Isolation of a conditional suppressor of leucine auxotrophy in *Saccharomyces cerevisiae*

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Phenotypically and genotypically (*leu2-3,112*) *Leu*[−] cells of *Saccharomyces cerevisiae* gave rise to small colonies on medium devoid of leucine. This only occurred on plates with a high density of *Leu*[−] cells or on medium supplemented with limiting quantities of leucine. Cells from these small colonies retained a growth advantage over their parent on limiting leucine supplements even after growth in a non-selecting (rich) medium. Therefore, the growth variants had acquired a heritable change. The phenotype was recessive and due to a change in a nuclear gene unlinked to the *LEU2* locus. The phenotype provided a growth advantage only during leucine starvation; growth of the variants was indistinguishable from their parent on medium lacking the other essential supplements (histidine and uracil) required for the growth of the strain. [¹⁴C]Leucine uptake assays demonstrated that the variants were better able than their parents to accumulate leucine from their environments, and this ability extended to other hydrophobic amino acids. These results suggest that in the variants an amino acid uptake system has been derepressed rather than there having been reversion or extragenic suppression of the mutation in leucine biosynthesis. We designate the mutant gene responsible for the phenotype *lup1* (for leucine uptake). The transport characteristics of the *lup1* mutants suggested that *LUP1* is a regulatory component of an ammonium-regulated hydrophobic amino acid uptake system.

Keywords: *Saccharomyces cerevisiae*, leucine, auxotrophy, amino acid transport, adaptive mutagenesis

INTRODUCTION

The *leu2-3,112* allele in the yeast *Saccharomyces cerevisiae* is a stable mutation. Because the allele does not revert, it has been used, for example, to demonstrate the delivery of large bacterial conjugative plasmids carrying the *LEU2* gene during matings between bacteria and yeast (Heinemann & Sprague, 1989). However, small colonies often do form during selection for *Leu*⁺ cells on medium devoid of leucine, even in controls without bacterial donors. Nevertheless, cells from these small colonies remain leucine auxotrophs. We considered the possibility that growth was due to variants that have regained a limited ability to synthesize leucine (by reversion or extragenic suppression of *leu2*). Alternatively, phenotypic suppression of the *Leu*[−] phenotype could be due to variants that have acquired the ability to better concentrate leucine from their environment by alterations of leucine uptake pathways.

Growth of *Leu*[−] cells might be due to leucine 'scavengers', variants within the population that are *Leu*[−] but grow because they accumulate sufficient quantities of leucine by some other means, such as transporting leucine released by dying neighbours. Leucine is presumed to enter *S. cerevisiae* cells by at least three routes (Horak, 1986; Ramos *et al.*, 1980; Wainer *et al.*, 1988). The first, called the general amino acid permease (GAP1) system after the permease gene *GAP1*, is a low-affinity, high-velocity pathway activated in response to nitrogen starvation (Grenson *et al.*, 1970). Depending on the yeast strain and nitrogen source, the GAP1 system is inducible 5 to 460-fold, and is thus classified as an adaptive system. The second pathway, also a low-affinity and high-velocity pathway, is specific for leucine and constitutive. The third pathway is a constitutive, leucine-specific, high-affinity and low-velocity system (Ramos *et al.*, 1980). The BAP (branched amino acid permease) system also transports leucine in a high-affinity, low-velocity manner and may be

identical to the high-affinity, low-velocity leucine transport system (Tullin *et al.*, 1991). Because the GAP1 pathway is negatively regulated by ammonium, its contribution to leucine transport is negligible in low leucine/high ammonium environments, and leucine must therefore enter by the other pathways under these conditions. There is some evidence to suggest that the constitutive leucine transport systems are also negatively regulated by ammonium salts (Kotliar *et al.*, 1990).

In this report we present evidence that the small colonies formed by *leu2-3,112* cells are not due to reversion or extragenic suppression of *leu2*. The mutation was recessive and did not map to the *leu2* locus. Rather, variant cells were better able to concentrate leucine in low leucine, high ammonium environments. The specificity of the variant phenotype for leucine and the rate of the uptake reaction indicated that derepression of the GAP1 pathway or increased activity of the low-affinity leucine uptake pathway were not the cause of the phenotype. However, the evidence is consistent with the inappropriate derepression of the high-affinity leucine uptake system. We have designated the mutant gene responsible for this phenotype, *lup1*, for leucine uptake.

METHODS

Relevant strain descriptions. Growth variants were derivatives of 329-6C (*Mata his3-537::TRP1::his3-513 leu2-3,112 trp1 ura3-52 ade6*). Strain 329-6C (Klein, 1988) was used as the parent, or wild-type, control in all experiments. Strain JY108 is a *Leu*⁺ derivative of 329-6C created by integration of the *LEU2* gene into the 329-6C genome. *LEU2* DNA was introduced by conjugation with bacteria as described previously (Heinemann & Sprague, 1991). JY117 and JY118 were selected on solid medium from populations of 329-6C by growth in a leucine-limiting (0.01 mg ml⁻¹) environment. JY126 is a spontaneous *His*⁺ derivative of JY117. JY107 (*Mata leu2-3,112 his3 gal2 can1*) was created by integration of the *URA3* gene into the genome of SY1229 (Heinemann & Sprague, 1989) following conjugation with bacteria as described (Heinemann & Sprague, 1991). JY127 (*MATa/α leu2-3,112/leu2-3,112 HIS3/his3 URA3/ura3*) was derived from a cross between JY126 and JY107. JY12714Aa (*MATa lup1 leu2-3,112 ura3 his3*) was isolated as a haploid segregant of JY127 and mated to SY2512 (*MATa LEU2 ura3 arg4 ade1*) to determine allelic identity between *lup1* and *LEU2*.

Media and nomenclature. Standard media preparations and genetic terminology are as described by Sherman (1991), except that the leucine concentration in synthetic medium was normally 0.08 mg ml⁻¹. SC medium contained adenine, arginine, tryptophan, methionine, uracil (all 0.02 mg ml⁻¹); 0.06 mg lysine ml⁻¹; 0.03 mg tyrosine ml⁻¹; 0.05 mg phenylalanine ml⁻¹; 0.2 mg threonine ml⁻¹; 0.08 mg leucine ml⁻¹ (Heinemann & Sprague, 1991). Several types of omission media were used. SC-leu, SC-ura, SC-his and SC-trp are synthetic complete medium lacking only leucine, uracil, histidine or tryptophan, respectively. MA medium was the equivalent of SD (Sherman, 1991). MP medium was similar to MA with 0.1% proline and 1 × yeast nitrogen base without amino acids or (NH₄)₂SO₄ (Difco) substituting for 1 × yeast nitrogen base without amino acids (Difco). MA and MP media were supplemented with leucine, histidine, uracil and adenine (ade) as appropriate at the concentrations listed above.

Culturing procedures. Yeast stocks were maintained at -70 °C in 15% (v/v) glycerol and streaked fresh onto solid YPD medium. Growth medium (10 ml) was inoculated with a single colony and grown to saturation. These cultures were either used directly or diluted into flasks so their growth could be monitored in a Klett-Summerson colorimeter. Before plating on SC-leu, saturated cultures were diluted into MA medium and incubated at 30 °C until their growth ceased. Markers were routinely tested by replica plating (Sherman, 1991).

Phenotype frequencies. Frequencies at which phenotypes were isolated from a population of cells was determined by dividing the titre of variants by the titre of cells in the input culture. Input titres were determined by applying dilutions to YPD medium.

Radio-labelled amino acid uptake assays. *S. cerevisiae* cultures were grown in MA + leu + his + ura + ade or MP + leu + his + ura + ade medium at 30 °C until the culture reached saturation. Cells were pelleted from 5 ml cultures by centrifugation, washed once with water and resuspended in 50 ml MA + his + ura + ade or MP + his + ura + ade. Cultures were incubated in MA + his + ura + ade or MP + his + ura + ade for 5 h at 30 °C to deplete the intracellular leucine and enter the cells into leucine starvation. At 5 h, dilutions of the cultures were plated on YPD plates to determine the c.f.u. ml⁻¹. Concentration-dependent uptake assays were initiated by adding varying amounts of radiolabelled amino acids to 200 µl aliquots of the different *S. cerevisiae* cultures and incubating at 30 °C with shaking. The amount of radiolabelled amino acids added yielded concentrations ranging from 0.3125–10 µM as indicated in the text and figure legends. Samples were removed after 15 min and filtered through 0.45 µm cellulose acetate filters (cat. no. OE67, Schleicher & Schuell) in a vacuum-drawn filter manifold (1225 sampling manifold, Millipore). Filters were hydrolysed in 2 M HCl at 100 °C for 1.5 h, scintillation cocktail was added to the hydrolysate, and the d.p.m. of the sample determined on a Beckman LS 6000LL scintillation spectrometer using the single label d.p.m. program with a ¹⁴C or ³⁵S quench curve calibration.

To assay for GAP1 activity, cells were grown in MA + leu + his + ura + ade at 30 °C until the culture reached saturation. The cells were washed once with water, diluted into 20 ml MA + leu + his + ura + ade or MP + leu + his + ura + ade, and incubated at 30 °C for 4 h. At 4 h, the cells were washed once with water to remove amino acids and placed into an identical volume of either MA + ade + ura or MP + ade + ura. The amino acids were removed, since their presence would interfere with subsequent analysis of GAP1 activity. Cells were incubated for an additional hour at 30 °C and then transport assays were done as described above, except that L-[¹⁴C]citrulline was used at a single concentration of 100 µM. All radiolabelled amino acids were obtained from New England Nuclear and were used at the following specific activities: L-[U-¹⁴C]leucine, specific activity, 157.15 mCi mmol⁻¹; L-[U-¹⁴C]phenylalanine, 100 mCi mmol⁻¹; L-[³⁵S]methionine, 400 mCi mmol⁻¹; L-[¹⁴C-ureido]citrulline, 22.5 mCi mmol⁻¹ (1 Ci = 37 GBq).

RESULTS

The *leu2-3,112* allele of the yeast *S. cerevisiae* was created by *in vivo* recombination between the two different frameshift mutations *leu2-3* and *leu2-112* (Hinnen *et al.*, 1978; Klein & Petes, 1981). A *leu2-3,112* genotype is recessive and it renders haploid and homozygous diploid strains completely dependent upon the addition of leucine to minimal growth medium. Strains with the double mutant allele revert to leucine prototrophy (*Leu*⁺) so infrequently that

Fig. 1. Growth in leucine-depleted environments. The progenitor *leu2-3,112* strain 329-6C (□), its *Leu*⁺ derivative, JY108 (○) and a representative *Leu*⁻ variant, JY117 (▲) were prepared as described in Methods, titred, and diluted into liquid SC-leu medium supplemented with various amounts of leucine. After 23 h incubation, titres were again determined and the number of generations calculated.

the allele is considered a functional equivalent of a deletion (Hinnen *et al.*, 1978). We have never observed a spontaneous leucine prototroph arising from strains harbouring the *leu2-3,112* allele [fewer than 1 in $\sim 10^{10}$ cells screened (Heinemann & Sprague, 1989, and unpublished observations)]. Prolonged incubation under selection was also ineffective at raising prototrophs (fewer than 1 in $\sim 10^9$ cells screened for approximately 3 weeks).

Leucine auxotrophs, however, formed colonies on medium lacking leucine. Their colonies remained small and they did not form colonies when transferred to fresh SC-leu plates. Appearance of these colonies was dependent on a heavy inoculum of yeast cells.

Small colonies were not contaminants. The parental strain, 329-6C, has a number of other auxotrophic markers. 329-6C is *Ura*⁻ (*ura3-52*), *Ade*⁻ (*ade6*), *His*⁻ (*his3-537* and *his3-513*; see Methods), and unstably *Trp*⁺ [*TRP1* is integrated between duplicated *his3* alleles and is lost at a frequency of $\sim 1 \times 10^{-6}$ by intrachromosomal recombination (Klein, 1988)]. The genotypes of small colonies were routinely confirmed for these auxotrophies, as was their ability to grow on YPD (rich) medium.

Several lines of evidence, discussed below, implied that the cells which gave rise to small colonies represented an altered amino acid uptake phenotype. As such, the variants were designated *Lup*⁺, for leucine uptake. *Lup*⁺ colonies were characteristically found on plates inoculated with approximately 10^7 to 10^8 (*Leu*⁻) cells. This suggested

that colony-formers possessed enhanced capacity to grow in a leucine-limiting, but not deprived, environment. When large numbers of cells are plated, the leucine released from dead cells might be utilized by 'scavengers'. Consistent with the variants having an enhanced capacity to acquire leucine from the environment, they grew in both liquid and solid medium supplemented with 0.01 mg leucine ml⁻¹, a concentration that did not support the growth of their progenitor (Fig. 1).

Genetic characterization of the *Lup*⁺ phenotype

These variants were genetically stable but were not *Leu*⁺ because they could not grow on SC-leu. Six independently selected variants were purified for further examination. They were either restreaked on low leucine medium (0.01 mg leucine ml⁻¹) and then passaged on rich medium or were streaked directly to YPD. Following sequential passage through solid and liquid YPD medium, variants retained the ability to grow on medium with low concentrations of leucine (Fig. 1). Their growth characteristic was therefore heritable. However, unlike *LEU2*-containing derivatives of the progenitor (such as JY108), growth of these variants was restricted to medium with at least 0.01 mg leucine ml⁻¹ (Fig. 1).

Variants were selected directly from populations of 329-6C by plating liquid YPD cultures onto solid medium supplemented with 0.01 mg leucine ml⁻¹. Tenfold dilutions of cultures beginning with approximately 10^7 cells ml⁻¹ were applied to the selective medium. Colonies appeared in 2–4 d at a frequency of $4 \times 10^{-6} \pm 1 \times 10^{-6}$. Based on the number of cells initially applied to plates, by day 12 of incubation the variant phenotype arose at a frequency as high as 3×10^{-2} .

The growth phenotype was recessive. Five independent diploids (e.g. JY127) derived from matings between a *Lup*⁺ (JY126) and an unrelated *Lup*⁻ *leu2* (JY107) strain were tested for their ability to grow on 0.01 mg leucine ml⁻¹. Diploids formed by mating JY126 and SY1229 were selected on MA + Leu medium (Table 1). In contrast to the *Lup*⁺ parent, none of the diploids grew on SC-Leu medium (Table 1). Therefore, the variant phenotype was not the result of partial reversion of the leucine biosyn-

Table 1. Recessive/dominant test of *lup1*

Results are representative of five independent heterozygotes.

Strain	Growth	
	MA + Leu	SC-Leu + 0.01 mg leucine ml ⁻¹
JY126	—	+
SY1229	—	—
JY126 × SY1229	+	—

Table 2. Tetrad analysis

	Linkage test	Parental ditypes	Non-parental ditypes	Tetratypes
A	<i>lup1-MAT</i>	10	5	37
B	<i>lup1-leu2</i>	1	3	7

thesis mutation because this class of mutants would be expected to be dominant.

The mutant phenotype segregated as a single nuclear gene. Fifty-two tetrads were dissected following sporulation of the JY126 × JY107 diploid. The mutant phenotype segregated 2:2 in all 52 tetrads. The mutation identified a single nuclear gene, which in the wild-type we designated *LUP1*.

lup1 was not allelic to *LEU2*. The *LEU2* locus is linked to *MAT* on chromosome III. As can be seen in Table 2 (line A), the ratio of parental ditype to non-parental ditype to tetratype tetrads with respect to *MAT* and *lup1* was approximately 1:1:4, showing that *lup1* is not linked to *MAT* (Sherman & Wakem, 1991). Moreover, *LUP1* was not linked to the *LEU2* locus. A *MATα lup1 leu2-3,112* segregant (JY12714Aa) from above (JY127) was mated to a *MATα LEU2* strain (SY2512), induced to sporulate, and 11 tetrads were dissected (Table 2, line B). From the ratio of the tetrad types we concluded that *LEU2* and *lup1* sorted independently. Finally, we isolated several *leu2 LUP1* haploids following sporulation of the above diploid. The *leu2 LUP1* haploids could only be recovered if *LEU2* and *lup1* were separate genes (i.e. the diploid genotype was *LEU2/leu2 LUP1/lup1*). Therefore, *LEU2* and *lup1* cannot be alleles of the same gene.

Physical characterization of the *Lup*⁺ phenotype

Gradient assays. *Lup*⁺ cells were examined for evidence of a general change in their ability to grow on limiting concentrations of other essential nutrients (histidine and uracil for strain 329-6C) as opposed to a change specific for leucine starvation. Several nutrient gradients were created on omission media, and the growth of *Lup*⁺ (*lup1*), the *Lup*⁻ progenitor (329-6C) and an isogenic *LUP1 LEU2* strain (JY108) was measured. SC-ura, SC-his or SC-leu plates were supplemented with 0.06 mg uracil, 0.06 mg histidine or 0.2 mg leucine, respectively, as a droplet in the centre of the plate (final concentration approximately 0.1 × standard for the entire plate). After the droplet dried, 100 to 1000-fold dilutions of saturated cultures of *Lup*⁺, *Lup*⁻ or *LEU2* strains were applied to the surface of the plate in a spoke-like fashion with the arms of the spoke extending outward from the centre. The extent to which growth extended from the plate centre after 18 h of incubation was measured. *Lup*⁺ cultures were indistinguishable from either the *Lup*⁻ or *LEU2* strain on uracil or histidine gradients (all grew

Fig. 2. Uptake of [¹⁴C]leucine by *S. cerevisiae* strain 329-6C (○) and growth variant JY117 (△). Assays were performed as described in Methods.

equal distances from the plate centre. In stark contrast, when assayed on a leucine gradient, the *Lup*⁺ cells grew twice as far along the gradient (2.8 ± 0.2 cm) as did *Lup*⁻ (1.6 ± 0.06 cm).

Leucine uptake and inhibition of leucine uptake. The ability of the *Lup*⁺ isolate JY117 to take up [¹⁴C]leucine was compared to that of the progenitor 329-6C. At concentrations of [¹⁴C]leucine ranging from 0.3125 to 10 μM, leucine-starved JY117 transported 10–20 times as much leucine as did the leucine-starved progenitor strain (Fig. 2). Similar results were obtained with another independently isolated *Lup*⁺ variant, JY118. A Lineweaver–Burk plot of leucine uptake kinetics of *Lup*⁺ cells showed a $V_{\max}$ of 4570 pmol leucine per 10⁷ cells per 15 min and a K_m of 3.7 μM ($r^2 = 1.0$), indicating that the uptake system was saturable.

In similar assays, the specificity of [¹⁴C]leucine uptake was also analysed (Table 3). The *Lup*⁺ variant, JY117, and the progenitor strain, 329-6C, were incubated in the presence of 10 μM [¹⁴C]leucine with or without 1 mM unlabelled competitor: leucine, methionine, isoleucine, phenylalanine, valine, histidine, lysine, tryptophan, threonine and serine, respectively. Leucine uptake was strongly inhibited by a 100-fold higher concentration of the various hydrophobic amino acids but not at all by histidine, lysine, threonine and serine when cells were grown in the presence of ammonium. A 100-fold higher concentration of unlabelled leucine caused >95% inhibition of [¹⁴C]leucine transport in all cases. Remarkably, phenylalanine was the next most effective competitor of leucine transport. This was unexpected because the alkyl side chains of isoleucine, valine and methionine more resemble leucine than the phenyl group of phenylalanine.

Uptake of other hydrophobic amino acids. The inhibition of [¹⁴C]leucine transport by various hydrophobic amino acids suggested that the *Lup*⁺ transport phenotype was not limited to the transport of leucine. Since phenylalanine and methionine were strong inhibitors of leucine transport, uptake of these amino acids by *Lup*⁺ and *Lup*⁻ cells was assayed. Like leucine, the transport of both phenylalanine (Fig. 3a) and methionine

Fig. 3. Uptake of $[^{14}\text{C}]$ phenylalanine (a) and $[^{35}\text{S}]$ methionine (b) by *S. cerevisiae* strain 329-6C (○) and growth variant JY117 (△). Assays were performed as described in Methods.

(Fig. 3b) into the Lup^+ strain JY117 was strongly elevated relative to 329-6C. These results correlate well with the inhibition assays (Table 3) and suggest that Lup^+ cells have an enhanced ability to transport hydrophobic amino acids. All hydrophobic amino acids are likely to be substrates for the system identified by the *lup1* allele (the 'LUP1' system) in view of the rapid uptake of three structurally distinct amino acids studied here – leucine, phenylalanine and methionine. Indeed, the phenotype exhibited by a *lup1 trp1* strain on SC-*trp* medium (data not shown) was similar to the Lup^+ phenotype exhibited by a *lup1 leu2* strain on SC-*leu*.

Ammonium regulation. When grown in the presence of ammonium, the Lup^+ strain JY117 transported leucine six times faster than did 329-6C (Table 3). However, 329-6C grown in MP medium, where proline is the sole nitrogen source, transported leucine at rates similar to JY117 (Table 3). Growth in MP medium derepresses the GAP1 system, thereby increasing the transport of all amino acids. Thus, we considered the possibility that GAP1 was inappropriately derepressed in the presence of ammonium in *lup1* strains.

Increased leucine transport into JY117 and MP-grown 329-6C, however, was not due to derepression of GAP1. The GAP1 system has a much higher affinity for basic amino acids than acidic, neutral or hydrophobic amino acids (Grenson *et al.*, 1970). Woodward & Cirillo (1977) have shown that a 10-fold higher concentration of unlabelled histidine or lysine inhibited > 95% of the transport of the hydrophobic amino acid valine via the GAP1 system. In the inhibition assays reported here, a

Table 3. Inhibition of leucine transport by competing amino acids

Results are representative of three experimental determinations. Cells were grown in MA (minimal with ammonium sulfate as nitrogen source) or MP (minimal with proline as nitrogen source). Potential inhibitors were added to assay mixtures at a concentration of 1 mM, 100 times the concentration of $[^{14}\text{C}]$ leucine. The concentration of $[^{14}\text{C}]$ leucine ($157.15 \text{ mCi mmol}^{-1}$) in the assays was $10 \mu\text{M}$.

Strain/ growth medium	Amino acid	v (pmol leucine per 10^7 cells per 15 min)	Percentage inhibition
329-6C/MA	None	442.0	0
	Leucine	13.5	97
	Methionine	50.5	89
	Isoleucine	27.5	94
	Phenylalanine	17.5	96
	Valine	76.0	83
	Histidine	440.5	0
	Lysine	461.5	0
	Tryptophan	213.0	52
	Threonine	349.0	21
	Serine	458.5	0
JY117/MA	None	2732.5	0
	Leucine	104.0	96
	Methionine	591.5	78
	Isoleucine	365.5	87
	Phenylalanine	194.0	93
	Valine	993.5	64
	Histidine	3316.5	0
	Lysine	3520.5	0
	Tryptophan	1897.0	31
	Threonine	2934.5	0
	Serine	3215.0	0
329-6C/MP	None	2048.5	0
	Leucine	71.5	97
	Methionine	409.0	80
	Isoleucine	218.0	89
	Phenylalanine	189.0	91
	Valine	580.0	72
	Histidine	1727.0	16
	Lysine	1964.0	4
	Tryptophan	1297.5	37
	Threonine	1704.5	17
	Serine	1725.5	16

100-fold higher concentration of histidine or lysine had no effect on leucine transport in JY117 or 329-6C grown in MA medium (Table 3). Further evidence that the GAP1 and LUP1 systems are distinct derived from studies of L- $[^{14}\text{C}]$ citrulline transport. L-Citrulline transport is mediated only by GAP1 (Grenson *et al.*, 1970). As can be seen when Lup^+ and Lup^- cells were grown in GAP1-repressing (MA) and GAP1-derepressing (MP) medium, L- $[^{14}\text{C}]$ citrulline uptake remained regulated by ammonium

Table 4. Induction of GAP1 system in *LUP1* and *lup1* strains

Results are representative of three experimental determinations. The concentration of L-[¹⁴C]citrulline (22.5 mCi mmol⁻¹) in the assays was 100 µM and uptake values are expressed as velocity (pmol L-[¹⁴C]citrulline per 10⁷ cells per 15 min). Cells were grown overnight in MA + his + leu + ura + ade (minimal with ammonium sulfate as nitrogen source), washed with water and then diluted into either MA + his + leu + ura + ade or MP + his + leu + ura + ade (minimal with proline as nitrogen source) and incubated at 30 °C for 4 h. At 4 h, the cells were washed once with water to remove amino acids from the medium and placed into an identical volume of either MA + ura + ade or MP + ura + ade and incubated for an additional hour prior to initiating citrulline uptake assays. Induction (-fold) is the ratio of citrulline transport by proline-grown cells over citrulline transport by ammonium sulfate-grown cells.

Nitrogen source	L-[¹⁴ C]citrulline uptake	
	329-6C	JY117
Ammonium sulfate	254	325
Proline	5266	17502
Induction (-fold)	21	54

in both wild-type and *Lup*⁺ cells (Table 4). Therefore, GAP1 is not responsible for the transport of leucine into *Lup*⁺ cells (or *Lup*⁻ cells grown in MA medium) and *LUP1* must be a regulatory component of a separate amino acid transport system.

Leucine transport into 329-6C (*Lup*⁻) cells grown in MP medium was to some extent inhibited by all amino acids. Following induction of GAP1, lysine, histidine, serine and threonine inhibited leucine transport into 329-6C from 4 to 17%. Therefore, the GAP1 system accounted for a maximum of 5–15% of the leucine transport observed in 329-6C grown in GAP1-derepressing conditions. The majority of leucine transport into these cells must occur by another pathway which is ammonium-repressible. Based on the similarities of leucine transport rates and the inhibition profiles (Table 3) of JY117 and MP-grown 329-6C, we conclude that *LUP1* is a regulatory component of an ammonium-repressible hydrophobic amino acid transport system in *S. cerevisiae*, distinct from GAP1. In *lup1* strains, the hydrophobic amino acid transport system has been liberated from ammonium repression. Consistent with the *Lup*⁺ phenotype being recessive, we suggest that *LUP1* is a repressor of the hydrophobic amino acid uptake pathway.

DISCUSSION

Lup⁺ colonies of *Leu*⁻ yeast form on minimal medium in the absence of leucine. Reconstruction experiments using plates supplemented with extra leucine (0.01 mg ml⁻¹) showed that growth of *Leu*⁻ cells on SC-leu medium could not have been due to trace amounts of leucine

contaminating the medium. On SC-leu medium supplemented with 0.01 mg leucine ml⁻¹, variants arose at a frequency of 4×10^{-6} . If growth were due to leucine in unsupplemented medium, lawns would have been expected on plates with 0.01 mg leucine ml⁻¹ and colonies would have been expected on SC-leu plates with fewer than 10⁷ input cells.

The variants had acquired a stable change. Unlike a *LEU2* strain, variants could not be cultured in the absence of leucine. They were therefore not true revertants. Because the trait was recessive (Table 1), it was unlikely to be a partial revertant of *leu2* (Horak, 1986).

The stability of the change, as evidenced by the variants' ability to grow on limiting concentrations of leucine after culture under non-selective conditions, suggested that it was genetic. That the trait was a gene mutation, as opposed to an epigenetic change (Novick & Weiner, 1957; Pillus & Rine, 1989), was confirmed when it segregated 2:2 among the meiotic offspring of a heterozygous diploid.

Mapping experiments further confirmed that the *Lup*⁺ phenotype was not a result of partial reversion of the *leu2-3,112* allele. *lup1* was not linked to either *MAT* (which is linked to *LEU2*) or *LEU2* (Table 2). Furthermore, *LUP1 leu2* spores were readily isolated from a diploid whose parents were either *LEU2* or *lup1*, respectively. If *lup1* had been allelic to *LEU2* (i.e. the diploid genotype had been *LEU2/lup1* vs *LEU2/leu2 LUP1/lup1*), only *LEU2* or *lup1* haploids would be recovered following sporulation.

The phenotypic change was confined to the uptake of leucine and other hydrophobic amino acids. Gradient assays were used to show that growth was enhanced only on medium limited for leucine and not on medium limited for other required supplements for strain 329-6C, in this case histidine and uracil.

Uptake assays using radiolabelled leucine demonstrated that *Lup*⁺ cells were significantly better at accumulating extracellular leucine than wild-type cells (Figs 1 and 2). This enhanced ability to concentrate leucine provided *Lup*⁺ cells with a growth advantage over wild-type cells in leucine-poor environments ranging from at least 0.005 to 0.04 mg leucine ml⁻¹ (Fig. 1). This permitted variants to form detectable colonies on leucine-deprived (at cell concentrations of $\geq 10^7$ cells per plate) and leucine-limiting (0.01 mg ml⁻¹) medium.

Competition experiments using various unlabelled amino acids demonstrated the transport specificity for hydrophobic amino acids. Moreover, these experiments indicated that the *Lup*⁺ character is not due to derepression of the GAP1 pathway in the presence of ammonium. Firstly, the GAP1 pathway strongly prefers the basic amino acids lysine and histidine to leucine (Woodward & Cirillo, 1977) and the competition assays indicated that neither of these was an effective competitor of leucine transport in *lup1* cells. Secondly, the *Lup*⁺ cells were not advantaged on histidine gradients. Thirdly, the amino acid citrulline, which is transported only by the GAP1 pathway, was still

regulated by ammonium in the *lup1* strain JY117 and the absolute amount of citrulline uptake was similar in the *LUP1* and *lup1* strains in the presence of ammonium.

The absolute rate and specificity of the transport phenotype displayed in *Lup*⁺ (compared to MP-grown 329-6C) cells, and the fact that the trait was recessive, suggested the inactivation of a repressor of the high-affinity leucine uptake system, but not of the GAP1 or the low-affinity leucine uptake system. The high transport velocities observed with the *Lup*⁺ phenotype at low concentrations of substrate ($\leq 10 \mu\text{M}$) indicate that this is a high-affinity system (Ramos *et al.*, 1980; Wainer *et al.*, 1988). The virtually identical leucine transport inhibition profiles of 329-6C (*Lup*⁻) and JY117 (*Lup*⁺) suggest that the same transport system is present in both strains and that this transport system is derepressed in JY117. We therefore propose that *LUP1* mediates ammonium repression of the high-affinity/low-velocity leucine uptake pathway.

There is significant phenotypic correlation between *Lup*⁺ cells and *aat1* mutants of *S. cerevisiae* (Garrett, 1989). Growth of *leu2 aat1* strains is inhibited by isoleucine, phenylalanine, methionine, valine and tyrosine (Garrett, 1989), a group of amino acids similar to the inhibitors of leucine transport reported here. Garrett (1989) suggested that *AAT1* is required for leucine transport in the presence of hydrophobic amino acids. Under such conditions, *aat1 leu2* cells are deprived of leucine because the other hydrophobic amino acids block leucine transport. We propose the following model to fit these observations. *AAT1* is required for function of the low-affinity/high-velocity leucine uptake system. In *aat1 leu2* cells, the only mechanism remaining for the uptake of leucine is the high-affinity, low-velocity pathway, which is probably controlled by *LUP1*. Since the hydrophobic amino acids are effective competitors of leucine for uptake by the *LUP1* pathway, they inhibit the transport of leucine and thereby the growth of *aat1 leu2* cells.

The inhibition profiles observed in this study do not correspond with the results obtained from studies on the BAP (branched-chain amino acid permease) system (Tullin *et al.*, 1991). In contrast to our results, leucine transport by the BAP system is not inhibited by phenylalanine or methionine, but was somewhat inhibited by histidine and lysine. We did not observe the transport phenotype of the BAP system in our strains, which may simply be a reflection of the different genetic backgrounds. The presence of similar high-affinity leucine/hydrophobic amino acid transport systems in both *Lup*⁻ (329-6C) and *aat1* (Garrett, 1989) cells suggests that the *LUP1* system is identical to the high-affinity leucine transport system reported by Ramos *et al.* (1980).

Determining the nature of the change (e.g. transposon insertion or point mutation) awaits isolation of *lup1*. The *Lup*⁺ phenotype was eventually displayed by a large percentage of the population (~ 1 per 100 input cells in 12 d). Even the initial frequency of ~ 1 variant per 10^6 input cells represents an unusually high mutation rate in yeast, making it unlikely that *Lup*⁺ is due to a point mutation. The *Lup*⁺ phenotype may even be stimulated

by leucine starvation because colonies continue to emerge over time. However, no effort was made to rule out continued DNA metabolism during leucine starvation (Mittler & Lenski, 1992).

Uptake variants raise interesting questions in light of work on 'adaptive' or 'Cairnsian' mutations. Adaptive mutations arise specifically in environments that reward the cell harbouring the mutation. Moreover, these mutations only occur in genes whose products or functions are relevant to the selecting environment. Adaptive changes may occur in both bacteria (*Escherichia coli*) and yeast (*S. cerevisiae*), although reports continue to generate controversy (Foster, 1992; Mittler & Lenski, 1992; Stahl, 1992). To absolutely eliminate the possibility of all growth or cell turnover in a limiting but non-lethal environment is difficult and thus exposes many claims of adaptive mutagenesis to criticism (Mittler & Lenski, 1992). Critics charge that undetected turnover or growth could result in sufficient rounds of DNA replication to account for the accumulation of mutations in relevant genes purely by random chance. Most notably, Mittler & Lenski (1992) have attributed the dramatic reversion frequency of a double mutant strain described by Hall (1991) to the partial growth of single revertants. Growth due to the activation of pathways like *LUP1* could further confuse the issue. Even granting the possibility that the *Lup*⁺ phenotype may be a product of adaptive change, the growth of *Lup*⁺ intermediates might mask the randomness of subsequent changes.

Alternatively, intermediate phenotypes might weaken the putative link between selective forces and their gene targets. Genes may not be susceptible to 'directed' changes unless growth has been completely halted, so selective pressures may be unfocused in slowly growing intermediates. Interestingly, Steele & Jinks-Robertson (1993) found that recombination between heterologous gene duplications in yeast, wherein both alleles were defective, was stimulated during nutrient starvation. This was true during starvation for a number of different nutrients except leucine. It is interesting to speculate that perhaps *Lup*⁺ cells arise at sufficient frequencies to prevent starvation-stimulated recombination.

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