CLONING AND CHARACTERIZATION OF YEAST *LEU4*, ONE OF TWO GENES RESPONSIBLE FOR α -ISOPROPYLMALATE SYNTHESIS

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ABSTRACT

By complementation of an α -isopropylmalate synthase-negative mutant of *Saccharomyces cerevisiae* (*leu4 leu5*), a plasmid was isolated that carried a structural gene for α -isopropylmalate synthase. Restriction mapping and subcloning showed that sequences sufficient for complementation of the *leu4 leu5* strain were located within a 2.2-kilobase *SalI-PvuII* segment. Southern transfer hybridization indicated that the cloned DNA was derived intact from the yeast genome. The cloned gene was identified as *LEU4* by integrative transformation that caused gene disruption at the *LEU4* locus. When this transformation was performed with a *LEU4^{tr} LEU5* strain, the resulting transformants had lost the 5',5',5'-trifluoro-D,L-leucine resistance of the recipient strain but were still *Leu⁺*. When it was performed with a *LEU4 leu5* recipient, the resulting transformants were *Leu⁻*. The α -isopropylmalate synthase of a transformant that carried the *LEU4* gene on a multicopy plasmid (in a *leu5* background) was characterized biochemically. The transformant contained about 20 times as much α -isopropylmalate synthase as wild type. The enzyme was sensitive to inhibition by leucine and coenzyme A, was inactivated by antibody generated against α -isopropylmalate synthase purified from wild type and was largely confined to the mitochondria. The subunit molecular weight was 65,000–67,000. Limited proteolysis generated two fragments with molecular weights of about 45,000 and 23,000. Northern transfer hybridization showed that the transformant produced large amounts of *LEU4*-specific RNA with a length of about 2.1 kilonucleotides. The properties of the plasmid-encoded enzyme resemble those of a previously characterized α -isopropylmalate synthase that is predominant in wild-type cells. The existence in yeast of a second α -isopropylmalate synthase activity that depends on the presence of an intact *LEU5* gene is discussed.

IN yeast, as in other microorganisms and plants, leucine biosynthesis is accomplished in four steps: (1) condensation of acetyl-CoA and α -ketoisovalerate to α -isopropylmalate (α -IPM), catalyzed by α -IPM synthase; (2) isomerization of α -IPM to β -IPM, catalyzed by IPM isomerase; (3) oxidative decarboxylation of β -IPM to yield α -ketoisocaproate, catalyzed by β -IPM dehydrogenase; and (4) transamination of α -ketoisocaproate, catalyzed by branched-chain amino acid

Abbreviations used: IPM, isopropylmalate; PMSF, phenylmethylsulfonyl fluoride; TPCK, tosylamino-2-phenylethyl chloromethyl ketone; TLCK, tosyl-L-lysine chloromethyl ketone; YEPD, yeast extract-peptone-dextrose; Kb, kilobasepairs.

aminotransferase. Evidence presented previously suggests that IPM isomerase and β -IPM dehydrogenase, which are encoded by *LEU1* and *LEU2*, respectively, are induced by α -IPM, the product of the first reaction, in conjunction with a regulatory element produced by *LEU3* (BAICHWAL *et al.* 1983). In this respect, leucine pathway regulation in *S. cerevisiae* is very similar to that described for another Ascomycete, *viz.*, *Neurospora crassa* (for reviews see GROSS 1969; KOHLHAW 1983). Because of the likely involvement of α -IPM in controlling subsequent biosynthetic steps, α -IPM synthase would be expected to play a key role in leucine pathway regulation. This is indeed the case and is particularly obvious with yeast which appears to have evolved a rather sophisticated " α -IPM synthase system." A recent genetic analysis showed that two genes, designated *LEU4* and *LEU5*, must be mutated in order to generate an α -IPM synthase-negative phenotype (BAICHWAL *et al.* 1983). *LEU4* was defined as a structural gene because an allele of *LEU4* produces a feedback-resistant α -IPM synthase (BAICHWAL *et al.* 1983). The nature of *LEU5* is uncertain, that is, it is not known whether it represents another structural gene or controls the expression of a structural gene. Available evidence suggests that yeast does contain a second α -IPM synthase activity that may account for up to 25% of the total synthase activity of wild-type cells (L. F. CHANG, P. R. GATZEK and G. B. KOHLHAW, unpublished results; see also DISCUSSION). The major α -IPM synthase activity is encoded by *LEU4* (BAICHWAL *et al.* 1983) and, as will be shown in this paper, possesses properties very much resembling those of the yeast α -IPM synthase characterized in previous studies. Those studies demonstrated the following. (1) α -IPM synthase is inhibited by leucine (SATYANARAYANA, UMBARGER and LINDEGREN 1968; ULM, BÖHME and KOHLHAW 1972). (2) The enzyme is subject to a highly specific inactivation by CoA (TRACY and KOHLHAW 1975, 1977). The CoA effect can be prevented or reversed by ATP and is viewed as a means that allows the yeast cell to channel acetyl-CoA away from biosynthetic pathways and into the citric acid cycle whenever the acetyl-CoA concentration reaches a certain low threshold value (HAMPSEY and KOHLHAW 1981). (3) The level of α -IPM synthase is regulated by the mechanism known as "general control" of amino acid biosynthesis (HSU, KOHLHAW and NIEDERBERGER 1982). (4) α -IPM synthase consists of two apparently identical subunits whose molecular weight is 65,000–67,000 (TRACY and KOHLHAW 1977). (5) Much of the α -IPM synthase activity of yeast is found in the mitochondrial matrix (HAMPSEY, LEWIN and KOHLHAW 1983). Import into the mitochondria does not result in a measurable molecular weight change (HAMPSEY, LEWIN and KOHLHAW 1983; GASSER, DAUM and SCHATZ 1982).

It is of interest from an evolutionary point of view that there are significant differences between the α -IPM synthase system of yeast and its *Neurospora* counterpart. The *Neurospora* enzyme, although inhibited by leucine, apparently is not inactivated by CoA and is localized in the cytosol; it also has much smaller subunits (molecular weight approximately 43,000) that aggregate to form trimers or tetramers (GROSS 1970; S. GROSS, personal communication). Expression of the *Neurospora leu-4* gene, the gene that encodes α -IPM synthase, is negatively controlled by leucine and appears to be affected also by the *leu-3* gene product (GROSS 1969).

The present communication is part of an ongoing effort to identify and

TABLE 1

Vectors and strains

Vector or strain	Description	Source or reference
YEpl3	Yeast episomal plasmid (10.6 kb) containing the 2.2-kb <i>Eco</i> RI fragment of the 2- μ m plasmid, yeast <i>LEU2</i> and pBR322	J. BROACH
YEpl24	Yeast episomal plasmid (7.7 kb) containing the 2.2-kb <i>Eco</i> RI fragment of the 2- μ m plasmid, yeast <i>URA3</i> and pBR322	D. BOTSTEIN
YIp5	Yeast-integrating plasmid (5.5 kb) containing yeast <i>URA3</i> and pBR322	R. DAVIS
pLFC8	Vector YIp5 containing the <i>Bam</i> HI fragment of <i>LEU4</i> ; see text	This work
<i>S. cerevisiae</i>		
S288c	<i>MATα</i> (wild type)	
HB190	<i>MATα</i> <i>leu4 leu5</i> ; α -IPM synthase-negative leucine auxotroph derived from S288c by EMS mutagenesis	H. E. UMBARGER; BAICHWAL <i>et al.</i> (1983)
CG219	<i>MATα</i> <i>ura3-52</i>	T. D. PETES, C. GIROUX
AB320	<i>HO ade2-1 lys2-1 trp5-2 leu2-1 can1-100 ura⁻ met4-1</i> ; derived from diploid W87	B. HALL; NASMYTH and REED (1980)
XK12-8A	<i>MATα</i> <i>leu5 ura3-52</i> ; recovered from cross between HB190 and CG219	BAICHWAL <i>et al.</i> (1983)
XK12-8C	<i>MATα</i> <i>leu4 ura3-52</i> ; recovered from cross between HB190 and CG219	BAICHWAL <i>et al.</i> (1983)
XK61-18	<i>MATα</i> <i>LEU4^{br} ura3-52</i> ; recovered from cross between XK14-13C (<i>a LEU4^{br} his4</i>) and CG219	This work
XK61-42	<i>MATα</i> <i>LEU4^{br} ura3-52 his4</i> ; recovered from cross between XK14-13C (<i>a LEU4^{br} his4</i>) and CG219	This work
SK902 } SK903 } SK904 }	<i>MATα</i> <i>leu4::URA3 ura3-52 his4</i> ; independent isolates resulting from transformation of XK61-42 with vector pLFC8; see text	This work
<i>E. coli</i>		
HB101	<i>leu⁻ pro⁻ thi⁻ thr⁻ lacY1 str^R r⁻ m⁻ recA</i>	

characterize the components of the leucine biosynthetic pathway in yeast. It defines the genomic region containing *LEU4* and establishes some basic properties of the *LEU4* gene product, including evidence for the existence of two structural domains. Part of this work has been published in abstract form (CHANG, CUNNINGHAM and KOHLHAW 1983).

MATERIALS AND METHODS

Strains and growth conditions: The plasmids and strains used in this investigation are listed in Table 1. Cells were grown aerobically at either 30° (yeast) or 37° (*E. coli*) and were harvested in late log phase. Growth media were YEPD and minimal medium with appropriate supplements for yeast (FINK 1970) and L broth and Vogel-Bonner minimal medium for *E. coli* (DAVIS, BOTSTEIN and ROTH 1980). Ampicillin was added to a final concentration of 50 μ g/ml.

Specific materials: 5',5',5'-Trifluoro-D,L-leucine was obtained from Fairfield Chemical Company, Blythwood, South Carolina. β -IPM and dimethylcitrate were gifts from H. E. UMBARGER.

Transformation procedures: Yeast cells were transformed essentially as described by HINNEN, HICKS and FINK (1978). *E. coli* cells (strain HB101) were transformed by the calcium chloride procedure according to MANIATIS, FRITSCH and SAMBROOK (1982).

DNA preparation: Rapid isolation of plasmid DNA from *E. coli* was performed by the "boiling method" of MANIATIS, FRITSCH and SAMBROOK (1982). Large-scale isolation of plasmid DNA was performed by an alkali lysis procedure followed by banding in CsCl/ethidium bromide (MANIATIS, FRITSCH and SAMBROOK 1982). Yeast DNA (a mixture of genomic and plasmid DNA) was prepared by the method of NASMYTH and REED (1980).

DNA digestion with restriction endonucleases and fragment separation: Purified DNA was digested for 1–3 hr at 37° in a total volume of 10 μ l, usually with 1–2 units of the desired restriction enzyme per μ g of DNA. Fragment separation was done by electrophoresis on 1% agarose gels in Tris-acetate/EDTA buffer (MANIATIS, FRITSCH and SAMBROOK 1982). DNA was stained with ethidium bromide (0.5 μ g/ml).

DNA fragment isolation and nick translation: The DNA preparation containing the fragment in question was subjected to preparative agarose gel electrophoresis. The desired band was then electroeluted onto a DEAE membrane (S & S NA-45) and eventually removed from the membrane by treatment with 20 mM Tris-HCl buffer, pH 8.0, containing 1 M NaCl and 0.1 mM EDTA. Ethidium bromide was extracted with water-saturated *n*-butanol, and the DNA was concentrated by ethanol precipitation. Nick translation was performed with α [³²P]-dCTP (Amersham) and resulted in an initial specific activity of approximately 10⁸ cpm/ μ g of DNA.

Southern and Northern blot analyses: The transfer technique of SOUTHERN (1975) was used as described by MANIATIS, FRITSCH and SAMBROOK (1982). For Northern blot hybridization, RNA was isolated by the method of SILVERMAN *et al.* (1982) from cells grown to a density of 2×10^7 cells/ml. The RNA was denatured with glyoxal in the presence of dimethylsulfoxide, subjected to electrophoresis on a 1% agarose gel and transferred to a nitrocellulose filter as described by MANIATIS, FRITSCH and SAMBROOK (1982).

Enzyme assays: The procedures for preparing cell-free extracts and for assaying IPM isomerase were those of BAICHWAL *et al.* (1983). β -IPM dehydrogenase was measured by the continuous assay that determines NADH production at 340 nm (HSU and KOHLHAW 1980). α -IPM synthase activity was measured by the sensitive fluorometric assay of CALVO, BARTHOLOMEW and STIEGLITZ (1969). Specific activities are expressed either as nanomoles of substrate utilized per minute per milligram of protein (IPM isomerase) or as nanomoles of product formed per minute per milligram of protein (β -IPM dehydrogenase, α -IPM synthase).

RESULTS

Isolation and characterization of plasmids able to complement an α -IPM synthase-negative mutant: The plasmid pool used for transformation of strain HB190, kindly provided by B. HALL, contained yeast DNA fragments of 5–20 kb that had been obtained by partial *Sau*3A digestion of DNA from strain AB320 and ligated into the *Bam*HI site of plasmid YEp13 (BROACH, STRATHERN and HICKS 1979). The recipient strain HB190, a *leu4 leu5* double mutant, spontaneously reverted to leucine prototrophy with a frequency of about 3×10^{-8} (both *LEU4 leu5* and *leu4 LEU5* strains have a *Leu*⁺ phenotype, see BAICHWAL *et al.* 1983). *Leu*⁺ transformants arose at a frequency of about 10²/ μ g of DNA. Plasmids of four such transformants were subjected to restriction analysis. Their restriction maps were very similar, with differences existing only in the length of the insert. The plasmids were capable of retransforming strain HB190 to *Leu*⁺ at high frequency (approximately 1×10^4 transformants/ μ g of DNA). One of them, designated pTSC2, was used for further study. It contains an insert of about 6.5 kb that was required for HB190 transformation (Figure 1). Since the presence of *LEU2* in pTSC2 was undesirable, a 2.25-kb *Kpn*I fragment was deleted that contained about $\frac{3}{4}$ of the *LEU2* structural gene (A. ANDREADIS, Y.-P. HSU, G. B. KOHLHAW, M. HERMODSON and P. SCHIMMEL, unpublished data). The resulting plasmid, pLFC1, exhibited unchanged transforming capacity with respect to

FIGURE 1.—Restriction map of plasmid pTSC2 drawn to scale. The inside arrow in pTSC2 indicates the presumed location of *LEU4* (see Figure 2).

strain HB190. To identify the DNA region capable of complementing strain HB190, additional plasmids were constructed and checked for their transforming capability. Plasmids pLFC2, pLFC3 and pLFC4 were derived from pLFC1 by deleting a 1.4-kb *Hind*III fragment, a 0.9-kb *Bam*HI fragment and a 2.3-kb *Sph*I fragment, respectively, and religating. Plasmids pLFC5 and pLFC6 were constructed by inserting portions of pLFC1 into vector YE24; pLFC5 contains a *Sal*I fragment of pLFC1; pLFC6 contains a *Pvu*II fragment of pLFC1. The plasmid constructions are summarized in Figure 2. It is possible to delimit the HB190-complementing sequence by comparing the transforming capability of plasmids pLFC4, pLFC5 and pLFC6. Plasmid pLFC4, whose insert extends from the left to the *Sph*I site, was unable to complement HB190. By contrast, plasmid pLFC6 was capable of high-frequency transformation of HB190 to leucine prototrophy. Its insert extends about 0.4 kb further to the right than that of pLFC4 (to the *Pvu*II site). Plasmid pLFC5, with an insert that extends from the right to the *Sal*I site, was also capable of high-frequency transformation of HB190. It then follows that the HB190-complementing sequence must be contained within the 2.2-kb piece extending from *Sal*I to *Pvu*II. The fact that plasmid pLFC3 cannot complement HB190 is consistent with this conclusion, since its insert lacks a 0.9-kb *Bam*HI fragment located between the *Sal*I and *Pvu*II sites in question.

Figure 3 shows the results of a Southern transfer analysis with genomic DNA (from strain AB320) and pTSC2 plasmid DNA that had been digested with

FIGURE 2.—Detailed restriction map of the 6.5-kb yeast DNA insert in plasmid pTSC2 and delimitation of the HB190-complementing region. See text for details regarding construction of the various inserts. Closed bars, open bars and thin lines have the same meaning as in Figure 1; dashed lines indicate deletions. Plus signs refer to the capability to transform strain HB190 to *Leu*⁺; minus signs indicate lack of such capability.

various restriction endonucleases and probed with the nick-translated 0.9-kb *Bam*HI fragment isolated from pLFC5. It can be seen that the 0.9-kb *Bam*HI fragment, the 4.0-kb *Pvu*II fragment and the 2.5-kb *Eco*RI/*Sal*I fragment of the plasmid are present in genomic DNA, indicating that the region capable of transforming strain HB190 was isolated intact. (Lane 2 of Figure 3A cannot be compared with lane 2 of Figure 3B because the restriction enzymes used were different.)

Identification of the cloned DNA as LEU4 by gene disruption: Genomic integration of cloned DNA by homologous recombination should allow identification of the cloned DNA. We decided to use the 0.9-kb *Bam*HI fragment present in the transforming plasmids (Figure 2), arguing that it might be internal to the protein coding region of the cloned gene and might, therefore, cause gene disruption (SHORTLE, HABER and BOTSTEIN 1982). This argument is based on the observation that α -IPM synthase produced by strain HB190/pLFC2 has a monomer molecular weight of about 65,000. A molecular weight of 65,000 corresponds to a protein-coding region of about 1.8 kb. A coding region of this size should overlap the 0.9-kb fragment even if the coding region started at either end of the 2.2-kb *Sal*I-*Pvu*II piece believed to contain the structural gene.

The plasmid used for genomic integration was pLFC8 (Figure 4). In addition to the 0.9-kb *Bam*HI fragment mentioned before, it contains pBR322 and yeast

FIGURE 3.—Southern transfer analysis. A, Genomic DNA from strain AB320 was digested with *Bam*HI (lane 1), *Hind*III (lane 2), *Pvu*II (lane 3) and *Eco*RI plus *Sal*I (lane 4). B, pTSC2 plasmid DNA was digested with *Bam*HI (lane 1), *Hind*III plus *Sph*I (lane 2), *Pvu*II (lane 3) and *Eco*RI plus *Sal*I (lane 4). In both series of experiments, the digests were electrophoresed on a 1% agarose gel and transferred to nitrocellulose. Nick-translated 0.9-kb *Bam*HI fragment isolated from plasmid pLFC5 was used as the hybridization probe. Fragments obtained from a *Hind*III digest of λ DNA and a *Hae*III digest of ϕ X174 DNA served as size standards.

FIGURE 4.—Predicted results of integrative transformation of a *LEU4*^{fbr} *ura3-52* strain, with plasmid pLFC8 integrating at the *LEU4* locus. Solid bars, Yeast DNA containing *LEU4* sequences; open bars, yeast *URA3* DNA; thin line, pBR322 sequences; S, *Sal*I; B, *Bam*HI; E, *Eco*RI; P, *Pvu*II; TFL, trifluoroleucine. *, The location of the mutation causing feedback resistance of the *LEU4* gene product is not known.

TABLE 2

Levels of leucine pathway-specific enzymes in various yeast strains

Strain	Relative activities of		
	α -IPM synthase	IPM isomerase	β -IPM dehydrogenase ^a
S288c (wt) ^b	1.0	1.0	1.0
HB190	<0.05	<0.1 ^c	<0.1 ^c
HB190/pTSC2	20.0	2.4	29.8
HB190/pLFC2	18.0	2.0	1.2
SK902	0.25	0.7	0.65
XK12-8C	0.22	0.9	0.7

All strains were grown in minimal medium at 30° with appropriate supplements. Strain HB190 was grown in minimal medium containing 0.2 mM leucine. Cells were harvested in late log phase. The values listed are averages of at least two independent experiments.

^a Note that plasmid pTSC2 contains an intact *LEU2* gene (encoding β -IPM dehydrogenase), whereas plasmid pLFC2 does not.

^b The specific activities were: α -IPM synthase, 4.0; IPM isomerase, 31.4; β -IPM dehydrogenase, 11.8.

^c The virtual absence of isomerase and dehydrogenase in strain HB190 is believed to be due to the lack of α -IPM production; the *LEU1* and *LEU2* genes are intact (BAICHWAL *et al.* 1983).

URA3 sequences. It was constructed by cleaving vector YIp5 at its unique *Bam*HI site and inserting the 0.9-kb *Bam*HI fragment isolated from plasmid pLFC1. Integrative transformation of recipient strain XK61-42 (*LEU4^{br} ura3-52 his4*; selection for Ura⁺) occurred with a frequency of about six transformants per microgram of plasmid DNA when pLFC8 that had been linearized by cleavage at the unique *Xba*I site in the insert was used. With intact, circular pLFC8 the transformation frequency was about 10-fold lower. All 20 transformants tested had acquired sensitivity to 2 mM trifluoroleucine (the recipient strain is trifluoroleucine resistant, owing to the presence of the feedback-resistant α -IPM synthase encoded by *LEU4^{br}*) but were still Leu⁺. Their trifluoroleucine-sensitive, Ura⁺ phenotype was stable during a 24-hr growth in YEPD liquid medium (about nine generations) followed by a 2-day growth on YEPD plates. One transformant (SK902) was analyzed with respect to the three leucine pathway-specific enzymes. Its cell-free extract contained approximately 25% of the wild-type level of α -IPM synthase and approximately two-thirds of the normal (wild type) complement of IPM isomerase and β -IPM dehydrogenase (Table 2). Crossing SK902 and two other independent isolates (SK903 and SK904) to a strain with genotype *LEU4 leu5 ura3-52* (Table 3) resulted in a segregation pattern of the Leu⁻ phenotype that resembled the one seen previously in crosses between *leu4 leu5* and *LEU4 LEU5* or *leu4 LEU5* and *LEU4 leu5* strains (BAICHWAL *et al.* 1983). The pattern (PD:NPD:T at an average ratio of 3:1:11) suggests that both *leu4* and *leu5* alleles are present in the diploid. In the same crosses, all spores were trifluoroleucine sensitive, the Ura⁻ marker segregated 2:2 and all Leu⁻ spores were Ura⁺. In a second series of experiments, SK902, SK903 and SK904 were crossed to a

TABLE 3

Tetrad analysis of crosses with integrative transformants

Cross ^a	No. of tetrads	Segregation of				
		Leu ⁺ /Leu ⁻			TFL ^S /TFL ^R	
		4 ⁺ :0 ⁻	2:2	3 ⁺ :1 ⁻	4 ^S :0 ^R	2:2
		PD	NPD	T		
SK902 × XK12-8A	16	2	1	13	16	0
SK903 × XK12-8A	16	4	1	11	16	0
SK904 × XK12-8A	13	4	1	8	13	0
SK902 × XK61-18	14	14	0	0	0	14
SK903 × XK61-18	12	12	0	0	0	12
SK904 × XK61-18	13	13	0	0	0	13

^a In the first series of crosses, all Leu⁻ spores were Ura⁺. In the second series of crosses, all TFL^S spores were Ura⁺ and all TFL^R spores were Ura⁻. TFL = trifluoroleucine. In both series, the His⁻ marker segregated 2:2.

LEU4^{fb} *ura3-52* strain (Table 3). In the 39 tetrads examined, all spore clones were Leu⁺, and trifluoroleucine resistance segregated 2:2. Significantly, all trifluoroleucine-sensitive spores were Ura⁺ and all trifluoroleucine-resistant spores were Ura⁻, that is to say, the *URA3* DNA segregated as an allele of *LEU4*^{fb}. The phenotype of the integrative transformants and the results of both types of crosses are consistent with insertion of a *URA3*-carrying element at the *LEU4*^{fb} locus. Disruptive integration at the *LEU4* locus with plasmid pLFC8 was performed also on a *LEU4 leu5 ura3-52* recipient (strain XK12-8A). In this case, a stable Ura⁺ Leu⁻ phenotype was obtained.

To further characterize the postulated disruptive integration event shown in Figure 4, a Southern transfer analysis was performed. Figure 5 shows the results of an experiment in which genomic DNA of strains SK902, SK903, SK904 and XK61-42/YIp5 was digested with *Eco*RI plus *Sal*I and probed with the nick-translated 0.9-kb *Bam*HI fragment isolated from plasmid pLFC5 (see Figure 2). Strain XK61-42/YIp5 was generated by integrating the *URA3*-carrying vector YIp5 at the *ura3* locus of XK61-42. The hybridization pattern obtained with DNA from strains SK902, SK903 and SK904 showed two strong bands of 2.4 and 1.7 kb, respectively. By contrast, only one strong band of about 2.5 kb appeared with DNA from XK61-42/YIp5 (relevant genotype, *LEU4*^{fb} *LEU5*). These results are in agreement with the integrative transformation process outlined in Figure 4. The lighter band of about 2 kb present in lanes 1 through 5 of Figure 5 is likely to represent a genomic region of weak homology to the *Bam*HI fragment; it largely disappears at higher stringency (lane 6 of Figure 5). The diffuse band in the lower part of lane 4 is probably the result of a 0.7-kb *Bam*HI fragment contamination present in the preparation of the 0.9-kb *Bam*HI probe. The contaminating fragment would contain the *Bam*HI-*Sal*I sequence of pBR322 (0.275 kb) and would be expected to hybridize to a YIp5-derived *Eco*RI-*Sal*I piece of 0.65 kb. This piece is not present in pLFC8.

FIGURE 5.—Southern transfer analysis of disrupted *LEU4* alleles. Genomic DNA from three independently isolated integrative transformants and a control strain were digested with restriction endonucleases *Eco*RI and *Sal*I. The digests were electrophoresed on a 1% agarose gel and transferred to nitrocellulose. The hybridization probe was the nick-translated 0.9-kb *Bam*HI fragment isolated from pLFC5. Lanes 1, 2, and 3, Genomic DNA from strains SK902, SK903 and SK904, respectively. Lane 4, Genomic DNA from strain XK61-42/YIp5. Lanes 5 and 6, Genomic DNA from strain SK903. The hybridization/washing temperatures were 63° (lanes 1–4), 58° (lane 5) and 68° (lane 6), respectively. Size standards were obtained from a *Hind*III digest of λ DNA and a *Hae*III digest of ϕ X174 DNA. See MATERIALS AND METHODS for other details.

Properties of the LEU4 gene product: Strain HB190/pLFC2 contained about 20 times more α -IPM synthase activity than wild type (Table 2), which is probably a consequence of the plasmid copy number. The introduction of intact *LEU4* genes into strain HB190 restored not only α -IPM synthase function but also IPM isomerase and β -IPM dehydrogenase activities [to levels slightly greater than those of wild-type cells (Table 2)]. This provides further support for the idea that α -IPM induces the formation of the *LEU* and *LEU2* gene products (BAICHWAL *et al.* 1983). The almost 30-fold increase of the β -IPM dehydrogenase level in strain HB190/pTSC2, which carries multiple copies of both *LEU4* and *LEU2*, possibly reflects the combined effect of high gene dosage and induction.

Several key properties of the α -IPM synthase produced by strain HB190/pLFC2 were compared with those of α -IPM synthase from wild-type yeast cells studied previously (ULM, BÖHME and KOHLHAW 1972; TRACY and KOHLHAW 1975, 1977; HAMPSEY and KOHLHAW 1981; HAMPSEY, LEWIN and KOHLHAW 1983). Figure 6 shows that the α -IPM synthase of strain HB190/pLFC2 was sensitive to inhibition by both CoA-Zn²⁺ (panel A) and L-leucine (panel B). Time-dependent inactivation by CoA in the presence of zinc ions and inhibition by leucine were quantitatively very similar to values obtained with enzyme from untransformed wild-type cells studied under comparable conditions (TRACY and

FIGURE 6.—Biochemical characterization of the α -IPM synthase of strain HB190/pLFC2. A, CoA inactivation: at time zero, 40 μ l of a mixture of CoA and ZnCl_2 were added to 320 μ l of a suspension of permeabilized cells prepared according to MIOZZARI, NIEDERBERGER and HÜTTER (1978). The final concentration of CoA and Zn^{2+} was 90 μ M. After incubation at 30° for the indicated times, aliquots were withdrawn and assayed at pH 7.2. B, Leucine inhibition: cell-free extract was assayed for α -IPM synthase activity in the presence of the indicated concentrations of L-leucine. The pH was 7.2. C, Immunoinhibition: the indicated amounts of an antibody preparation were added to a fixed amount of cell-free extract (38 μ g of protein). After incubation at room temperature for 30 min, aliquots were withdrawn and assayed. IgG refers to antiserum fractionated according to KAWAMURA (1977). Filled circles, Antiserum prepared against highly purified α -IPM synthase from *S. cerevisiae* SK101 (HAMPSEY 1982); open circles, control serum.

KOHLHAW 1975; ULM, BÖHME and KOHLHAW 1972). Furthermore, all of the measurable α -IPM synthase present in cell-free extracts from HB190/pLFC2 was inactivated by antiserum prepared against highly purified enzyme from *LEU4 LEU5* cells (Figure 6, panel C). The apparent molecular weight of the monomer of the *LEU4* gene product was determined by polyacrylamide gel electrophoresis of antiserum-precipitable material obtained after *in vitro* translation of RNA isolated from strain HB190/pLFC2; it was found to be approximately 65,000 (W.-J. CHEN, unpublished results). This value is identical with that obtained with wild-type enzyme (TRACY and KOHLHAW 1977). A very similar value for the size of the subunit was found when highly purified α -IPM synthase, isolated from the overproducing transformant HB190/pLFC2 (see Table 2), was denatured and subjected to polyacrylamide gel electrophoresis (Figure 7). Exposure of highly purified α -IPM synthase to small amounts of trypsin led to the appearance of two fragments with molecular weights of approximately 45,000 and 23,000, long before the onset of more extensive digestion. This suggests that yeast α -IPM synthase contains an especially sensitive or exposed peptide bond, possibly at the junction between two domains (see also DISCUSSION).

The results of a Northern blot hybridization experiment (Figure 8) were consistent with the idea that the cloned *LEU4* gene encodes a protein with a molecular weight of about 65,000 (corresponding to a structural gene, without introns, of about 1.8 kb). The one major RNA species that hybridized to the

FIGURE 7.—Limited proteolysis of α -IPM synthase isolated from strain HB190/pLFC2. The enzyme was purified to near homogeneity from 60 g of cells grown in minimal medium. The purification procedure was that of HAMPSEY (1982) except that chromatography on leucine-Sepharose was used only once. Purified α -IPM synthase was subjected to trypsin digestion in a solution containing 100 mM Tris-HCl buffer, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 0.45 μ g of bovine pancreatic trypsin and 45 μ g of α -IPM synthase in a final volume of 50 μ l. Incubation was performed at 22° for the periods indicated below. At each interval, 10 μ l of the solution was removed and protease inhibitors (PMSF, TPCK, TLCK) were added to a final concentration of 2 mM each. To each aliquot, SDS and β -mercaptoethanol were added to give a final concentration of 2 and 1%, respectively, and the mixtures were kept at 100° for 3 min. They were then electrophoresed on polyacrylamide gel slabs (10% acrylamide) as described by LAEMMLI (1970). Protein bands were made visible by staining with Coomassie brilliant blue R250. Bovine serum albumin, ovalbumin and chymotrypsin A were used as standards. Lane 1, Highly purified α -IPM synthase, no trypsin digestion; lane 2, trypsin digestion for 1 min; lane 3, trypsin digestion for 5 min; lane 4, trypsin digestion for 20 min; lane 5, trypsin digestion for 60 min.

*Bam*HI fragment believed to be internal to *LEU4* migrated to a position corresponding to about 2.1 kb. Figure 8 also shows that the concentration of the hybridizing RNA species was much greater in the transformant than in wild-type cells. This probably reflects the increased dosage of *LEU4* in the transformed strain.

With respect to subcellular localization, preliminary results indicate that in cells of strain HB190/pLFC2, as in wild-type cells, α -IPM synthase is largely confined to the mitochondria. The distribution between a crude mitochondrial preparation and the postmitochondrial supernatant was 62 and 38%, respectively (average of two experiments). The corresponding values for citrate synthase were 64 and 36%, respectively.

DISCUSSION

The results presented in this paper clearly establish that the gene that has been cloned by complementation of an α -IPM synthase-negative mutant of yeast (*leu4 leu5*) is *LEU4*. The properties of the product of the cloned *LEU4* gene that were

FIGURE 8.—Northern blot hybridization of total RNA from strains S288c and HB190/pLFC2, respectively. The RNA was hybridized to the 0.9-kb *Bam*HI fragment isolated from plasmid pLFC5. Lane 1, 5 μ g of total RNA from strain S288c. Lane 2, 5 μ g of total RNA from strain HB190/pLFC2. Standard sizes were obtained from a *Hind*III digest of phage λ .

examined (inhibition by leucine, inactivation by CoA, reaction with antibody against α -IPM synthase, apparent molecular weight, subcellular localization) very much resemble those of a wild-type yeast α -IPM synthase that was characterized previously (ULM, BÖHME and KOHLHAW 1972; TRACY and KOHLHAW 1975, 1977; HAMPSEY and KOHLHAW 1981; HAMPSEY, LEWIN and KOHLHAW 1983). A new finding is that the *LEU4* gene product is likely to contain two structural domains (possible implications will be presented later).

The question arises whether the results reported here also provide information about the *LEU5* gene and its product. It should be remembered that *LEU4* and *LEU5* must both be mutant for the α -IPM synthase-negative phenotype to appear, i.e., both *LEU4 leu5* and *leu4 LEU5* strains are *Leu*⁺ (BAICHWAL *et al.* 1983). Of special interest is the observation reported here that a *LEU4^{br} LEU5* strain in which the *LEU4* gene was disrupted lost its trifluoroleucine resistance but retained a *Leu*⁺ phenotype and, with it, a low but significant amount of α -IPM synthase. Two possibilities may be considered to explain this observation. (1) The segments of the *LEU4* gene that were generated by gene disruption (see Figure 4) contain enough sequence to produce a weakly active enzyme. This case requires the additional assumption that *LEU4* expression is dependent on an intact *LEU5* gene, since a *Leu*⁻ phenotype results when gene disruption is performed with a *LEU4 leu5* strain. (2) There exists a second α -IPM synthase that is encoded either by *LEU5* itself or by another gene whose expression depends on *LEU5*. We favor the second possibility, i.e., the existence of two α -IPM synthase activities, since the levels of α -IPM synthase and of the *LEU1* and *LEU2* gene products as well as the growth rate of strain SK902 do not differ very much from the corresponding parameters of a *leu4 LEU5* mutant identified previously (XK12-8C; see Table 2 and BAICHWAL *et al.* 1983). It is unlikely that the two very different *leu4* lesions should produce nearly the same weakly active enzymes. This argument is reinforced by the properties of a large *leu4* deletion that has recently been constructed in this laboratory (L. F. CHANG and G. B. KOHLHAW, unpublished data). The deletion was constructed by replacing the 0.9-kb *Bam*HI fragment believed to be internal to *LEU4* with a 1.7-kb *Bam*HI fragment carrying the yeast *HIS3* gene. Southern blotting and tetrad analysis confirmed that the desired one-step gene disruption had occurred. The resulting strain (*leu4-Δ1::HIS3 LEU5*) was again *Leu*⁺ and again contained about 20% of α -IPM synthase activity when compared to wild-type cells. If indeed there are two genetically different α -IPM synthases, then what could be the origin of the second enzyme? It is possible that the second activity is the coincidental property of another enzyme. However, it is also tempting to speculate that the second α -IPM synthase might be an evolutionary relic corresponding to the type of α -IPM synthase seen in *N. crassa* (which in turn is similar in subunit size and certain other properties to the enzyme found in enteric bacteria). This hypothesis assumes that there is a common ancestor for the yeast and *Neurospora* α -IPM synthases and that the principal α -IPM synthase found in present-day yeast was endowed with its distinctive properties (mitochondrial import, CoA-mediated inactivation) by gene duplication, gene fusion and subsequent modification. The presence in the yeast genome of a region of weak homology to a *LEU4*-derived

*Bam*HI Fragment (see Figure 5) may, in fact, be a reflection of the existence of two related α -IPM synthase genes. It is of considerable interest in this context that the larger of the two fragments generated by limited proteolysis of the *LEU4* gene product (molecular weight about 45,000; see Figure 7) is almost identical in size with the subunit of the *Neurospora* α -IPM synthase.

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