KOR. JOUR. MICROBIOL. December, 1986 p. 357-363 Vol. 24, No 4

Molecular Cloning and Restriction Endonuclease Mapping of Homoserine Dehydrogenase gene (*HOM6*) in Yeast, *Saccharomyces cerevisiae*

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Aspartate계 아미노산 대사 효모 유전자 *HOM6*의 Cloning 및 구조분석

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Abstract: Synthesis of threonine and methionine in yeast, *Saccharomyces cerevisiae* shares a common pathway from aspartate via homoserine. *HOM6* gene encodes homoserine dehydrogenase (HSDH) which catalyzes the inter-conversion of beta-aspartate semialdehyde and homoserine. The level of HSDH is under methionine specific control. A recombinant plasmid (pEK1 : 13.3 kb), containing *HOM6* gene, has been isolated and cloned into *E. coli* by complementary transformation of a homoserine auxotrophic yeast strain M20-20D (*hom6*, *trp*1, *ura3*) to a prototrophic M20-20D/pEK1, using a library of yeast genomic DNA fragments in a yeast centromeric plasmid, YCp50 (8.0kb). Isolation of *HOM6* has been primarily confirmed by retransformation of the original yeast strain M20-20D, using the recombinant plasmid DNA which was extracted from M20-20D/pEK1 and subsequently amplified in *E. coli*. Eleven cleavage sites in the insert (5.3kb) have been localized through fragment analysis for 8 restriction endonucleases; Bgl II (1 site), Bgl II (1), Cla I (3), Eco RI (1), Hind III (2), Kpn I (1), Pvu II (1) and Xho I (1).

Key words: *Saccharomyces cerevisiae*, Homoserine dehydrogenase gene (*HOM6*), Restriction map.

Biosynthesis of amino acid in yeast is regulated at two levels; the regulation of enzyme synthesis by control of gene expression and the regulation of enzyme activity by control of metabolite flow between cell compartments. The enzymes for amino acid biosynthesis also can be under general and specific control, affected by metabolites of end products of the cross and specific pathway, respectively. Regulation by general control system for amino acid biosynthesis is mediated by positive (GCN) and negative (GCD) regulatory genes. Specific control system is mediated by trans-acting and cis-dominant regulatory elements(Jones and Fink, 1982).

Classical genetic and biochemical techniques do not allow us distinguish a promoter mutation from nonsense or frameshift mutations located early within that gene. Resolution power of genetic fine-structure analysis is not strong enough to delineate the coding and non-coding regions. A cloned segment of the gene therefore is required to identify regulatory mutations defective in transcription through

measurement of mRNA levels by hybridization, draw a picture of gene composition and to localize the mutation by DNA sequence analysis. A cloned gene also permits in vitro mutagensis. Cis-dominant regulatory mutations at *HIS3* and *HIS 4* were identified, using these recombinant DNA procedures (Jones and Fink, 1982).

Cloned yeast genes for amino acid biosynthesis have been playing a key role in studies of gene composition and regulatory mechanisms at the molecular level; among them are *ARG4* (Hsiao and Carbon, 1979), *GDH1* (Moye *et al.*, 1985), *HIS2* (Malone and Cramton, 1985), *HIS3* (Hope and Struhl, 1985), *HIS4* (Roeder *et al.*, 1980), *ILV1* and *ILV5* (Holmberg *et al.*, 1985), *ILV2* (Falco and Dumas, 1985), *LEU2* (Chinault and Carbon, 1979), *LEU4* (Beltzer *et al.*, 1986), and *TRP1* (Tschumper and Carbon, 1980).

Three reactions leading from aspartate to homoserine are shared for synthesis of threonine and methionine. A catalytic reaction of aspartokinase encoded by *HOM3* gene activates the beta-carboxyl group of aspartate, followed by reduction of beta-aspartyl phosphate to aspartate semialdehyde catalyzed by aspartate semialdehyde dehydrogenase (*HOM2*). *HOM6* gene encodes homoserine dehydrogenase, which catalyzes the interconversion of aspartate semialdehyde and homoserine. The pathways for methionine and threonine biosynthesis diverge from this point. The mechanism of regulatory control is complex and interwoven for this common part of the pathway. Aspartokinase responds to a threonine-specific signal or to multivalent repression (by threonine and methionine), which might reflect a possible cross-pathway control system. Aspartate semialdehyde dehydrogenase dose not respond to a specific singnal since neither end product represses. Addition of methionine but not threonine represses the level of homoserine dehydrogenase (HSDH) by a factor of two. Starvation of a methionine auxotroph increases enzyme level by derepression, but starvation of a threonine auxotroph does not alter enzyme levels (de Robichon-Szulmajster *et al.*, 1973). The evidence suggests that HSDH is controlled by a methionine-specific signal but not by cross pathway signals (Jones and Fink, 1982).

Knowledge on the regulation of enzymes on the methionine and threonine pathway is largely indirect and inferential. Cloned DNA fragments of regulatory regions and structual genes for the corresponding enzymes should provide an opportunity to investigate the regulatory mechanisms related to structural composition, transcription and regulation of the genes on this pathway. As the first step to approach the above aim, we have isolated the *HOM6* gene in a centromeric yeast-*E. coli* shuttle vector (YCp50) in a 5.3kb insert and have analyzed digestion fragments to delineate the cleavage sites of restriction endonucleases.

MATERIALS AND METHODS

Strains and culture conditions

The sources and genotypes of yeast, *Saccharomyces cerevisiae* strains used in this work are compiled in Table 1. The *E. coli* strain HB101 was from ATCC, Amercian Type Culture Collection 12301 Parklawn Drive, Rockville, Maryland 20852, USA, and used for transformation and amplification of plasmids. Yeast strains were obtained from the Yeast Genetic Stock Center, University of California Berkeley, Berkeley, California 94720, USA. Media and cultrue conditions were the same as described in Fink and Hicks (1982) and Maniatis *et al.* (1982). Homoserine auxotrophic strains were selected on homoserine dropout plates; defined complete medium without methionine and threonine. Cell growth was monitored with a Klett-Summerson colorimeter; 100 Klett units is approximately equivalent to 3×10^7 cells/m*l*.

Plasmids and enzymes

Plasmids and yeast genomic libraries were obtained from Francois LaCroute (Strasbourg, Cedex, France), and Ronald Davis (Stanford, California, USA). The original genomic library

was constructed by Gerald Fink (Cambridge, Massachusetts, USA). Sau3A fragments of the yeast chromosomal DNA were inserted into the BamHI site of a centromeric vector YCp50 (8.0kb) to construct the genomic library. Enzymes were purchased from New England BioLabs (Beverly, Massachusetts, USA).

DNA preparations

Plasmid DNA was purified as described by Tschumper and Carbon (1980). Bank DNA for yeast transformation and crude yeast plasmids for *E. coli* transformation were prepared following procedures by Nasmyth and Reed (1980). *E. coli* transformants were screened for harbored plasmids by a scaled down (5m*l*-culture) DNA preparation (mini-prep) of procedures as described by Tschumper and Carbon (1980). The CsCl-ultracentrifugation was omitted in mini-preps, and the supernatent was precipitated with ethanol before restriction enzyme digestion and agarose gel electrophoresis.

Yeast and *E. coli* transformation

Yeast cells were transformed by the procedures of Hinnen *et al.* (1978) with modifications as described by Tschumper and Carbon (1980). *E. coli* transformations were a modification of Mandel and Higa's method as described by Tschumper and Carbon (1980).

Table 1. *Strain list*

Mating No.	Strain	Genotype	Source[a]
M11	RH218	MATa trp1 gal2 mal SUC2	YGSC
	STX272-4D	MAT@ ade1 ade6 his2 his4 CUP1 hom6 leu1 ura1 ura3	YGSC
M17	RH218	MATa trp1	YGSC
	M11-7D	MAT@ hom6 ura3	This work
M20	RH218	MATa trp1	YGSC
	M17-37D	MAT@ hom6 trp1 ura3	This work
	M20-20D	MAT@ hom6 trp1 ura3	This work

[a]YGSC; Yeast Genetic Stock Center, University of California Berkeley, Berkeley, California 94720, USA.

Strain construction

Yeast strain with the target gene (*HOM6*) for molecular colning was mated with strain RH218 with high transformation efficiency and the diploid cells were sporulated. The cell wall of tetrads was partially digested with zymolase-100T (Kirin Brewery Co., Tokyo, Japan) and dissected with a micromanipulator. The progeny spores were germinated on YPD complex medium plates and replicated to screen for cells with the desired genetic markers. The progeny strain was further backcrossed twice to RH218, according to the methods of Sherman *et al.* (1974).

RESULTS AND DISCUSSION

Construction of a yeast strain M20-20D; homoserine auxotroph with high transformation frequency

Transformation efficiency in yeast is variable with strains (Kingsman *et al.*, 1979). The strain RH218 is transformed with high frequency by supercoiled plasmid DNAs. The yeast strain STX272-4D has the target gene marker (*hom6*), but is transformed with low frequency. To date, no satisfactory explanations are available for this observation. Strain STX272-4D was backcrossed to RH218 twice to obtain progeny strains M11-7D, M17-37D and M20-20D in each generation, respectively (Table 1). The strain M20-20D demonstrated high frequency transformation and possessed all the desired genetic markers; *hom6* (target gene for cloning), *trp1* (yeast marker for YRp plasmids), and *ura3* (yeast marker for YCp50 and YIp plasmids). YIp and YRp plasmids are shuttle vectors to be used in further studies for functional analysis and subcloning.

Isolation of recombinant plasmids containing the yeast HOM6 gene

The plamid vector YCp50 is composed of a 1.1kb Hind III fragment of yeast *URA3* gene in the Ava I site of pBR322 and a 2.5kb Hind III-Pvu II fragment of yeast replicator *ARS1* and yeast centromere 4 (*CEN4*) in Pvu II site of pBR322. Plasmid YCp50 is stably

maintained during both mitosis and meiosis because it has the *CEN 4* sequence. This useful shuttle vector provides *URA 3* function for selection in *ura 3* yeast strain, has antibiotic resistance genes (*amp*^{r} and *tet*^{r}) of pBR322 for selection in *E. coli,* and also can replicate autonomously in both yeast and *E. coli.* A library KC1 of yeast genomic sequences consists of pooled recombinant plasmids in each of which a partial Sau3A fragment of the yeast sequence is inserted into the BamHI site of YCp50 (Parent *et al.,* 1985). The library KC1 was used to transform yeast strain M20-20D, selecting for Ura$^+$ and Hom$^+$ transformants. While a single step double selection was unsuccessful, two consecutive tandem selection (Ura$^+$ selection followed by Hom$^+$ selection) has yielded 81 Hom$^+$ colonies out of about 5×10^4 Ura$^+$ transformants. These Hom$^+$ colonies may not represent individual transformants, because the Ura$^+$ transformants colonies were not individually picked but mixed, smashed, and plated onto homoserine selection plates. Yeast plasmid DNA was extracted from the collective culture of Hom$^+$ transformants in homoserine dropout liquid medium. Yeast DNA preparation was used to transform *E. coli* HB101 to ampicilline resistant. *E. coli* transformants were replicated onto tetracycline plates to confirm that they harbor recombinant plasmids with inserts at the BamHI site of pBR322. *E. coli* plasmid DNA was extracted from 5m*l*/each cultures of 80 *E. coli* transformants with recombinant plasmids, electrophoresed in 1% agarose gels, and grouped into 5 different sized plasmids. These were designated as pEK1-pEK5, and digested with EcoRI. EcoRI fragment patterns indicated that the inserts in pEK1-pEK5 were originated from the same chromosomal locus. The sizes of pEK1-pEK5 were estimated to be approximately 13.3, 15.2, 18.5, 17.0, and 14.0, as calibrated against size markers of Lambda phage HindIII/EcoRI fragments, respectively. The recombinant pEK1 was chosen to represent the cloned yeast sequence containing *HOM 6* gene in its insert (5.3 kb). Plasmid

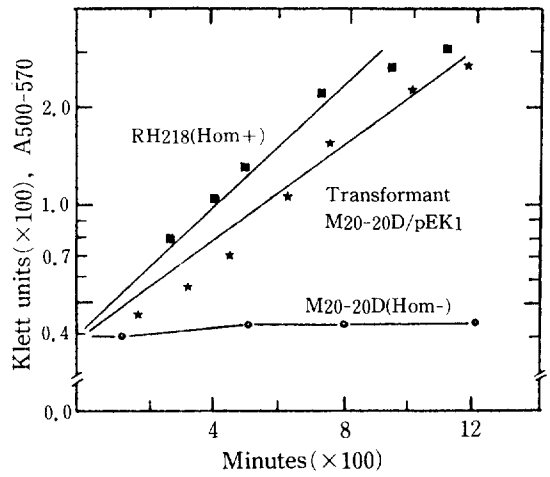

Fig. 1. *Cell growth of yeast strains in homoserine dropout liquid medium.*

pEK1 was amplified in HB101, extracted, and purified on a CsCl gradient. To confirm *hom 6* complementing activity of pEK1, it was reintroduced into the original yeast strain

Table 2. *DNA fragments generated by restriction endonuclease digestion of pEK1.*

Double digestion		
First	Second	Fragment (kb)
Bgl II	*Apa* I	5.4, 4.5, 3.4
	Kpn I	6.2, 4.5, 1.4, 1.2
	Pst I	4.2, 3.8, 3.4, 1.8
	Pvu II	5.9, 5.5, 2.1
	Sal I	5.5, 4.8, 3.1
	Sma I	5.3, 5.0, 2.9
	Xho I	6.2, 3.9, 2.2, 0.7
Cla I	*Pst* I	3.8, 2.4, 2.0, 1.8, 1.6, 0.9, 0.8
	Pvu II	8.5, 2.4, 1.5, 0.9, 0.1
	Sal I	7.5, 2.4, 1.6, 1.0, 0.9
	Sma I	5.6, 2.8, 2.4, 1.6, 0.9
Pst I	*EcoR* I	4.1, 3.8, 3.0, 1.8, 0.7
	Hind III	4.6, 3.8, 1.8, 1.4, 0.9, 0.8
	Kpn I	5.1, 2.7, 2.5, 1.8, 1.2
Pvu II	*Kpn* I	5.5, 4.7, 3.1
	Xho I	5.1, 4.2, 3.9
Sal I	*Hind* III	7.5, 3.6, 1.4, 0.9
	Kpn I	5.6, 4.1, 3.7
Xho I	*Kpn* I	7.8, 4.0, 1.2, 0.5
	Pst I	6.3, 3.1, 1.8, 1.4, 0.7
Bgl I	–	6.0, 4.5, 2.6, 0.2

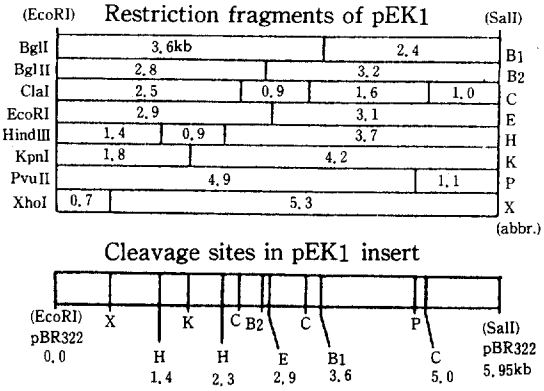

Fig.2. *Restriction endonuclease cleavage sites in the insert of pEK1.*

M20-20D. At high frequency, the pEK1 transformed Hom- M20-20D to Hom$^+$ M20-20D/pEK1. Subsequently, cell growth of the yeast retransformant M20-20D/pEK1 was compared with those of Hom$^+$ strain RH218 and the original recipient strain M20-20D in homoserine dropout liquid medium. Cell growth of M20-20D/pEK1 was comparable with that of RH218, while no growth of M20-20D was detected. The doubling time of M20-20D/pEK1 was about 6 hours which was about 1 hour longer than that of RH218, as shown in Fig.1. Average doubling time of wild type in a complex medium (YPD) is about 2 hours.

Restriction fragment analysis and mapping

A restriction map for the 5.3 kb insert of pEK1 must be established because no information is available on the physical organization of the insert fragment which was primarily identified to contain *hom6*-complementing property by means of retransformation and bioassay. pEK1 was first subjected to 24 hexanucleotide recognizing restriction enzymes to decide which enzyme has recognition sites in the insert. Enzymes with more than 4 cleavage sites in the insert have been omitted in the second digestion (double digestion pairs) to avoid ambiguity of the position of the fourth site. Enzymes were chosen to digest in pairs and the fragments were generated, as

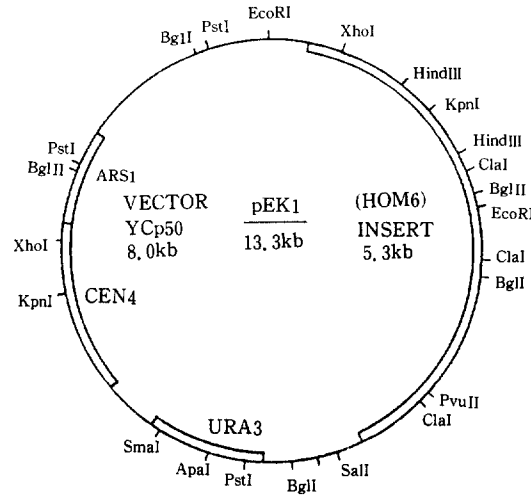

Fig.3. *Restriction map of the recombinant plasmid pEK1. Single line; pBR322. Double line; yeast sequences.*

shown in Table 2. Fragments were analyzed to delineate the relative position of the cleavage sites for restriction enzymes with respect to each other in double digestions. The size of 5.3 kb for the insert was the best estimate, considering error factors in reading the location of bands on a agarose gel and in establishing the standard line of size markers. Small discrepancy (0.1-0.3 kb) between sums of fragments from each pair digestion was adjusted to match the total size of the best estimate 13.3 kb. Cleavage sites in the pEK1 insert were summarized in Fig.2. The restriction map of pEK1 has been established, as shown in Fig. 3. The 2.9 kb EcoRI fragment and fragments of Cla I would be good candidates to be inserted without tailing into YRp and YIp plasmids for functional analysis and further integration test.

ACKNOWLEDGEMENT

We wish to thank Woo-Young Lea for restriction enzyme digestion. This work was partly supported by th Basic Science Research Institute Program, Ministry of Education, 1985.

적 요

효모에 있어서 아스파테이트계 아미노산인 트레오닌과 메치오닌은 호모세린을 공통경로로 하여 생합성된다.

*HOM6*는 베타—아스파테이트—세미알데하이드와 호모세린의 가역반응을 촉매하는 Homoserine dehydrogenase (HSDH) 효소의 유전자이며 제 10번 염색체의 우완에 위치한다.

HSDH의 세포내 양적수준은 메치오닌 첨가에 의하여 억제되지만 트레오닌 첨가에는 변화를 보이지 않는 특성이 있다.

HOM6 유전자의 발현 조절기작을 연구하기 위한 첫번째 단계로 본 유전자를 영양요구성의 상보현상을 이용한 효모 형질전환의 방법으로 분자클로닝하고 그 유전자를 삽입부위내에 포함하고 있는 재조합플라스미드를 pEK1 (13.3Kb=Vector-YCp50+Insert 5.3kb)이라 명명하였다.

그 삽입절편에는 8 가지 제한효소가 11군데에 반응하고 있었으며 그 작용부위가 결정된 제한효소 지도가 작성되었다.

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(Received Nov. 20, 1986)