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Regulation of isoleucine-valine biosynthesis in *Saccharomyces cerevisiae*

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Summary. The threonine deaminase gene (*ILV1*) of *Saccharomyces cerevisiae* has been designated "multifunctional" since Bollon (1974) indicated its involvement both in the catalysis of the first step in isoleucine biosynthesis and in the regulation of the isoleucine-valine pathway. Its role in regulation is characterized by a decrease in the activity of the five isoleucine-valine enzymes when cells are grown in the presence of the three branched-chain amino acids, isoleucine, valine and leucine (multivalent repression). We have demonstrated that the regulation of AHA reductoisomerase (encoded by *ILV5*) and branched-chain amino acid transaminase is unaffected by the deletion of *ILV1*, subsequently revealing that the two enzymes can be regulated in the absence of threonine deaminase. Both threonine deaminase activity and *ILV1* mRNA levels increase in mutants (*gcd2* and *gcd3*) having constitutively derepressed levels of enzymes under the general control of amino acid biosynthesis, as well as in response to starvation for tryptophan and branched-chain amino acid imbalance. Thus, the *ILV1* gene is under general amino acid control, as is the case for both the *ILV5* and the transaminase gene. Multivalent repression of reductoisomerase and transaminase can be observed in mutants defective in general control (*gcn* and *gcd*), whereas this is not the case for threonine deaminase. Our analysis suggests that repression effected by general control is not complete in minimal medium. Amino acid dependent regulation of threonine deaminase is only through general control, while the branched-chain amino acid repression of AHA reducto isomerase and the transaminase is caused both by general control and an amino acid-specific regulation.

Key words: General amino acid control – *ilv* – multivalent repression – *gcn* – *gcd*

Introduction

Five enzymes catalyze the parallel biosynthesis of isoleucine and valine in the yeast *Saccharomyces cerevisiae*. The first enzyme in the pathway for isoleucine is threonine deaminase (EC 4.2.1.16), an allosteric enzyme converting threonine to α-ketobutyrate. The other four enzymes are shared with the biosynthetic pathway for valine, converting α-ketobutyrate to isoleucine and pyruvate to valine (Fig. 1; Kakar and Wagner 1964; Jones and Fink 1982). The immediate precursor of valine, α-ketoisovaleric acid, serves as the starting point for the synthesis of the third branched-chain amino acid, leucine (Satyanarayana et al. 1968). In yeast, threonine deaminase is encoded by the *ILV1* gene. Mutations in *ILV1* lead to auxotrophy for isoleucine. Acetohydroxy acid (AHA) synthase (EC 4.1.3.18) is coded for by *ILV2*, AHA reductoisomerase (EC 1.1.1.86) by *ILV5* and dihydroxy acid dehydrase (EC 4.2.1.9) by *ILV3*. Mutations in any of the latter three genes lead to auxotrophy for both isoleucine and valine (Kakar and Wagner 1964; Robichon-Szulmajster and Magee 1968). No gene has been assigned to the last enzyme, a branched-chain amino acid aminotransferase. This enzyme may also catalyze the last step in leucine biosynthesis.

The levels of the isoleucine-valine enzymes are regulated. Taking as basal levels the specific activities found in cells growing in minimal medium, repression of the five enzymes is effected by the simultaneous addition

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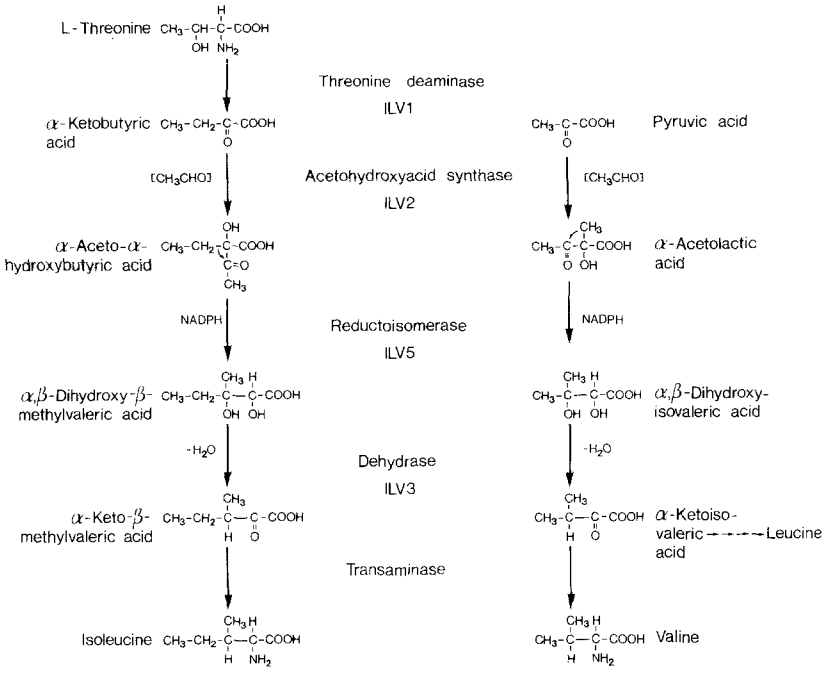


Fig. 1. The biosynthetic pathway for isoleucine and valine in *Saccharomyces cerevisiae*

of isoleucine, valine and leucine (Bussey and Umbarger 1969; Magee and Hereford 1969). This repression, which resembles multivalent repression in bacteria (Freundlich et al. 1962), may be mediated by a specific regulatory system acting in the isoleucine-valine pathway. Since alterations in the regulatory pattern of isoleucine-valine enzymes have been observed in some *ilv1* mutants, it has been suggested that threonine deaminase, in addition to its role in catalysis, has a function as a positive regulator of the other enzymes of the pathway (Bollon and Magee 1971; Bollon and Magee 1973; Bollon 1974; Bollon 1975). Briefly, strains with a threonine deaminase 100-fold less sensitive to feed-back inhibition by Ile have elevated levels of AHA synthase, AHA reductoisomerase and dihydroxy acid dehydrase. Also, strains with nonsense mutations mapping to the middle of *ILV1*, but not strains with carboxyterminal mutations, have altered regulation of the same three enzymes: on minimal medium the levels were the same as in repressed wild-type cells and they were not further repressed by the addition of Ile, Val and Leu.

At least 30 amino acid biosynthetic genes are regulated by general control of amino acid biosynthesis (Hinnebusch 1986). General control is a transcriptionally acting regulatory system which, upon limitation of a single amino acid, leads to the induction of genes coding for amino acid biosynthesis enzymes in many different pathways (Wolfner et al. 1975; Niederberger et al. 1981; Struhl and Davis 1981; Donahue et al. 1983). In isoleucine-valine biosynthesis, AHA reductoisomerase and branched-chain amino acid transaminase activities are increased under amino acid starvation conditions (Delforge et al. 1975; Petersen and Holmberg 1986), whereas the specific activity of AHA synthase does not go up in response to histidine starvation (Wolfner et al. 1975).

The present paper reports on a study of threonine deaminase, AHA reductoisomerase and transaminase regulation. This study was made in order to see if the observed multivalent repression of the enzyme activities by Ile, Val and Leu is caused by a pathway-specific repression, by general control or possibly by an interplay between pathway-specific repression and general control. To examine the proposed regulatory role of *ILV1* in multivalent repression, haploid strains have been constructed in which the *ILV1* coding region has been deleted or disrupted.

Materials and methods

Materials, media and strains. All chemicals used were analytical grade. Restriction endonucleases, T4 DNA ligase and *E. coli* DNA polymerase I were purchased from Boehringer Mannheim or New England Biolabs. All enzymes were used as recommended by the suppliers. [α-^{32}P]dATP was from New England Nuclear (specific activity $\geq$ 600 Ci/mmol).

E. coli strain HB101 was used for transformation according to Cohen et al. (1972).

S. cerevisiae strain X2180-1A (*MAT*a *SUC2 mal mel CUP1*) and the general control mutants RH558-1 (*gcd2*), RH771 (*gcd3*) and RH375-1α (*MAT*α *gcn1*) were kindly provided by P. Niederberger, Zürich, Switzerland. The general control mutant strains were derived by mutation from the isogenic wild-type strains X2180-1A (*MAT*a) and X2180-1B (*Mat*α) (Miozzari et al. 1978; Niederberger et al. 1981; Niederberger et al. 1986). Strain M1-2B (*MAT*α *ura3 trp1*) was kindly provided by J. Polaina. Both the *ilv1* disruption strain (C83-P328, *MAT*α

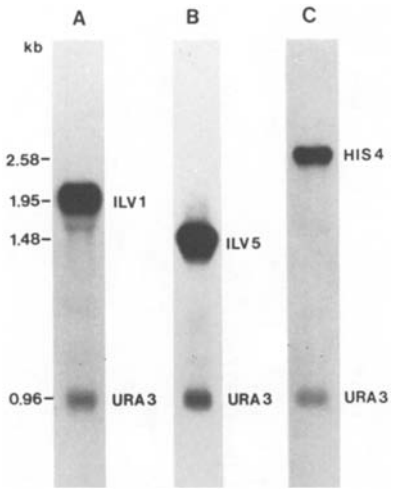


Fig. 2A–C. Northern analysis. RNA was prepared from strain X2180-1A grown in minimal medium, electrophoresed, blotted and probed with radiolabeled DNA fragments containing **A** *ILV1* and *URA3* sequences, **B** *ILV5* and *URA3* sequences and **C** *HIS4* and *URA3* sequences

ura3 trp1 ilv1::URA3 (Petersen et al. 1983)) and the *ilv1* deletion strain (C85-P460, *MATα ura3 trp1 Δilv1::URA3*) were constructed in strain M1-2B. Transformation of yeast followed the cation method of Ito et al. (1983) using lithium acetate. Minimal medium for yeast was 0.67% Bacto Yeast Nitrogen Base without amino acids and 2% glucose, buffered with 10 g succinic acid and 6 g NaOH per liter (pH 5.8). The branched-chain amino acids were used at a final concentration of 2 mM unless otherwise stated. For derepression by general control, the amino acid analogs 5-methyl-DL-tryptophan (MeTrp) and O-methyl-L-threonine (MeThr) were added to the growth medium at a density of $1-3 \times 10^6$ cells per ml at the concentrations indicated in the text, and incubation continued for 6 h.

Preparation of nucleic acids. The *ILV1* containing plasmids pC516, pC517, pC518 and pC521 have been described previously (Petersen et al. 1983; Holmberg et al. 1985). Plasmid pE17/11–B1 containing *ILV5* has been described by Polaina (1984). Plasmid p074 containing part of the *HIS4* coding region was a gift from R. Piñon, San Diego, CA, USA. Plasmid pMB1068 (pBR322-*URA3*) was kindly provided by T. D. Petes, Chicago, IL, USA. Plasmid DNA from *E. coli* was prepared in small scale according to Birnboim and Doly (1979); large scale plasmid preparations were by a scaled-up version of the same procedure followed by CsCl-ethidium bromide equilibrium centrifugation. Total yeast DNA was prepared in small scale as described by Davis et al. (1980). DNA restriction fragments were isolated from agarose gels according to Yang et al. (1979) and further purified by phenol extraction and microcentrifuge gel filtration on Sephadex G-50 (Pharmacia, Uppsala, Sweden). Total yeast RNA was prepared from exponentionally growing cells ($0.5-1.5 \times 10^7$ cells per ml) as described earlier (Holmberg et al. 1985).

Gel transfer hybridizaton. Electrophoresis of total yeast RNA was in 1.5% agarose-formaldehyde gels (Maniatis et al. 1982). DNA or RNA was transferred from agarose gels to nitrocellulose paper (Millipore HAWP 00010 or Schleicher and Schuell BA85) and hybridized to ^{32}P-labeled DNA according to standard methods (Maniatis et al. 1982). Autoradiographic exposure using Kodak RP1 film was for 1–2 days with or without Kodak X-Omatic regular intensifying screens at −80 °C.

Preparation of DNA probes. The *ILV1* probe used in Southern analysis was the purified 5.8 kilo base pair (kb) HindIII-SalI insert of plasmid pC516 isolated after cloning into pBR322 (giving pC519). In Northern analysis, pC519 hybridizes to three mRNA's (Petersen et al. 1983). For the regulatory studies, the following gene-specific probes were used: the *ILV1* probe was a 2.8 kb HindIII-SalI fragment from pC516 subcloned into pBR-322 (pC517); the *ILV5* probe was a 1.3 kb BamHI-PvuII fragment from pE17/11-B1 (Polaina 1984) subcloned in pUC19; the *HIS4* probe was a 0.56 kb EcoRI-HindIII fragment from pC503 (Holmberg et al. 1979) subcloned in pBR322 (giving p074) and the *URA3* probe was a 1.1 kb HindIII fragment from pMB1068. The isolated fragments were nick-translated according to Maniatis et al. (1982).

Determination of relative levels of individual mRNA species by Northern blots. Samples containing 20 μg total yeast RNA were loaded on the gels. Each blot was hybridized to the *URA3* probe as well as to either the *ILV1*, *ILV5* or the *HIS4* probe. Since the amount of *URA3* mRNA does not vary with the growth conditions used (Silverman et al. 1982), it was used as an internal standard for the amount of RNA. The Northern blots in Fig. 2 show the *ILV1* mRNA of 1,950 nucleotides, the *ILV5* mRNA of 1,480 and the *HIS4* mRNA of 2,580 nucleotides, as well as the 960 nucleotide long *URA3* mRNA. The mRNAs were quantified by using the autoradiograms as a matrix for cutting out the bands from the nitrocellulose filter and the radioactivity present in the bands was determined by counting in a Beckmann LS3155T liquid scintillation counter. The number of counts present in the *URA3* mRNA band was used for normalization.

Enzyme assays. Enzyme assays were performed with cells made permeable by treatment with either Triton X100 (Miozzari et al. 1978) or toluene (Magee and Hereford 1969). Cells were grown and harvested as already described for RNA isolation. Threonine deaminase was determined according to Robichon-Szulmajster and Magee (1968), reductoisomerase according to Magee and Hereford (1969), branched-chain amino acid transaminase according to Aki and Ichihara (1970) using L-valine as the substrate, and histidinol dehydrogenase as described by Fink and Styles (1974) using [^{14}C]histidinol. Total protein was measured with the Bio-Rad protein assay after boiling the cell samples in 0.5 M NaOH for 5 min and using bovine serum albumin as the standard. Threonine deaminase activity was measured as nmol α-ketobutyrate formed/min per mg protein; reductoisomerase as nmol α,β-dihydroxy isovaleric acid formed/min per mg protein; transaminase as nmol α-ketoisovaleric acid formed/min per mg protein and histidinol dehydrogenase activity as cpm [^{14}C]-histidine formed/min per $OD_{550} \times ml^{-1}$.

Results

Multivalent repression of isoleucine and valine enzymes

We have analyzed threonine deaminase, encoded by *ILV1*, acetohydroxy acid (AHA) reductoisomerase, encoded by *ILV5*, and branched-chain amino acid transaminase (see Fig. 1) with respect to repression by isoleucine, valine and leucine in the wild-type strain X2180-1A. The upper parts of Table 1A and B show the relationship

Table 1. Regulation in isoleucine and valine biosynthesis. *ILV1* and *ILV5* enzyme activity and mRNA level and branched-chain amino acid transaminase activity in exponentially growing cells of wild type and isogenic general control mutant strains are given. *Numbers in parenthesis* are the activities relative to the levels found in the wild-type strain X2180-1A grown in minimal medium (*MA*). The concentration of 5-methyltryptophan (*MeTrp*) was 0.5 mM and O-methylthreonine (*O-MeThr*) was used at 2 mM

Table 1A

Strain	Medium	Threonine deaminase[a]						Mean of relative levels	*ILV1* mRNA[b]						Mean of relative levels
		Experiment no							Experiment no						
		1	2	3	4	5	6		1	2	3	4	5	6	
X2180-1A	MA	26.2 (1)	23.0 (1)	16.5 (1)	12.0 (1)	43.3 (1)	46.9 (1)	1	153 (1)	806 (1)	51 (1)	83 (1)	309 (1)	156 (1)	1
	MA + Ile	–	–	–	–	46.6 (1.08)	48.7 (1.04)	1.06	180 (1.18)	1,028 (1.28)	54 (1.06)	77 (0.93)	–	–	1.11
	MA + IleValLeu	23.3 (0.89)	21.5 (0.93)	8.8 (0.53)	8.7 (0.73)	38.5 (0.89)	45.2 (0.96)	0.82	119 (0.78)	691 (0.86)	36 (0.71)	52 (0.63)	–	–	0.75
	MA + MeTrp	45.5 (1.74)	36.0 (1.57)	25.3 (1.53)	23.7 (1.98)	– –	– –	1.71	253 (1.65)	1,869 (2.32)	69 (1.35)	–	555 (1.80)	214 (1.37)	1.70
	MA + IleValLeu + MeTrp	49.2 (1.88)	49.1 (2.13)	27.6 (1.67)	25.8 (2.15)	–	–	1.96	–	–	–	–	534 (1.73)	263 (1.69)	1.71
	MA + MeThr	44.4 (1.69)	50.8 (2.21)	–	–	99.8 (2.30)	74.7 (1.59)	1.95	–	–	–	–	–	–	
	MA + Leu (20 mM)	43.7 (1.67)	42.3 (1.84)	–	–	86.3 (1.99)	75.7 (1.61)	1.78	192 (1.25)	1,343 (1.67)	63 (1.24)	95 (1.14)	–	–	1.33
X2180-1A	MA	34.0 (1)	9.5 (1)	40.6 (1)	56.2 (1)	53.0 (1)	50.3 (1)	1	751 (1)	61 (1)	83 (1)	62 (1)			1
RH558-1	MA	62.3 (1.83)	13.9 (1.46)	–	–	76.6 (1.45)	89.9 (1.79)	1.63	1,567 (2.09)	120 (1.97)	–	117 (1.89)			1.98
	MA + IleValLeu	–	–	–	–	80.5 (1.52)	110.9 (2.20)	1.86							
RH771	MA	51.2 (1.51)	13.8 (1.45)	–	–	78.5 (1.48)	108.6 (2.16)	1.65	1,300 (1.73)	148 (2.43)	115 (1.39)	153 (2.47)			2.01
RH375-1α	MA	28.7 (0.84)	8.9 (0.94)	44.6 (1.10)	48.0 (0.85)	46.3 (0.87)	49.5 (0.98)	0.93	878 (1.17)	58 (0.95)	–	–			1.06
	MA + IleValLeu	30.3 (0.89)	8.0 (0.84)	39.6 (0.98)	44.8 (0.80)	46.2 (0.87)	46.5 (0.92)	0.88							

Table 1B

Strain	Medium	Reductoisomerase activity[a]					Mean of relative levels	*ILV5*mRNA[b]			Mean of relative levels
		Experiment no						Experiment no			
		1	2	3	4	5		1	2	3	
X2180-1A	MA	2.27 (1)	2.31 (1)	3.61 (1)	2.05 (1)	2.03 (1)	1	1292 (1)	112 (1)	142 (1)	1
	MA + Ile	1.84 (0.81)	2.01 (0.87)	3.00 (0.83)	–	–	**0.84**	–	–	–	
	MA + IleValLeu	1.17 (0.52)	1.43 (0.62)	1.94 (0.54)	0.66 (0.32)	0.77 (0.38)	**0.48**	938 (0.73)	80 (0.71)	–	**0.72**
	MA + MeTrp	2.49 (1.10)	2.32 (1.00)	4.26 (1.18)	1.99 (0.97)	1.71 (0.84)	**1.02**	1454 (1.13)	127 (1.13)	157 (1.11)	**1.12**
	MA + IleValLeu + MeTrp	1.92 (0.85)	2.23 (0.97)	–	–	–	**0.91**	1533 (1.19)	–	–	**1.19**
X2180-1A	MA	3.41 (1)	4.19 (1)	3.92 (1)	2.48 (1)		**1**				
RH558-1	MA	4.44 (1.30)	3.93 (0.94)	4.58 (1.17)	5.05 (2.04)		**1.36**				
	MA + IleValLeu	2.93 (0.86)	3.50 (0.84)	–	–		**0.85**				
RH771	MA	5.30 (1.55)	4.79 (1.14)	6.34 (1.62)	5.24 (2.11)		**1.61**				
RH375-1α	MA	3.33 (0.98)	3.87 (0.92)	5.17 (1.31)	3.24 (1.31)		**1.13**				
	MA + IleValLeu	1.96 (0.57)	1.78 (0.42)	2.84 (0.72)	1.41 (0.57)		**0.57**				

between enzyme activities and mRNA levels for *ILV1* and *ILV5* after growth in minimal medium and in minimal medium supplemented with either Ile, or Ile, Val and Leu. The enzyme activity of threonine deaminase is repressed to 82% (mean of 6 independent experiments) when Ile, Val and Leu are added to the minimal medium. The steady-state level of the *ILV1* mRNA is repressed to the same small extent (about 75%), suggesting that the repression is transcriptional. The addition of Ile alone has no effect. The repression by Ile, Val and Leu of the AHA reductoisomerase is about 2-fold and appears also to be effected transcriptionally (Table 1B). Included in Table 1C are data for the transaminase showing that Ile, Val and Leu in combination repress its acitivity to a mean value of 47%. The observed multivalent repression of these enzyme activities is in aggreement with published results (Bussey and Umbarger 1969).

Threonine deaminase is dispensable for multivalent repression of reductoisomerase and transaminase

Altered regulation of isoleucine-valine enzymes have been reported in different *ilv1* mutant strains (Bollon and Magee 1973; Bollon 1974). This has raised the question whether a domain of threonine deaminase is essential for multivalent repression. This was tested by destroying the chromosomal copy of *ILV1* in strain M1-2B by two different methods. An *ilv1* deletion mutant, strain C85-P460, was constructed by the one-step gene replacement technique (Rothstein 1983) replacing two EcoRV fragments (3.0 and 0.2 kb) of the *ILV1* chromosomal region with a 1.1 kb HindIII fragment containing the *URA3* gene (Fig. 3). In this strain, the promoter region and 1.6 kb of the coding region are deleted, leaving only 135 bp of the 3′ end of the coding region (7.8%). In the previously constructed (Petersen et al. 1983) disruption strain, C83-P328, 5.4 kb DNA consisting of pBR322 and the *URA3* gene is inserted at position + 1258 in the *ILV1* coding region (Fig. 3). Both constructions were confirmed by Southern blot

Table 1C

Strain	Medium	Transaminase activity[a] Experiment no 1	2	3	4	5	6	Mean of relative levels
X2180-1A	MA	93.9 (1)	38.1 (1)	57.2 (1)	16.5 (1)	12.0 (1)	55.5 (1)	1
	MA + Ile	118.3 (1.26)	48.1 (1.26)	–	–	–	–	1.26
	MA + IleValLeu	29.1 (0.31)	16.8 (0.44)	27.3 (0.48)	8.8 (0.53)	8.7 (0.73)	19.0 (0.34)	0.47
	MA + MeTrp	–	44.4 (1.17)	112.5 (1.97)	25.3 (1.53)	23.7 (1.98)	–	1.66
	MA + IleValLeu + MeTrp	–	–	–	27.6 (1.67)	25.8 (2.15)	–	1.91
	MA + MeThr	–	70.2 (1.84)	83.9 (1.47)	31.4 (1.90)	18.8 (1.57)	–	1.70
	MA + Leu (20 mM)	–	–	55.8 (0.98)	20.3 (1.23)	18.7 (1.56)	61.8 (1.11)	1.22
X2180-1A	MA	87.3 (1)	63.4 (1)	57.2 (1)	55.5 (1)	54.9 (1)	47.1 (1)	1
RH558-1	MA	130.2 (1.49)	127.1 (2.00)	83.9 (1.47)	77.9 (1.40)	94.8 (1.73)	102.1 (2.17)	1.71
	MA + IleValLeu	–	–	–	–	70.6 (1.29)	65.2 (1.38)	1.34
RH771	MA	–	–	72.9 (1.27)	70.1 (1.26)	109.9 (2.00)	90.2 (1.92)	1.61
RH375-1α	MA	75.2 (0.86)	69.2 (1.09)	42.7 (0.75)	44.9 (0.81)	42.6 (0.78)	37.5 (0.80)	0.85
	MA + IleValLeu	24.9 (0.29)	–	20.6 (0.36)	21.6 (0.39)	19.4 (0.35)	21.6 (0.46)	0.37

[a] Enzyme activities expressed as nmol product formed/min per mg protein

[b] Cpm per 100 cpm in the *URA3* band. The number of cpm measured in the *URA3* band was between 197 and 580

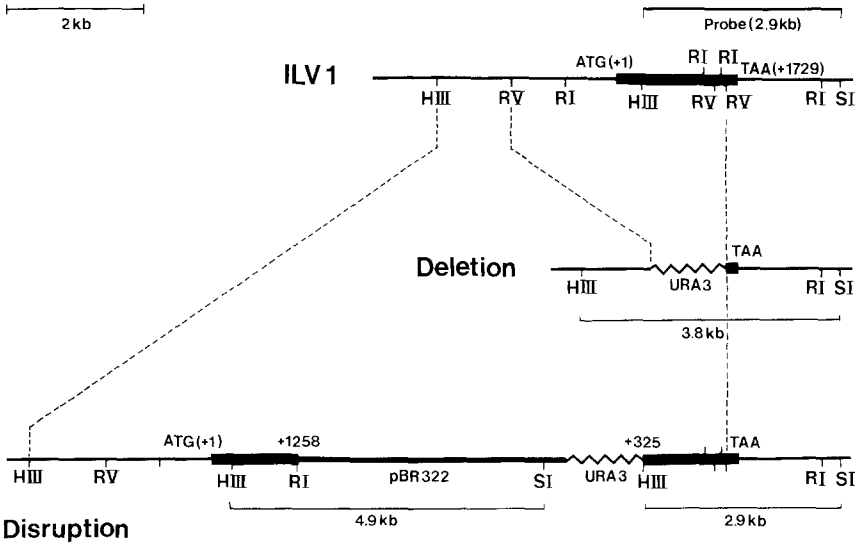
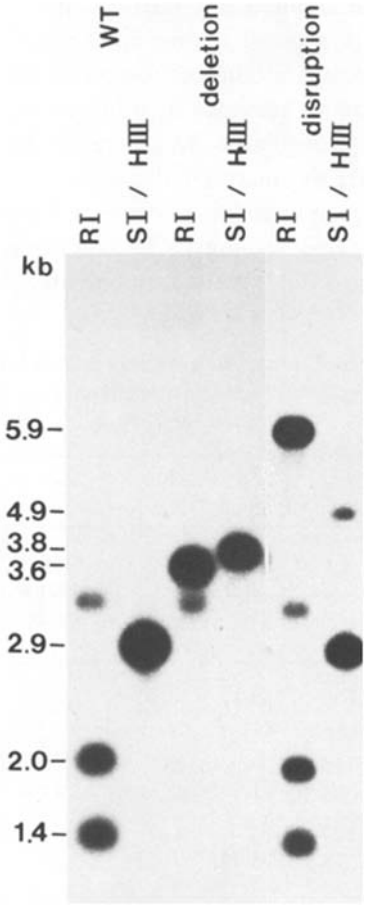


Fig. 3. Gene replacement and gene disruption of *ILV1*. The *ILV1* chromosomal region is shown. The *box* indicates the position of the *ILV1* coding region. The *ilv1* deletion was constructed by replacing two EcoRV fragments (3.0 and 0.2 kb) with a 1.1 kb HindIII fragment harboring the *URA3* gene. The *ilv1* disruption was constructed by the integration of plasmid pC521 into the *ILV1* coding region (Petersen et al. 1983). The DNA fragments generated by HindIII and SalI digestion of genomic DNA – which should hybridize to the 2.9 kb *ilv1* probe used in Southern analysis (Fig. 4) – are indicated. EcoRI = *RI*, EcoRV = *RV*, HindIII = *HIII*, SalI = *SI*

Table 2. Repression by isoleucine, valine and leucine of AHA reductoisomerase and branched-chain amino acid transaminase in wild type, and in isogenic *ilv1* deletion and disruption strains. The strains were grown in minimal medium supplemented with uracil and tryptophan. Enzyme activities are expressed as nmol of product formed/min per mg protein. *Numbers in parenthesis* show the enzyme levels relative to growth in medium with isoleucine for each of the three strains

Strain	Medium	Reductoisomerase				Mean of relative levels	Transaminase				Mean of relative levels
		Repeated series					Repeated series				
		1	2	3	4		1	2	3	4	
M1-2B (wt)	+Ile	1.32 (1)	1.61 (1)	3.7 (1)	3.6 (1)	1	70.6 (1)	82.0 (1)	60.5 (1)	63.4 (1)	1
	+ IleValLeu	1.00 (0.76)	0.85 (0.53)	2.7 (0.73)	2.6 (0.72)	0.69	29.8 (0.42)	30.4 (0.37)	28.2 (0.47)	26.6 (0.42)	0.42
C85-P460 (*Δilv1::URA3*)	+ Ile	1.60 (1)	1.61 (1)	1.85 (1)	2.25 (1)	1	94.7 (1)	68.0 (1)	80.5 (1)	78.6 (1)	1
	+ IleValLeu	1.43 (0.89)	1.69 (1.05)	1.22 (0.66)	1.42 (0.63)	0.81	48.2 (0.51)	38.3 (0.56)	42.6 (0.53)	36.9 (0.47)	0.52
C83-P328 (*ilv1::URA3*)	+ Ile	4.8 (1)	6.6 (1)	5.1 (1)	6.4 (1)	1	60.2 (1)	68.5 (1)	55.1 (1)	60.6 (1)	1
	+ IleValLeu	4.2 (0.88)	4.6 (0.70)	3.7 (0.73)	4.0 (0.63)	0.74	37.1 (0.62)	37.0 (0.54)	32.0 (0.58)	35.3 (0.58)	0.58



analysis (Fig. 4). We then tested whether reductoisomerase and transaminase are repressed by the branched-chain amino acids in the two strains (Table 2). Since the strains are auxotrophic for Ile, we compared enzyme levels in cells grown in supplemented minimal medium with the addition of Ile, Val and Leu, and with Ile alone. It was observed that the simultaneous presence of Ile, Val and Leu decreases the levels of the two enzymes in both the deletion and the disruption strain and to about the same degree as found in the parent strain. This result argues strongly against any regulatory role of the *ILV1* gene product in the repression of reductoisomerase and transaminase by the branched-chain amino acids.

◀ **Fig. 4.** Southern blot analysis of *ilv1* deletion and disruption strains. EcoRI and HindIII + SalI digests of DNA from the deletion strain C85-P460, the disruption strain C83-P328 and the *ILV1* wild type strain M1-2B were probed with the nick-translated 2.9 kb HindIII-SalI fragment from the *ILV1* region (see Fig. 3). DNA from the deletion strain shows hybridization to the predicted 3.8 kb HindIII-SalI gene-replacement band indicated in Fig. 3. The disruption strain shows hybridization to the predicted HindIII-SalI fragments of 2.9 and 4.9 kb indicated in Fig. 3. The molecular structures of the *ilv1* mutations are also confirmed by the EcoRI digests. EcoRI = *RI*, HindIII = *HIII*, SalI = *SI*

General control of isoleucine-valine enzymes

The specific activities of AHA reductoisomerase and transaminase are increased under amino acid starvation conditions (Delforge et al. 1975; Petersen and Holmberg 1986), indicating regulation by general amino acid control (Wolfner et al. 1975; Niederberger et al. 1981). We examined whether threonine deaminase is also regulated by general control. Enzyme and mRNA levels were determined in wild-type cells and in the isogenic constitutively derepressed mutants, RH558-1 [*gcd2* (Miozzari et al. 1978)] and RH771 [*gcd3* (Niederberger et al. 1986)], which produce derepressed levels of enzymes known to be under general control.

In strains RH558-1 and RH771, the level of threonine deaminase activity is about 1.7 times higher than that found in the wild type (Table 1A). A similar derepression of *ILV1* mRNA is observed in both strains. In wild-type cells, both the *ILV1* enzyme and mRNA levels are increased 1.7-fold after the addition of 5-methyltryptophan (upper part of Table 1A). This tryptophan analog acts as a false feed-back inhibitor of anthranilate synthase (Miozzari et al. 1977), resulting in tryptophan starvation, which then provides a signal for derepression by general control. Similarly, the addition of the threonine analog O-methyl-threonine (Cervone and Iaccarino 1972) gives a 2-fold increase in threonine deaminase activity. Branched-chain amino acid imbalance brought about by excess Leu (Niederberger et al. 1981) also derepresses both threonine deaminase and its mRNA. Finally, derepression takes place when 5-methyltryptophan is added to minimal medium supplemented with Ile, Val and Leu. Thus, the *ILV1* gene is subject to general control of amino acid biosynthesis.

Analysis of the reductoisomerase in strains RH558-1 and RH771 gave enzyme levels which are about 1.4 and 1.6 times the wild-type levels, respectively (Table 1B). However, no derepression on either enzyme or mRNA is seen in wild-type cells growing in the presence of 5-methyltryptophan. On the other hand, addition of 5-methyltryptophan to minimal medium containing Ile, Val and Leu increases the levels of both reductoisomerase and *ILV5* mRNA, but not above the level found in unsupplemented minimal medium. Taken together, the *ILV5* gene is subject to general control.

The relative levels of the transaminase are increased both in the wild-type strain X2180-1A under amino acid limitation and in the *gcd* mutants, as compared to the levels found in the wild-type strain in minimal medium (Table 1C). This confirms and extends the result of Delforge et al. (1975) that the transaminase is under general amino acid control.

Branched-chain amino acid repression and general control

As pointed out above, 5-methyltryptophan fails to derepress the *ILV5* gene in wild-type cells grown in minimal medium, but is effective in minimal medium supplemented Ile, Val and Leu. This can be explained by assuming that wild-type cells growing in minimal medium are limited for one or more of the branched-chain amino acids, thus leading to almost full derepression of the *ILV5* gene by general amino acid control. If multivalent repression is simply a result of preventing derepression by general control, it should be observable for at least some genes encoding enzymes in other amino acid biosynthetic pathways regulated by general amino acid control. To test this, we analyzed the expression of the *HIS4* gene. This gene encodes an enzyme functioning in three of the steps leading to the synthesis of histidine (the *HIS4 A*, *B* and *C* regions), and is subject

Table 3. Histidinol dehydrogenase (*HIS4C* activity (cpm/min per $OD_{550} \times ml^{-1}$)) and *HIS4* mRNA steady-state levels in strain X2180-1A under various growth conditions. *Numbers in parenthesis* show the *HIS4* mRNA levels relative to the levels found when cells are grown in minimal medium

Medium	Histidinol dehydrogenase activity								*HIS4* mRNA[a]					
	Individual measurements						Mean	Relative value of the mean	Repeated series					Mean of relative levels
									1	2	3	4	5	
MA	158	146	164	184	191	168	169	**1**	2,395	191	150	113	99	
	175								(1)	(1)	(1)	(1)	(1)	**1**
MA + MeTrp	368	316	291	308	257	308	308	**1.82**	8,329	293	315	–	256	
									(3.48)	(1.53)	(2.10)		(2.59)	**2.43**
MA + IleValLeu	112	139	151	145	141	101	135	**0.80**	1,930	114	127	81	83	
	153								(0.81)	(0.60)	(0.85)	(0.72)	(0.84)	**0.76**

[a] Cpm per 100 cpm in the *URA3* band. The number of cpm measured in the *URA3* band was between 188 and 788

to general control (Wolfner et al. 1975; Silverman et al. 1982). The result is given in Table 3. Both histidinol dehydrogenase activity (*HIS4C*) and *HIS4* mRNA are derepressed in wild-type cells starved for tryptophan by adding 5-methyltryptophan. When Ile, Val and Leu are added to minimal medium, the levels of *HIS4C* enzyme and *HIS4* mRNA are repressed to 80% and 76% (mean values), respectively.

To further test the relationship between general amino acid control and the repression by Ile, Val and Leu, we looked at whether repression of threonine deaminase, AHA reductoisomerase and the transaminase could be observed in the isogenic general control mutant strains RH375-1α (*gcn1*) and RH558-1 (*gcd2*). This analysis is shown in Table 1. In both the *gcn1* and *gcd2* strain no repression of threonine deaminase activity is seen by the addition of Ile, Val and Leu to minimal medium (Table 1A), suggesting that the slight repression observed in wild-type cells is brought about by the general control system. In contrast, the addition of Ile, Val and Leu causes the repression of both AHA reductoisomerase and the transaminase in the *gcn1* strain; this repression is comparable to that found in wild-type cells (Table 1B and C). In the *gcd2* strain also, the levels of the two enzyme activities are lowered although the degree of repression is somewhat reduced as compared to that of the wild-type. Thus, the repression of reductoisomerase and transaminase by the three branched-chain amino acids seems not to be mediated solely by the general control system.

Discussion

The repression of isoleucine-valine biosynthetic enzymes has been observed to occur in yeast with the addition of Ile, Val and Leu to minimal medium (multivalent repression) (Bussey and Umbarger 1969; Magee and Hereford 1969). We find that in the wild-type yeast strain X2180-1A, this repression is about 20% for threonine deaminase and 50% for both AHA reductoisomerase and branched-chain amino acid aminotransaminase in comparison to enzyme levels found in cells grown in minimal medium (Table 1). We have not been able to see repression of threonine deaminase by Ile alone as reported by Bollon (1975). This and other minor differences to earlier studies, e.g., the degree of repression (Bussey and Umbarger 1969; Magee and Hereford 1969), may reflect strain differences.

The *ILV1* gene has been termed a multifunctional gene (Bollon 1975). It has been proposed that threonine deaminase, in addition to its catalytic activity, plays a direct role in multivalent repression of the other isoleucine-valine enzymes, possibly exerted by the binding of a charged leucyl-tRNA to the carboxyterminal end of the enzyme (Bollon and Magee 1971; Bollon 1974). If this is correct, repression by the simultaneous presence of Ile, Val and Leu should be abolished in a strain in which the chromosomal copy of *ILV1* has been deleted. Using isogenic strains, we have shown that at least for AHA reductoisomerase and the transaminase, repression by the branched-chain amino acids is unaffected by delection or disruption of *ILV1* (Table 2), indicating that *ILV1* plays no role in the regulation of gene expression. A truncated protein may be produced by the disrupted *ilv1* gene, whereas it is unlikely that a polypeptide active in regulation is produced in the *ilv1* deletion strain. In addition, in the latter construction, direction of transcription of the *URA3* gene is away from the remaining 135 bp of the *ilv1* coding region. The observed derepression of AHA synthase and AHA reductoisomerase in mutants with a feed-back resistant threonine deaminase (Bollon and Magee 1971) could be a consequence of induction through general control brought about by threonine starvation. Such strains would be expected to be low in threonine and high in isoleucine pools. Strains with centrally mapping nonsense mutations in *ILV1* were reported to have unregulated repressed levels of AHA synthase, reductoisomerase and dehydrase (Bollon and Magee 1973). Jones and Fink (1982) discussed the possibility that the abnormally slow transport of the threonine deaminase ochre fragment into the mitochondrion, where isoleucine-valine synthesis takes place (Ryan and Kohlhaw 1974), could have effects on regulation, assuming that the enzymes form a complex. An explanation along these lines does not seem likely as normal regulation of reductoisomerase and transaminase is observed in the *ilv1* deletion strain (Table 2). In contrast, our results suggest that the isoleucine-valine enzymes do not form a stringently organized complex in the mitochondrion. Moereover, such a complex is difficult to envisage, since the *ILV5* gene appears to be highly expressed, reductoisomerase being at least 10-fold more abundant than threonine deaminase (Petersen and Holmberg 1986).

In this paper it is shown that the *ILV1* gene encoding threonine deaminase is regulated by general control of amino acid biosynthesis (Table 1). In agreement with this, DNA sequences upstream of *ILV1* contain several copies of the general control repeat consensus sequence TGACTC (Kielland-Brandt et al. 1984). This sequence has been directly implicated as the common recognition signal for genes under general control (Struhl 1982; Donahue et al. 1983; Hinnebusch et al. 1985). *GCN4* protein is a positive effector of general control (Hinnebusch and Fink 1983) and its in vitro translation product binds specifically to the *HIS3* regulatory site (Hope and Struhl 1985). It has recently been shown that purified *GCN4* protein binds to the general control repeat se-

quences found in the *ILV1* promoter region (Arndt and Fink 1986). In agreement to an earlier study (Petersen and Holmberg 1986) we have found that the reductoisomerase gene (*ILV5*) also appears to be subject to general control (Table 1). However, the regulation of *ILV5* is different from that of other genes subject to general control since derepression of reductoisomerase is not observed in cells starved for tryptophan in normal ammonium-containing minimal medium; it is observed when wild-type cells are starved for tryptophan in the presence of Ile, Val and Leu. In the case of AHA synthase (encoded by *ILV2*), histidine starvation, as well as a branched-chain amino acid imbalance brought about by the addition of Leu to ammonium-containing minimal medium, does not lead to an increase in enzyme activity (Bussey and Umbarger 1969; Wolfner et al. 1975). In analogy with *ILV5*, it would be interesting to see whether amino acid starvation in the presence of Ile, Val and Leu could bring about derepression of the AHA synthase. The *ILV2* promoter region contains a single copy of the general control consensus sequence. This sequence has an affinity for the *GCN4* protein in vitro (Falco et al. 1985; Arndt and Fink 1986), suggesting that *ILV2* is subject to cross-pathway control. This might also be the case with the dihydroxy acid dehydrase (encoded by *ILV3*) as Bussey and Umbarger (1969) observed a small derepression in wild-type cells upon the addition of Leu to minimal medium. Thus, there is evidence that all five enzymes in isoleucine-valine biosynthesis are regulated by general amino acid control.

The analysis of *HIS4* (Table 3) shows that repression by Ile, Val and Leu is not restricted to Ile-Val biosynthesis, i.e., it is not a result of pathway-specific regulation. The observed degree of repression of *HIS4* is comparable to that of *ILV1*. Since repression of threonine deaminase has not been seen in the *gcn1* and *gcd2* strains, regulation by general control may be responsible for the slight repression of *ILV1* and *HIS4*. We propose that wild-type cells growing in minimal medium undergo a shortage for one or more of the branched-chain amino acids, thereby eliciting a derepression of enzymes under general control. This is supported by the following observations by Niederberger et al. (1981). (i) Wild-type cells grow slightly slower when Ile, Val and Leu are not present in minimal medium and (ii) arginiosuccinate lyase activity (encoded by *ARG4* and regulated by general amino acid control) decreases when wild-type cells are grown under multivalent repression conditions.

The analysis of reductoisomerase and transaminase in both *gcn1* and *gcd2* strains showed that the simultaneous presence of Ile, Val and Leu lead to repression of the enzymes' activities (Table 1). This suggests that the genes encoding the two enzymes are subject to regulation by an amino acid dependent system in addition to regulation by the general control.

Leucine alone causes a 2- to 3-fold repression of *LEU1* and *LEU2* enzymes, whereas leucine plus threonine result in a more than 10-fold decrease in the enzyme levels (Satyanarayana et al. 1968; Brown et al. 1975). This is accompanied by repression of the corresponding mRNA levels (Andreadis et al. 1984; Hsu and Schimmel 1984). A short GC-rich palindromic sequence, GCCGGA-ACCGGCTT, has been identified upstream of *LEU2* affecting both expression and leucine regulation of the gene (Martinez-Arias et al. 1984). Homologous sequences have also been noted upstream of *LEU1* (Hsu and Schimmel 1984) and *LEU4* (Beltzer et al. 1986). The effect of leucine on *LEU1* and *LEU2* may be an indirect one, as it has been suggested that the expression of the two genes is controlled by a complex between α-isopropylmalate and the *LEU3* gene product (Baichwal et al. 1983). Interestingly, a related DNA element is present in the 5′ noncoding region of *ILV5* (Petersen and Holmberg 1986), implying that this gene has the potential to be regulated by leucine-specific signals in addition to being subject to general control. We have also observed this sequence motif in front of *ILV2* (Falco et al. 1985) but we have not been able to identify a similar sequence element upstream of *ILV1* (Kielland-Brandt et al. 1984) or *HIS4* (Donahue et al. 1982). We suggest that in the case of *ILV5* and *ILV2*, the repression by Ile, Val and Leu is effected both by general amino acid control and regulation by leucine specific signals. It is tempting to spectulate that this is also the case with *ILV3* and the gene encoding the branched-chain amino acid transaminase, as all four gene products have metabolic links to the leucine pathway. In contrast, the *ILV1* gene encoding an enzyme which is not a prerequisite for leucine biosynthesis seems to be regulated solely by the general control system.

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