Isolation of a conditional suppressor of leucine auxotrophy in \textit{Saccharomyces cerevisiae}

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Phenotypically and genotypically (\textit{leu2-3,112}) Leu\textsuperscript{-} cells of \textit{Saccharomyces cerevisiae} gave rise to small colonies on medium devoid of leucine. This only occurred on plates with a high density of Leu\textsuperscript{+} 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 \textit{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. \textsuperscript{[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 \textit{lup1} (for leucine uptake). The transport characteristics of the \textit{lup1} mutants suggested that \textit{LUP1} is a regulatory component of an ammonium-regulated hydrophobic amino acid uptake system.

\textbf{Keywords:} \textit{Saccharomyces cerevisiae}, leucine, auxotrophy, amino acid transport, adaptive mutagenesis

\section*{INTRODUCTION}

The \textit{leu2-3,112} allele in the yeast \textit{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 \textit{LEU2} gene during matings between bacteria and yeast (Heinemann & Sprague, 1989). However, small colonies often do form during selection for Leu\textsuperscript{+} 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 \textit{leu2}). Alternatively, phenotypic suppression of the Leu\textsuperscript{-} 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\textsuperscript{-} cells might be due to leucine 'scavengers', variants within the population that are Leu\textsuperscript{+} 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 \textit{S. cerevisiae} cells by at least three routes (Horak, 1986; Ramos \textit{et al.}, 1980; Wainer \textit{et al.}, 1988). The first, called the general amino acid permease (\textit{GAP1}) system after the permease gene \textit{GAPI}, is a low-affinity, high-velocity pathway activated in response to nitrogen starvation (Grenson \textit{et al.}, 1970). Depending on the yeast strain and nitrogen source, the \textit{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 nitrogan starvation. The third pathway is a constitutive, leucine-specific, high-affinity and low-velocity system (Ramos \textit{et al.}, 1980). The \textit{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 (Kotljar 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 was 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, lap1, for leucine uptake.

**METHODS**

**Relevant strain descriptions.** Growth variants were derivatives of 329-6C (Mata his3-357::TRP1::b13-513 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 32-6C 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 cant) was created by integration of the UR3A3 gene into the genome of SY1229 (Heinemann & Sprague, 1989) following conjugation with bacteria as described (Heinemann & Sprague, 1991). JY127 (Mata/a leu2-3,112 leu2-3,112 HIS3/b13 UR3A3/ura3) was derived from a cross between JY126 and JY107. JY12714Aa (Mata lap1 leu2-3,112 ura3 b13) was isolated as a haploid segregant of JY127 and mated to SY2512 (Mata LEU2 ura3 arg4 ade6) to determine allelic identity between lap1 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, and histidine (all 0.02 mg ml–1). 52 was grown at 30 °C. 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. 758, 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 32P 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-[14C]phenylalanine, 100 mCi mmol–1; L-[35S]methionine, 400 mCi mmol–1; L-[14C-ornithine]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 vitro 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 homologous 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...
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 passed 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 10⁷ cells ml⁻¹ were applied to the selective medium. Colonies appeared in 2-4 d at a frequency of 4 x 10⁻⁶ ± 1 x 10⁻⁸. 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 x 10⁻⁴.

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>
<thead>
<tr>
<th>Strain</th>
<th>Growth Strain</th>
<th>Growth SC-Leu + 0.01 mg leucine ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>JY126</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>SY1229</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>JY126 x SY1229</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 1. Recessive/dominant test of lup1

Results are representative of five independent heterozygotes.
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.

\textit{lup}1 was not allelic to \textit{LEU}2. The \textit{LEU}2 locus is linked to \textit{MAT} on chromosome III. As can be seen in Table 2 (line A), the ratio of parental ditotype to non-parental ditotype to tetratype tetrads with respect to \textit{MAT} and \textit{lup}1 was approximately 1:1:4, showing that \textit{lup}1 is not linked to \textit{MAT} (Sherman & Wakem, 1991). Moreover, \textit{LUP1} was not linked to the \textit{LEU2} locus. A \textit{MATa} \textit{lup}1 \textit{leu}2-3,112 segregant (JY12714Aa) from above (JY127) was mated to a \textit{MATa} \textit{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 \textit{LEU2} and \textit{lup}1 sorted independently. Finally, we isolated several \textit{leu}2 \textit{LUP1} haploids following sporulation of the above diploid. The \textit{leu}2 \textit{LUP1} haploids could only be recovered if \textit{LEU2} and \textit{lup}1 were separate genes (i.e. the diploid genotype was \textit{LEU2/leu}2 \textit{LUP1/lup}1). Therefore, \textit{LEU2} and \textit{lup}1 cannot be alleles of the same gene.

\textbf{Physical characterization of the Lup+ phenotype}

\textbf{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 \textit{329-6C}) as opposed to a change specific for leucine starvation. Several nutrient gradients were created on omission media, and the growth of Lup+ (\textit{lup}1), the Lup+ progenitor (329-6C) and an isogenic \textit{LUP1} \textit{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 \textit{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 \textit{LEU2} strain on uracil or histidine gradients (all grew 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).

\textbf{Leucine uptake and inhibition of leucine uptake}. The ability of the Lup+ isolate JY117 to take up \textit{[14C]leucine} was compared to that of the progenitor 329-6C. At concentrations of \textit{[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 10^7 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 \textit{[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 \textit{[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 \textit{[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.

\textbf{Uptake of other hydrophobic amino acids.} The inhibition of \textit{[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

\begin{table}[h]
\centering
\caption{Tetrad analysis}
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Linkage} & \textbf{Parental} & \textbf{Non-} & \textbf{Tetratypes} \\
\textbf{test} & \textbf{ditypes} & \textbf{parental} & \\
\hline
A & \textit{lup}1-M\textit{AT} & 10 & 5 & 37 \\
B & \textit{lup}1-\textit{leu}2 & 1 & 3 & 7 \\
\hline
\end{tabular}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig2.png}
\caption{Uptake of \textit{[14C]leucine} by \textit{S. cerevisiae} strain 329-6C (○) and growth variant JY117 (△). Assays were performed as described in Methods.}
\end{figure}
Leucine uptake in yeast

(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 lupl 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 GAPI system, thereby increasing the transport of all amino acids. Thus, we considered the possibility that GAPI 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 GAPI. The GAPI system has a much higher affinity for basic amino acids than acidic, neutral or hydrophobic amino acids (Grenson et al., 1970). Woodward & Grillo (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 GAPI system. In the inhibition assays reported here, a 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 GAPI and LUP1 systems are distinct derived from studies of L-[¹⁴C]citrulline transport. L-Citrulline transport is mediated only by GAPI (Grenson et al., 1970). As can be seen when Lup⁺ and Lup⁻ cells were grown in GAPI-repressing (MA) and GAPI-derepressing (MP) medium, L-[¹⁴C]citrulline uptake remained regulated by ammonium.

### 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 [¹⁴C]leucine. The concentration of [¹⁴C]leucine (157·15 mCi mmol⁻¹) in the assays was 10 μM.

<table>
<thead>
<tr>
<th>Strain/growth medium</th>
<th>Amino acid</th>
<th>v (pmol leucine per 10⁶ cells per 15 min)</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>329-6C/MA</td>
<td>Leucine</td>
<td>442·0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Methionine</td>
<td>13·5</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Isoleucine</td>
<td>50·5</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Phenylalanine</td>
<td>27·5</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Valine</td>
<td>17·5</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Histidine</td>
<td>76·0</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>461·5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Tryptophan</td>
<td>213·0</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Threonine</td>
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<td></td>
<td>Serine</td>
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<td>JY117/MA</td>
<td>Leucine</td>
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</tr>
<tr>
<td></td>
<td>Methionine</td>
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<td>96</td>
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<tr>
<td></td>
<td>Isoleucine</td>
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<td></td>
<td>Phenylalanine</td>
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<tr>
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<td>Valine</td>
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<tr>
<td></td>
<td>Histidine</td>
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<td></td>
<td>Lysine</td>
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</tr>
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<td></td>
<td>Tryptophan</td>
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</tr>
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<td></td>
<td>Threonine</td>
<td>2934·5</td>
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</tr>
<tr>
<td></td>
<td>Serine</td>
<td>3215·0</td>
<td>0</td>
</tr>
<tr>
<td>329-6C/MP</td>
<td>Leucine</td>
<td>2048·5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Methionine</td>
<td>71·5</td>
<td>97</td>
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<tr>
<td></td>
<td>Isoleucine</td>
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<td>Lysine</td>
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<td></td>
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</tr>
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<td></td>
<td>Threonine</td>
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</tr>
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<td></td>
<td>Serine</td>
<td>1725·5</td>
<td>16</td>
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Table 4. Induction of GAP1 system in LUP1 and lup1 strains

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>$\text{l-}[^{14}\text{C}]\text{citrulline uptake}$</th>
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</thead>
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<tr>
<td></td>
<td>329-6C</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>254</td>
</tr>
<tr>
<td>Proline</td>
<td>5266</td>
</tr>
<tr>
<td>Induction (-fold)</td>
<td>21</td>
</tr>
</tbody>
</table>

Results are representative of three experimental determinations. The concentration of $\text{l-}[^{14}\text{C}]\text{citrulline}$ was 225 μCi/mmol, and uptake values are expressed as velocity (pmol $\text{l-}[^{14}\text{C}]\text{citrulline}$ per 10^6 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.

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 $4 \times 10^{-4}$. 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 10^7 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 radiolabeled 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 GAPI or the low-affinity leucine uptake system. The high transport velocities observed with the Lup+ phenotype at low concentrations of substrate (< 10 μM) 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+ 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, they inhibit the transport of both 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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