CLOWING OF THE *ILV2*, *ILV3* AND *ILV5* GENES OF SACCHAROMYCES CEREVISIAE

by

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The molecular analysis of the *ILV* genes which code for enzymes catalyzing steps in the biosynthesis of isoleucine and valine, is a prerequisite for the elucidation of the pathway-specific and general amino acid control mechanisms regulating isoleucine and valine biosynthesis in *Saccharomyces*. The *ILV2*, *ILV3* and *ILV5* genes of *Saccharomyces cerevisiae* have been cloned by complementation of mutations in these genes. Southern hybridization analysis shows that the cloned genes represent unique sequences of the wild-type genome of *S. cerevisiae*. The identity of the cloned genes is demonstrated by their specificity in the complementation of a collection of mutant alleles of the *ILV* genes and by site-directed integration of the cloned fragments at the *ILV* loci.

1. INTRODUCTION

In yeast, as in other organisms, the biosynthesis of the branched-chain amino acids isoleucine and valine presents the unusual feature of a set of enzymes which, acting in parallel reactions, lead to the formation of different end products. The third branched-chain amino acid, leucine, is synthesized by a series of four reactions starting from α -ketoisovaleric acid, the last intermediate in the synthesis of valine (Figure 1). The enzymes of the isoleucine and valine pathways in *Saccharomyces* are repressed by the combined action of isoleucine, valine and leucine (3, 18). This phenomenon is analogous to the multivalent repression observed in bacteria (7). In addition to this pathway-specific control mechanism, the general amino acid control system (6, 12, 25, 28) operates on two enzyme activities: Threonine deaminase (S. HOLMBERG

and J.G.L. PETERSEN, personal communication) and transaminase (6). Two other enzymes seem not to be under general control, these are acetohydroxyacid synthase (28) and reductoisomerase (J.G.L. PETERSEN, personal communication).

The identification of the genes coding for most of the enzymes of the isoleucine and valine biosynthesis (Figure 1) was done by KAKAR and WAGNER (13) and by MAGEE and DE ROBICHON-SZULMAJSTER (19). No gene has been identified for the last pair of reactions. Probably, more than one transaminase can catalyze these reactions, as is suggested by the fact that despite extensive hunts mutants blocked at this step have not been found (13, 24). The *ILV1* gene has been cloned (23) and its nucleotide sequence determined (14). The *ILV2* gene has been cloned by exploiting increased resistance to the herbi-

Figure 1. The biosynthetic pathways of isoleucine and valine in *Saccharomyces cerevisiae*.

cide sulfometuron methyl conferred to wild-type *Saccharomyces* strains by multiple copies of plasmids carrying this gene. Its nucleotide sequence has been determined (S.C. FALCO, personal communication).

The study of the genetic regulation of the isoleucine and valine pathways in *Saccharomyces* is of practical interest in relation to beer production. A defined goal in the improvement of brewers yeast is the construction of strains that will produce beer with minimal levels of off-flavor compounds several of which are formed from intermediates of these pathways (4, 17, 21). Considerable progress has been achieved in the genetic manipulation of brewers yeast despite the difficulties imposed by their genomic configuration (8, 9, 16, 27). A combination of conventional and molecular genetic techniques seems to be the most promising approach for the improvement of these strains. The availability of cloned *ILV* genes may represent a useful tool for the elimination of off-flavor compounds of beer.

2. MATERIALS AND METHODS

2.1. Strains and plasmids

The strains of *Saccharomyces cerevisiae* used in this study are listed in Table I. *Escherichia coli* HB101 (2) was used in all cloning experiments carried out with bacteria. Plasmid YRp17 which contains the *URA3* and *TRP1* genes of *S. cerevisiae* (1) was used as cloning vector. This plasmid as well as the related YRp14 were used for subcloning.

2.2. Cloning procedures

Total yeast DNA was purified by the guanidinium chloride method (15) from strain S288C. The DNA was partially digested with the restriction endonuclease MboI to generate fragments of about 5-10 kb. These fragments were ligated into the BamHI site of the YRp17 vector. The ligation mixture was used to transform *E. coli* for amplification of the recombinant molecules. Hybrid plasmid pools were prepared from *E. coli* and used to transform appropriate *Ilv* strains of

Table 1.
***Saccharomyces cerevisiae* strains**

Strain	Genotype	Source
Source of DNA		
S288C	<i>MATα</i>	J. CONDE (MORTIMER)
Recipient strains		
IVPX2-1C	<i>MATα ura3 trp1 ilv2-11</i>	This study
IVPX5-2B	<i>MATα ura3 leu2 his⁻ ilv3-12</i>	This study
C82-1613	<i>MATα ura3 trp1 hom7 ilv5-1</i>	J.G.L. PETERSEN
Other strains		
C80-1158	<i>MATα trp1 his4 arg4 lys1 ura4 ilv2-7</i>	J.G.L. PETERSEN
C80-1148	<i>MATα trp1 his4 arg4 lys1 ura4 ilv2-48</i>	J.G.L. PETERSEN
C80-1133	<i>MATα trp1 his4 arg4 lys1 ura4 ilv3-33</i>	J.G.L. PETERSEN
C80-1153	<i>MATα trp1 his4 arg4 lys1 ura4 ilv3-53</i>	J.G.L. PETERSEN
C80-1140	<i>MATα trp1 his4 arg4 lys1 ura4 ilv5-40</i>	J.G.L. PETERSEN
C80-1151	<i>MATα trp1 his4 arg4 lys1 ura4 ilv5-51</i>	J.G.L. PETERSEN
IVPX1-4B	<i>MATα ura3 hom3 ilv2-11</i>	This study
M1-2B	<i>MATα ura3 trp1</i>	M. McDONELL (THOMAS)
4836-8C	<i>MATα leu1 thr1</i>	J. CONDE (FINK)

S. cerevisiae (10, 11). Plasmids containing the different *ILV* genes were identified by complementation of the corresponding *ilv* mutations. These plasmids were purified from the *S. cerevisiae* transformant clones (5), amplified in *E. coli* and subsequently characterized. As a guide for cloning procedures the manual by MANIATIS et al. (20) was used.

2.3. Southern hybridization

Samples of total yeast DNA were digested to completion with different restriction endonucleases, electrophoresed in agarose gels and blotted to nitrocellulose filters. Probes consisted of linear DNA fragments containing all or part of the gene being analyzed. These fragments were generated by restriction endonuclease digestion and fractionated by gel electrophoresis in low-melting-temperature agarose. The desired DNA fragment was purified from the agarose by phenol and heat treatment and labeled by nick-translation (20).

2.4. Genetic analysis

Tetrad dissections and other yeast procedures were carried out as described by SHERMAN et al. (26).

3. RESULTS

3.1. Cloning of *ILV2*, *ILV3* and *ILV5*

Upon transformation of strains IVPX2-1C (*ilv2-11*), IVPX5-2B (*ilv3-12*) and C82-1613 (*ilv5-1*) with the pools described in Materials and Methods, hybrid plasmids were isolated which complemented the *ilv* mutations of these strains. Figure 2 shows the restriction endonuclease site maps of the insertions found in these plasmids. Plasmid pE16/8-c4 complemented the *ilv2* mutation of strain IVPX2-1C. Plasmids pE30/5-2-1 and pE30/5-6-3 complemented the *ilv3* mutation of strain IVPX5-2B. The insertions of these two plasmids contained a homologous region but differed at one end as to the restriction sites present. Plasmids pE6/7-4-1 and pE6/7-13-3, which were indistinguishable, complemented the *ilv5* mutation of strain C82-1613.

Figure 2 also shows the fragments of the original plasmids which had been subcloned to localize the position of the *ILV* genes. Subcloning was carried out by assembling the desired fragment in a selectable plasmid able to replicate in both *E. coli* and *S. cerevisiae*. Vectors YRp17 and YRp14 were used in these constructions. Each one of the resulting plasmids was tested for its ability to complement the corresponding *ilv* mutation.

Figure 2. Restriction maps of the insertions present in the original *ilv* complementing plasmids and their subclones. Symbols: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; K, KpnI; P, PstI; Pv, PvuII; S, SalI; X, XbaI.

Figure 3. Southern hybridization analysis of *Saccharomyces cerevisiae* S288C DNA, digested with different restriction endonucleases, and probes derived from the *ilv* complementing plasmids. A, *ILV2* probe; B, *ILV3* probe; C, *ILV5* probe. Symbols: B, BamHI; E, EcoRI; H, HindIII; Pv, PvuII; S, Sall; X, XbaI.

3.2. Southern hybridization analysis

Figure 3 shows the patterns obtained upon hybridization of probes derived from the *ilv* complementing plasmids to total DNA from *S. cerevisiae* S288C. The probe used for the hybridizations shown in Figure 3A was the 0.7 kb EcoRI-PvuII fragment internal to plasmid pE16/8-c4, also present in plasmid pE16/4 (see Figure 2A). The probe used for Figure 3B was the 1.1 kb KpnI-BamHI fragment internal to all four

plasmids presented in Figure 2B. The probe used for Figure 3C was a 3.2 kb Sall-BamHI fragment covering the insertion of plasmid pE17/11-B1 (see Figure 2C) plus a contiguous 0.3 kb stretch of bacterial DNA originally derived from plasmid pBR322.

The observed hybridization patterns show that the fragments used as probes correspond to unique sequences of the wild-type genome of *S. cerevisiae*.

Table II.

Complementation pattern of *ilv* mutations by the isolated plasmids.

strain	IVPX2-1C	C80-1158	C80-1148	IVPX5-2B	C80-1133	C80-1153	C82-1613	C80-1140	C80-1151
mutation	<i>ilv2-11</i>	<i>ilv2-7</i>	<i>ilv2-48</i>	<i>ilv3-12</i>	<i>ilv3-33</i>	<i>ilv3-53</i>	<i>ilv5-1</i>	<i>ilv5-40</i>	<i>ilv5-51</i>
plasmid									
pE16/8-c4	+	+	+	-	-	-	-	-	-
pE30/5-2-1	-	-	-	+	+	+	-	-	-
pE30/5-6-3	-	-	-	+	+	+	-	-	-
pE6/7-4-1	-	-	-	-	-	-	+	+	+
pE6/7-13-3	-	-	-	-	-	-	+	+	+

3.3. Identification of the cloned genes

Two approaches were used to demonstrate that the *ilv* complementing plasmids contain the *ILV* genes which were sought and that their properties are not due to genetic or physiological suppression. First, the plasmids were used to transform a collection of strains marked with different mutant alleles of *ilv2*, *ilv3* and *ilv5*. The observed complementation pattern is presented in Table II. The second approach made use of the observation by ORR-WEAVER et al. (22) that transformation mediated by linear fragments of DNA, which are unable to replicate autonomously, frequently leads to chromosomal integration of the fragments at sites homologous to their ends. Transformants resulting from integration can be distinguished from those harbouring an autonomously replicating plasmid by their stable phenotype. Ura⁺ Ilv⁺ colonies were selected upon transformation of the *ura3 trp1 ilv2* strain IVPX2-1C with the *ilv2* complementing plasmid pE16/8-c4 digested with HindIII. This plasmid contains the genes *URA3* and *TRP1* from vector YRp17. The *TRP1* sequence is disrupted by HindIII which also cuts the insertion of pE16/8-c4 once. A single Ura⁺ Ilv⁺ stable transformant (Y7/10-7) obtained in this experiment was Trp⁻. This transformant was crossed with the *TRP1 ura3 ilv2* strain IVPX1-4B and with the *TRP1 URA3 ILV2* strain 4836-8C. Tetrad analysis of the first cross resulted in a 2+:2- segregation for Trp, Ura and Ilv and revealed linkage between *URA3* and *ILV2*. The second cross segregated 2+:2- for Trp, 4+:0- for Ilv and gave three types of tetrads (4+:0-, 3+:1- and 2+:2-) with regard to Ura. These

results show that in Y7/10-7 the *URA3* gene and the *ilv2* complementing fragment from pE16/8-c4 are integrated at the *ilv2* locus. Ura⁺ Trp⁺ stable clones were obtained by transformation of the *trp1 ura3 ILV3* strain M1-2B with the *ilv3* complementing plasmid pE30/5-2-1 digested with BamHI. The diploid clones resulting from crossing eight such stable transformants with the *TRP1 ura3 ilv3* strain IVPX5-2B were sporulated and tetrads were dissected. The site at which integration had occurred could not be determined because spore viability was extremely low in all cases. In a similar experiment, M1-2B was transformed with the *ilv5* complementing plasmid pE17/11-B1, after digestion with KpnI, to a stable Ura⁺ Trp⁺ phenotype. Genetic analysis of the transformants (designated Y10/7I) was carried out by crossing them with the *trp1 ura3 ilv5* strain C82-1613. Two diploids analyzed showed 2+:2- cosegregation for Trp, Ura and Ilv, indicating integration at the *ILV5* locus.

4. DISCUSSION

The presence of the *ILV2*, *ILV3* and *ILV5* genes in the cloned fragments of *S. cerevisiae* DNA has been shown in two ways. First, each one of the fragments containing a presumed *ILV* gene can complement different mutant alleles of a single *ILV* gene but not those of the other two. This provides evidence against the possibility that the cloned fragments contain suppressor genes. The second type of experiments provides conclusive proof for *ILV2* and *ILV5*. Plasmids containing the cloned fragments integrated at

these *ILV* loci, which reveals sequence homology. Since Southern hybridization analyses show that the cloned genes are unique in the genome, they must be the *ILV* genes.

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