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BAP2, a gene encoding a permease for branched-chain amino acids in *Saccharomyces cerevisiae*

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Abstract

To select the gene coding for an isoleucine permease, an isoleucine dependent strain (*ilv1 cha1*) was transformed with a yeast genomic multicopy library, and colonies growing at a low isoleucine concentration were selected. Partial sequencing of the responsible plasmid insert revealed the presence of a previously sequenced 609 codon open reading frame of chromosome II with homology to known permeases. Deletion, extra dosage and C-terminal truncation of this gene were constructed in a strain lacking the general amino acid permease, and amino acid uptake was measured during growth in synthetic complete medium. The following observations prompted us to name the gene *BAP2* (branched-chain amino acid permease). Deletion of *BAP2* reduced uptake of leucine, isoleucine and valine by 25–50%, while the uptake of 8 other L-α-amino acids was unaltered or slightly increased. Introduction of *BAP2* on a centromere-based vector, leading to a gene dosage of two or slightly more, caused a 50% increase in leucine uptake and a smaller increase for isoleucine and valine. However, when the 29 C-terminal codons of the plasmid-borne copy of *BAP2* were substituted, the cells more than doubled the uptake of leucine, isoleucine and valine, while no or little increase in uptake was observed for the other 8 amino acids.

Keywords: Yeast; Amino acid transport; Leucine; Isoleucine; Valine

1. Introduction

The yeast *Saccharomyces cerevisiae* takes up amino acids from the medium through amino acid permeases, driven by proton symport [1]. So far, 12 known or putative L-α-amino acid permease genes from *S. cerevisiae*, sharing homology, have been cloned and sequenced, and transport specificities have been indicated for 7 of them, the general amino acid permease, Gap1p, [2], and the permeases for arginine (Can1p) [3], lysine (Lyp1p) [4], proline (Put4p) [5], histidine (Hip1p) [6], tyrosine (Tat1p) and tryptophan (Tat2p) [7]. All the genes contain features corresponding to a suggested 12-transmembrane alpha helix structure.

The activity of at least some amino acid permeases is regulated by the availability of nitrogen sources in the medium. Some of them are active only if the nitrogen is present in a form which is relatively difficult to utilize, e.g., proline or urea. These permeases include Gap1p (the general amino acid permease), a low specificity, high capacity permease that transports L-α-amino acids, including some that do not occur in proteins, such as citrulline and ornithine [8], many D-amino acids, several toxic amino acid analogs and other amino acid-related compounds. Other members of this group are Uga4p, which transports GABA (4-aminobutyric acid), and the proline permease, Put4p.

The regulation of Gap1p activity has been studied to some extent. Upon addition of ammonia, the permease synthesis is repressed at the transcriptional level, and the permease is inactivated at the protein level. The inactivation seems to involve dephosphorylation of the molecule near the C-terminal [9,10]. Thus an intact C-terminus is probably important for the regulation of activity, but not for the transport activity as such. The *NPR1* gene product is necessary for reactivation of Gap1p and other ammonia-sensitive permeases on de-repressing medium [11,12]. The *NPR1* gene has been cloned and encodes a putative protein kinase [13].

In media rich in ammonia or glutamate, Gap1p has poor activity, while other, more specific permeases are active, such as lysine permease [14], arginine permease [15], and

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histidine permease [16]. Regulation of the amino acid permeases that are active in the presence of easily assimilable nitrogen sources has been studied to a lesser extent than regulation of Gap1p, and the mechanisms of regulation are unknown. Two main patterns have so far appeared. The presence of an amino acid, such as serine, in the medium may induce the uptake of the same amino acid [17,18]. On the other hand, preloading of yeast cells with an amino acid may decrease further uptake of the same amino acid [16,19].

We are interested in uptake of the branched-chain amino acids leucine, isoleucine and valine, which can take place under conditions that inhibit and repress Gap1p. Uptake kinetics can be interpreted as consisting of two Michaelis-Menten-obeying systems in addition to Gap1p [20,21], but the number of involved permeases and their (its) regulation are not known. An approach to these questions is isolation of the structural gene(s) for the permease(s). Tullin et al. [20] isolated a mutant, *bap1*, exhibiting decreased uptake and somewhat simplified kinetics of uptake of leucine, isoleucine and valine. However, further work (M.U. Jørgensen, C. Gjermansen and M.C. Kielland-Brandt, unpublished) has shown that the *bap1* mutation is not in the structural gene for a permease and that it affects the uptake of a broad range of amino acids. In the present work a different approach was taken to isolate a structural gene for a permease for one or more of the three amino acids. It was envisaged that the growth deficiency of an isoleucine requiring strain on a medium low in isoleucine might be compensated by overexpression of the gene encoding a permease for this amino acid. Selection of cells transformed with a genomic library yielded a gene which indeed has homology to known amino acid permeases. Deletion and extra dosage, respectively, of the gene gave phenotypes indicating that the encoded permease is rather specific for the branched-chain amino acids. Deletion of the codons for the 29 most C-terminal amino acid residues stimulated uptake, suggesting that these residues have a regulatory role.

2. Materials and methods

2.1. Strains and media

Minimal ammonia (MA) medium contains per litre: 10 g succinic acid, 6 g NaOH, 5 g $(NH_4)_2SO_4$, 1.7 g Bacto yeast nitrogen base without amino acids and without ammonium sulfate (Difco), and 22 g D-glucose monohydrate. Minimal proline (MP) medium is MA with 2 g/l proline instead of $(NH_4)_2SO_4$. Synthetic complete (SC) medium is MA supplemented with the following nutrients per litre: 20 mg adenine, 20 mg L-arginine, 300 mg L-aspartic acid, 100 mg L-glutamic acid, 20 mg L-histidine, 30 mg L-isoleucine, 30 mg L-leucine, 30 mg L-lysine, 60 mg L-methionine, 50 mg L-phenylalanine, 375 mg L-serine, 600 mg L-threonine, 20 mg L-tryptophan, 30 mg L-tyrosine, 20 mg uracil and 30 mg L-valine. FOA medium contains per litre: 7 g yeast nitrogen base without amino acids (Difco), 1 g 5-fluoroorotic acid, 50 mg uracil, 20 g D-glucose and 2% agar [22].

S. cerevisiae strains used in this study are: M2409 (*MATα ura3-52 ilv1-10 cha1-1 trp1*) [23], M3750 (*MAT***a** *gal2 ura3*) [24], M4054 (*MAT***a** *gal2 ura3 gap1*) (this study) and M4056 (*MAT***a** *gal2 ura3 gap1 bap2*) (this study). With the exception of M2409, the strains are isogenic to S288C [25].

2.2. Amino acid uptake

An overnight culture was diluted and grown to a cell density corresponding to OD_{600} 0.5–0.7 (0.065–0.90 mg dry weight/ml) on the indicated medium at 30°C. Cells were harvested from 20 ml of culture and resuspended in 700 μl SC medium, and 100 μl of this suspension were added to 150 μl SC containing 0.1 μCi radiolabeled amino acid. After 5 min, uptake was stopped by addition of 0.5 ml ice-cold 150–250 mM of the corresponding non-radioactive amino acid. The cells were collected on a glass fiber filter (GC50, Advante Toyo), washed three times with cold SC medium, dried and counted in a Beckman L8S 6000IC scintillation counter. For alanine uptake, all SC media were supplemented with 30 mg/l L-alanine. Radiolabeled amino acids were obtained from New England Nuclear and were; L-[^{14}C(U)]leucine (NEC279E), L-[^{14}C(U)]isoleucine (NEC278E), L-[^{14}C(U)]valine (NEC291E), L-[^{14}C(U)]glutamic acid (NEC290E), L-[side chain-3-^{14}C]tryptophan (NEC367E), L-[^{14}C(U)]tyrosine (NEC289E), L-[^{14}C(U)]phenylalanine (NEC284E), L-[^{14}C(U)]histidine (NEC277E), L-[methyl-^{14}C]methionine (NEC165E) and L-[^{14}C(U)]alanine (NEC266E). All uptake measurements were made in duplicate, and average values are given.

2.3. Construction of the Δgap1 and Δbap2 alleles

Plasmid pJCJ252 (kindly supplied by M. Grenson) [2] was digested with *Sph*I, and the fragment containing the *GAP1* gene was inserted into the *Sph*I site of pBR322 [26], generating plasmid pTD4. Plasmid pTD4 was digested with *Kpn*I and *Nsp*V, removing a 1396 bp fragment of the open reading frame of the *GAP1* gene. The plasmid was resealed by blunting the ends with T4 DNA polymerase and ligation, resulting in plasmid pTD5. The 2.5 kb *Sph*I fragment containing the *Δgap1* allele was excised from pTD5 and subcloned into the unique *Sph*I site in plasmid pTD1, a YIp5 [27] derivative where the *Eco*RI and *Hind*III sites have been removed by cutting with both enzymes, Klenow filling and ligation. One of the resulting plasmids (pTD6) was linearized with *Eco*RI and used to transform the *ura3* strain M3750. Selection for uracil independence resulted in integration of the *Δgap1* allele into the chromosomal locus of *GAP1*, and subsequent selection on FOA

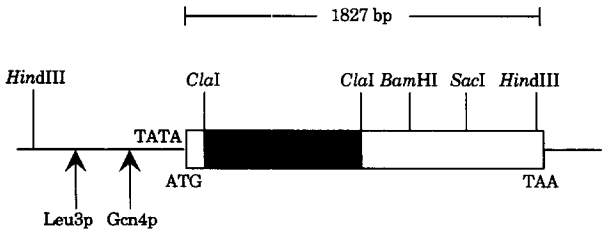

Fig. 1. The *BAP2* gene. Based upon the published sequence [32], positions of relevant restriction sites, coding region (1827), putative TATA boxes and putative binding sites for transcription activators are indicated. Shaded area indicates the 786 bp stretch deleted in the *Δbap2* construct.

plates yielded colonies having lost the vector and either allele [28,29]. A clone containing only the *Δgap1* allele was identified by Southern analysis and [^{14}C]citrulline uptake measurement (data not shown) and designated M4054. Also *BAP2* was deleted by the loop in/loop out strategy. A 786 bp *Cla*I fragment situated 60 bp from the start codon was removed (cf. Fig. 1), and the *Hin*dIII-*Sac*I fragment was inserted into the integration vector pRS306 [30] to form the plasmid pMG12. This construct offers about 750 bp upstream and 450 bp downstream of the deletion for integration. It was opened at the *Bam*HI site before transformation of the *Δgap1* strain M4054. The Ura$^+$ colonies were plated on FOA medium and some of the 5-FOA-resistant colonies were replica-plated to SC + 100 mg/l metsulfuron methyl (MM), which inhibits the actohydroxyacid synthase (Ilv2p), thereby blocking biosynthesis of isoleucine and valine. Colonies with a growth defect on this medium were selected, and verified by Southern hybridization to carry the *bap2* deletion (data not shown).

3. Results

3.1. Isolation of the BAP2 gene

To isolate the gene for a permease for one (or more) of the three branched-chain amino acids, leucine, isoleucine and valine, we assumed that cells under limitation for isoleucine could overcome growth retardation when provided with extra copies of the permease gene. To accom-

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          1
Bap2p   MlssedFgss gkketspdsI sIrS.Fsagn nfqssSsekt ySkqksGSdk lihrFaDSFK
Gap1p   MsntssYekn npdnlkhngI tIdSeFltqe pitipSngsa vSidetGSgs kwqdFkDSFK

         61
Bap2p   Raegsttrtk qIneNtSDlE dgVesITsds kLKksMKsRH vvMmslGtgI GTGLLVanak
Gap1p   R.....vkpi eVdpNlSEaE k.VaiITaqt pLKhhLKnRH lqMiaiGgaI GTGLLVgsgt

        121
Bap2p   gLhyGGPAaL iIGYilvsfe tYfMIqAaGE MAVtYPtlpa nFnaYssiFI skSFGFAtvW
Gap1p   aLrtGGPAsL lIGWgstgtm iYaMVmAlGE LAViFP.isg gFttYatrFI deSFGYAnnF

        181
Bap2p   lYcFQWLtVL PLElItASmT IqF.GnDkin pDiYIliFYv flVfIhfFGV KaYGEtEFIF
Gap1p   nYmLQWLvVL PLEiVsASiT VnFwGtDpky rDgFValFWl aiViInmFGV KgYGEaEFVF

        241
Bap2p   nccKIlmIaG FIILsIViNC GGagndGYIG atYWHnPGAF AGDTsigrFK nVCyILVTAy
Gap1p   sfiKVitVvG FIILgIIlNC GGgptgGYIG gkYWHdPGAF AGDTpgakFK gVCsVFVTAa

        301
Bap2p   FSFgGmELfa LsvqEqsnPR KStPvAAKrs iYRIvviYlL tMiLIGFnVP YNDdqLmGAg
Gap1p   FSFaGsELvg LaasEsvePR KSvPkAAKqv fWRItlfYiL sLlMIGLlVP YNDksLiGAs

        361
Bap2p   gsathASPYV lAasiHGVKi vPhIINaVIL IsVvSVaNSs lYAgpRlics LAqQgYaPkf
Gap1p   svdaaASPFV iAiktHGIKg lPsVVNvVIL IaVlSVgNSa iYAcsRtmva LAeQrFlPei

        421
Bap2p   LdYVDReGRP LraliVccvF GvIAFVAASs KEeiVFtWLa AiaGLSeLFT WtsImlsHlR
Gap1p   FsYVDRkGRP LvgiaVtsaF GlIAFVAASk KEgeVFnWLl AlsGLSsLFT WggIcicHiR

        481
Bap2p   FRqAMkvQGR sLDELgYKat TGIWGSiYGv FfnIlvFVAQ FWVAlaPlGn ggkcdAEsFF
Gap1p   FRkALaaQGR gLDELsFKsp TGVWGSyWGl FmvIimFIAQ FYVAvfPvGd sp..sAEgFF

        541
Bap2p   qnYLaFPiwL afYfGymVYn RdFtLLnPlD KiDlDfhRRi yDpELMrQEd eEnKekLrnm
Gap1p   eaYLsFPlvM vmYiGhkIYk RnWkLFiPaE KmDiDtgRRe vDlDLLkQEi aEeKaiMatk

        601
Bap2p   slmrkaYhFW C
Gap1p   prwyriWnFW C
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Fig. 2. Alignment of amino acids residues of the Bap2p permease and the general amino acid permease, Gap1p. Conserved amino acids are indicated with capital letters. The alignment was made by the Pileup program, with a gap weight of 3.0 and a gap length weight of 0.1.

modate the possibility of highly specific permeases, growth limitation was performed by starvation for only one of the three amino acids, and isoleucine was chosen in this work. *ilv1* mutants require isoleucine for growth, while they require neither valine nor leucine, being defective in the anabolic threonine deaminase which converts threonine to the first committed intermediate in isoleucine biosynthesis, 2-oxobutyric acid. A yeast strain carrying a mutation in *ilv1* was chosen as the host strain for screening a multi-copy genomic library [31] with selection for growth on low (20 μM) concentration of isoleucine. However, *ilv1* mutants are subject to the occurrence of spontaneous suppressor mutations, which act by de-repressing *CHA1*, encoding a catabolic threonine deaminase, i.e., an isoenzyme of Ilv1p [23]. The strain was therefore chosen to also carry a *cha1* mutation, and the presence of the *ura3-52* marker allowed concomitant selection for vector sequences. Among 50 000 Ura^+ transformants, about 200 were able to grow on 20 μM isoleucine. These transformants were tested for concomitant loss of this phenotype and the Ura^+ phenotype, and it was ascertained that they were still unable to grow on plates without isoleucine. Eight clones passed these screens. Uptake of amino acids was measured during logarithmic growth, and 7 transformants were found to have increased uptake of all three branched-chain amino acids (data not shown). From three transformants the plasmids were propagated through *E. coli* and tested for the retransformation phenotype. Two of the plasmids had inserts with identical restriction maps, and one of these was chosen for further analysis. The third plasmid has not yet been analysed.

3.2. Nucleotide sequence analysis of *BAP2*

The cloned fragment was about 8 kb, and sequencing of about 300 bp from both ends of an approx. 3 kb *Hin*dIII fragment (cf. Fig. 1) revealed identity with a sequence including the stop codon of an ORF called YBR068c, located on the right arm of chromosome II [32], and with a derived amino acid sequence having strong homology to other known amino acid permeases. This ORF is situated only 888 bp downstream of the gene for another amino acid permease, *TAT1* [7]. *TAT1* is supposed to be a permease for tyrosine, and the two genes are transcribed in the same direction.

BAP2 has an open reading frame of 1827 bp and encodes a polypeptide with a calculated molecular mass of 78 446 kDa. There are three potential glycosylation sites, Asn-Xxx-Ser or Asn-Xxx-Thr, at positions Asn74, Asn396 and Asn597. The promoter has three closely situated putative TATA-boxes, and contains the recognition sequence 5′TGACTC, for the transcription activator Gcn4p mediating general amino acid control [33]. The promoter also contains the Leu3p recognition sequence 5′CCGGNNC-CGG at position −385 bp. Leu3p is a transcription activator of a group of genes involved in leucine biosynthesis

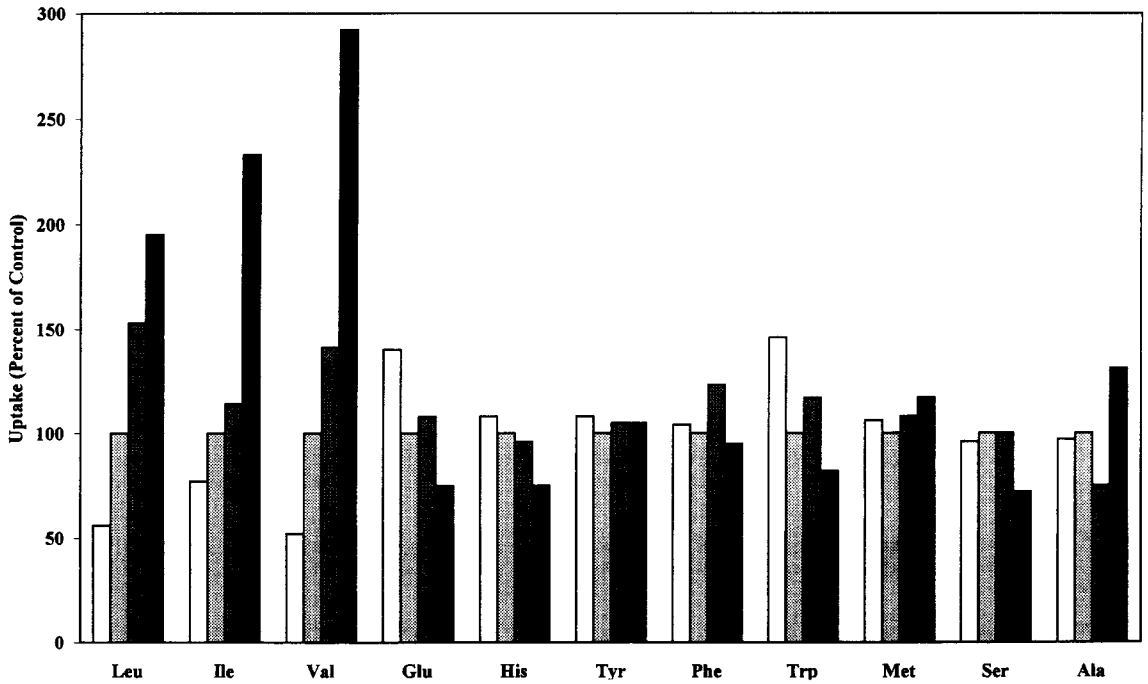

Fig. 3. Uptake of ^{14}C-labeled amino acids as influenced by genetic changes of *BAP2*. Uptake was measured during exponential growth on SC or SC without uracil, the latter for strains carrying *URA3*-containing plasmids. All strains were isogenic and deficient in the general amino acid permease. Open bars, strain M4056, deficient in Bap2p. Light shading, control strain M4054, wild-type for *BAP2*. Darker shading, same strain, except that it carries the centromere-based plasmid pMG1 with *BAP2*. Closed bars, same strain, except that the plasmid-borne *BAP2* gene is truncated for the 29 most C-terminal codons.

[34]. The 3′ non-coding end has the 5′TAG...TAG...(A/T rich)...TTT signal for transcription termination of mRNA in yeast [35]. The 5′AATAAA sequence for polyadenylation in higher eukaryotes cannot be found.

In a comparison of the deduced amino acid sequence of *BAP2* with known permease sequences, the best homology is found to Gap1p [2] (68%). An even higher homology (84%) is seen to a putative amino acid permease, Pap1p [36]. Fig. 2 shows the derived amino acid sequence of Bap2p aligned with the general amino acid permease, Gap1p.

3.3. Phenotype of a bap2 deletion

From a physiological point of view, the capacity, regulation, etc., of the more specific amino acid permeases are of primary interest on media that repress and/or inhibit the general amino acid permease, Gap1p, such as synthetic media which have ammonium as nitrogen source, e.g., MA and SC, simply because of the high capacity of Gap1p. SC (synthetic complete medium) was chosen in most of this work. However, somewhat dependent on the strain, Gap1p is not necessarily negligible on ammonium-containing media. To eliminate transport through Gap1p, a *gap1* deletion was constructed, and the resulting strain was used as the control strain in all uptake experiments and the parent for isogenic derivatives affected in *BAP2*, analysed in these experiments.

A deletion in *BAP2* was made by removing a 786 bp fragment, situated 60 bp into the gene from the start codon. Fig. 3 shows the amino acid uptake in the *bap2* strain in percentage of the control, i.e., the M4054 strain (*Δgap1 BAP2*). For this strain, uptake values in nmol/min × mg dry weight of cells were: leucine, 0.5; isoleucine, 0.2; valine, 0.1; glutamic acid, 0.1; histidine, 0.4; alanine, 0.04; phenylalanine, 0.6; tyrosine, 0.4; tryptophan, 0.1; methionine, 0.4; and serine, 1.8. Uptake in M4056 (*Δgap1 Δbap2*) of leucine and valine decreases to about 55%, and the uptake of isoleucine to about 75% of the control with the intact *BAP2* gene. Uptake of all other measured amino acids is unaltered or moderately increased, suggesting a high specificity of Bap2p. This is taken to mean that Bap2p is responsible for a large part of the uptake of the three branched-chain amino acids. Considering the size of the *bap2* deletion, it is likely that the remaining uptake goes via one or more other permeases.

3.4. The effect of moderately increased gene dosage

To measure the effect on amino acid uptake of an extra copy of the *BAP2* gene, the *BAP2* (*Δgap1*) control strain, M4054, was transformed with plasmid pMG1, consisting of the centromere-based vector pRS316 [30], with a 4.1 kb *Sca*I fragment inserted into the *Sma*I site. pMG1 contains an intact *BAP2* gene, with about 2 kb upstream sequences and 0.3 kb sequences downstream of the coding region. The transformant shows about 50% increase in the uptake of leucine and a smaller increase in the uptake of isoleucine and valine (Fig. 3). Uptake of the other measured amino acids changed less. When the *Δbap2* strain (M4056) was transformed with the same construct, uptake of the branched-chain amino acids was restored to the level seen in the *BAP2* control strain (M4054) (data not shown).

3.5. Phenotype of a C-terminal truncation

During a separate study (H.A. Andersen, unpublished) involving the closely linked *TAT1* gene [7], a 6 kb *Sau*3A fragment in the centromere-based vector pRS316 was found to markedly increase branched-chain amino acids uptake. The insert turned out to contain *BAP2* except for the last 87 nucleotides of the coding region, resulting in the substitution of the 29 most C-terminal amino acids, with a sequence of vector-encoded amino acids. In order to study the effect of this truncation without adding an extra copy of *TAT1*, plasmid pMG20 was constructed, consisting of a 3.1 kb *Spe*I-*Sau*3A fragment ligated into the *Spe*I and *Bam*HI sites of pRS316. When introduced into the *BAP2* control strain (M4054), the plasmid caused a doubling in the uptake of leucine, more than a doubling in the uptake of isoleucine and nearly a three times higher uptake of valine (Fig. 3). Again, uptake of the other measured amino acids was only modestly affected.

4. Discussion

In the present paper we identify a gene, *BAP2*, encoding an amino acid permease involved in the transport of the three branched-chain amino acids in *S.cerevisiae*. Eight other common amino acids, including those (phenylalanine, methionine, alanine) which, in hydrophobicity, etc., resemble the branched-chain amino acids, showed no clear sign of being transported by Bap2p, indicating a high specificity of this permease.

For uptake of L-leucine, three systems have previously been described; *GAP1*, the general amino acid permease, and two more specific transport systems, one with high affinity and low capacity (S1) and another with low affinity and high capacity (S2) [20,21]. Bap2p might well correspond to one of the two kinetically described leucine transport systems. This study shows that Bap2p is involved in leucine transport, and suggests that at least one more uptake system in addition to Bap2p and Gap1p exists. In the present study, deletion of *BAP2* reduces leucine uptake to about 50%. It is not known to what extent the remaining uptake goes via another permease specific for the branched-chain amino acids. It is possible that permeases with preference for other amino acids can transport leucine, isoleucine and valine to some extent, and that the uptake by these permeases increases when *GAP1* and *BAP2* are deleted. In analogy with this idea, we see

somewhat increased uptake of a few other amino acids in the deleted strain (cf. Fig. 3), suggesting slight activation of other permeases.

BAP2 and *TAT1* are located on chromosome II only 888 by apart. A similar close position of two permease genes is seen for *APL1*, a putative amino acid permease [37], and the lysine permease, *LYP1*. These genes are separated by 881 bp. However, whereas *BAP2* and *TAT1* are transcribed in the same direction, *APL1* and *LYP1* are transcribed in opposite directions. It is tempting to suggest a coordinated regulation of transcription of closely linked permease genes, but so far no experimental data can support this idea. In a comparison with other permease genes we found about 84% homology at the amino acid level between Bap2p and the putative permease Pap1p [36], which differs from Bap2p primarily at the termini. When compared to Bap2p, Pap1p is missing 28 amino acids at the C-terminus and 12 amino acids at the N-terminus. We hypothesize that the increased activity caused by a 29 amino acid C-terminal truncation of Bap2p reflects a regulatory role of this segment. In the general amino acid permease, Gap1p, phosphorylation and dephosphorylation, probably near the C-terminus, has a decisive effect on activity [9,10]. Also, yeast and plant proton translocating ATPases have regulating, inhibitory domains near their C-terminus [38]. However, it is conceivable that the C-terminal truncation of the Bap2p permease stimulates uptake by alternative mechanisms, e.g., affecting targeting or turnover of the permease.

The *BAP2* promoter region has putative binding sites for the transcription activators Leu3p and Gcn4p. Potential Gcn4p recognition sequences are also found in promoters of the amino acid permease genes *HIP1* [6] and *TAT2* [7]. The importance of the putative Gcn4p binding sites in these two promoters is different; increased levels of Gcn4p did not influence *TAT2* expression [7], while it stimulated *HIP1* expression [39]. We do not yet know roles for the Gcn4p binding site and the Leu3p binding site in the *BAP2* promoter.

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