to determine the relative position of the cleavage sites in the insert. The size of pSC3 insert was estimated to be 7.5kb. Small descrpancy (0.1-0.5kb) between sums of fragments from each double digestion was adjusted to equal the total size of the best estimate 15.5kb. Physical organization of the pSC3 insert fragment has been characterized by the determination of the cleavage sites of restriction endonuclease as shown in Fig. 2.

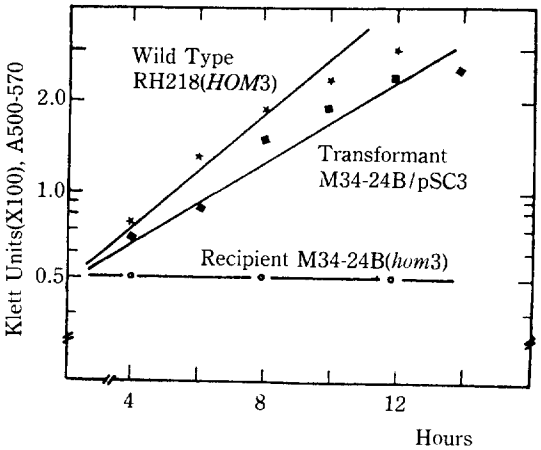

Fig. 1. *Cell growth of yeast strains in synthetic complete liquid medium without threonine and methionine at 30C.*
RH218; Wild type(*HOM3*)-homoserine prototroph, M34-24B; Recipient strain for transformation-homoserine auxotroph(*hom3*), M34-24B/pSC3; Yeast transformant harboring recombinant plasmid pSC3. Cell turbidity was measured with a Klett-Summerson colorimeter.

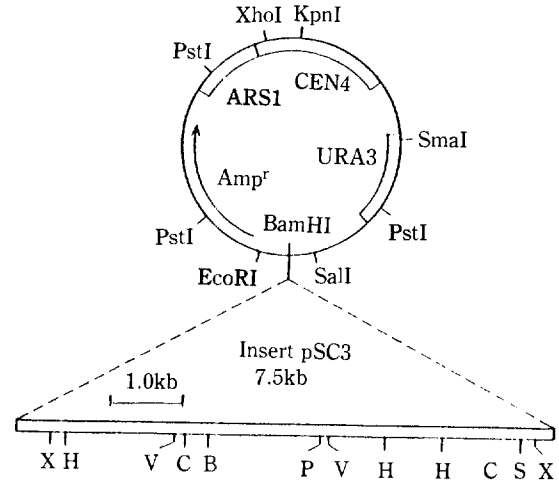

Fig. 2. *Restriction map of the recombinant plasmid pSC3 (15.5kb) containing yeast DNA sequence with hom3 complementing activity in the insert (7.5kb).*
Single line; pBR322. Double line; yeast sequence. B; BamHI, C; ClaI, H; HindIII P; PstI, S; SacI, V; PvuII, X; XbaI.

적 요

효모의 *HOM3*는 트레오닌과 메치오닌 생합성의 공통경로의 첫단계인 아스파테이트를 베타 아스파틸 포스페이트로 전환시켜 주는 아스파토카이네이즈 효소의 구조유전자이며, 제 5번 염색체의 우완에 위치한다. *HOM3* 유전자의 발현은 트레오닌 첨가에 의하여 4-7배로 억제되며 메치오닌 첨가에 의하여 약간의 억제효과를 보인다. 한편, 라이신 첨가에 의하여 억제현상을 보이는 것으로 보아 아미노산 생합성 과정의 일반조절기작도 또한 이 유전자의 발현조절에 관여하는 것으로 생각되어 왔다. 본 연구에서는 영양요구성의 상보현상을 이용한 효모 형질전환의 방법으로, 전체 크기가 약 15.5kb인 재조합 플라스미드 pSC3의 삽입부위내에 효모의 *HOM3* 유전자를 분자 클로닝하고 이에 대한 제한효소지도를 작성하였다.

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