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Two FK506 Resistance-Conferring Genes in *Saccharomyces cerevisiae*, *TAT1* and *TAT2*, Encode Amino Acid Permeases Mediating Tyrosine and Tryptophan Uptake

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The macrocyclic lactone FK506 exerts immunosuppressive effects on T lymphocytes by interfering with signal transduction leading to T-cell activation and also inhibits the growth of eukaryotic microorganisms, including *Saccharomyces cerevisiae*. We reported previously that an FK506-sensitive target in *S. cerevisiae* is required for amino acid import and that overexpression of two new genes, *TAT1* and *TAT2* (formerly called *TAP1* and *TAP2*), confers resistance to the drug. Here we report that *TAT1* and *TAT2* encode novel members of the yeast amino acid permease family composed of integral membrane proteins that share 30 to 40% identity. TAT1 is the tyrosine high-affinity transporter, which also mediates low-affinity or low-capacity uptake of tryptophan. TAT2 is the tryptophan high-affinity transporter. FK506 does not reduce the levels of *TAT1* and *TAT2* transcripts, indicating that the inhibition of amino acid transport by the drug is posttranscriptional.

The immunosuppressive drugs FK506, cyclosporin A, and rapamycin block the activation and proliferation of quiescent T lymphocytes (39). The effects of FK506 and cyclosporin A are mediated by a *cis-trans*-prolyl isomerase–drug complex that specifically interacts with and inhibits components of the signal transduction pathways leading to T-cell activation. The immunosuppressants also inhibit the growth of *Saccharomyces cerevisiae* by binding to the yeast homologs of the prolyl-isomerases (29). Cyclosporin A binds cyclophilin; rapamycin and FK506 bind FKBP12. We previously demonstrated that for FK506, yeast cells contain a second, FKBP12-independent target which is involved in the uptake of amino acids (17, 18). FK506 blocks the import of tryptophan, histidine, and leucine. Thus, strains auxotrophic for these three amino acids are sensitive to the drug, whereas prototrophy for one of these amino acids or an excess of tryptophan or histidine added exogenously to the growth medium is sufficient to overcome toxicity. The drug presumably interferes with the amino acid permeases mediating the uptake of these amino acids. Only 6 of the surmised ca. 20 amino acid transporters in *S. cerevisiae* have been cloned and sequenced: the general amino acid permease GAP1 (27), the arginine high-affinity permease CAN1 (1, 24), the lysine high-affinity permease LYP1 (42), the proline high-affinity permease PUT4 (44), the histidine high-affinity permease HIP1 (43), and the GAP1 homolog of unknown specificity YCC5 (35). All appear to be derived from a common molecular ancestor, as they all encode integral membrane proteins that share 30 to 40% identity and contain 12 membrane-spanning regions. With the exception of GAP1, which possesses broad specificity for all L amino acids and most D isomers, the other transporters are highly specific for individual amino acids or small groups of structurally related amino acids. On the basis of biochemical and genetic data, there is evidence that the entry of most amino acids is mediated by a specific high-affinity system which can also import other similar amino acids with low affinity or low capacity (5, 26). Thus, uptake of an amino acid can occur by at least three distinct routes: via the general amino acid permease GAP1, via a specific high-affinity permease, and via a low-affinity permease. Little is known about the regulation of amino acid uptake other than for import mediated by GAP1 and PUT4. These transporters belong to the uptake systems that are subject to nitrogen source regulation and are thus repressed during growth on a good nitrogen source such as ammonium (6, 13, 26, 28, 31, 47). Inhibition by ammonium ions involves at least two distinct control mechanisms: repression of transcription of the transporter genes mediated by the *URE2* gene product (31, 47) and posttranscriptional inactivation of the transporters by the action of the nitrogen permease inactivators NPI1 and NPI2 (13). In the absence of ammonium ions, permease inactivation is counteracted by the putative Ser-Thr kinase NPR1 (14, 45, 46). Regulation of the other, "constitutive" amino acid permeases at the transcriptional level has not been demonstrated; it has been described only as feedback inhibition and transinhibition at the protein level (5). Feedback inhibition explains the observation that high intracellular levels of a transported amino acid prevent the further uptake of this amino acid, perhaps by occupying the transporter. In the case of transinhibition, the inhibiting molecules are not transported by the inhibited uptake system and show little or no structural similarity to the physiological substrate.

Here we describe the characterization of two new genes, *TAT1* and *TAT2*, overexpression of which confers resistance to FK506 (17). We find that the TAT1 and TAT2 proteins are novel members of the yeast amino acid permease family and that they mediate tyrosine and tryptophan high-affinity uptake, respectively. *TAT1* and *TAT2* were formerly called *TAP1* and *TAP2* (16, 17); the names were changed to avoid conflict with a previous use of the *TAP* designation for a yeast gene (2).

MATERIALS AND METHODS

Strains, media, and plasmids. *S. cerevisiae* strains used in this study are listed in Table 1. Media were as described previously (40). Proline medium was made by using Bacto Yeast Nitrogen Base (Difco) without amino acids and ammonium sulfate (according to the Difco manual), adding glucose to a final concentration of 3% and proline to a final concentration of 1 mg/ml, and supplementing the medium with

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TABLE 1. Yeast strains used in this study

Strain	Genotype
JK9-3da/α	*MAT***a**/*MAT*α *trp1/trp1 his4/his4 leu2/leu2 ura3/ura3 rme1/rme1 HML***a**/*HML***a**
JK9-3dα	*MAT*α *trp1 his4 leu2 ura3 rme1 HML***a**
JK9-3da	*MAT***a** *trp1 his4 leu2 ura3 rme1 HML***a**
MH339	JK9-3da/α *TRP1/TRP1 HIS4/HIS4 LEU2/LEU2*
MH338-2a	JK9-3dα *TRP1 HIS4 LEU2*
MH338-7b	JK9-3da *HIS4 LEU2*
AS1	MH339 *tat1::URA3/TAT1*
AS1-3c	MH338-2a *tat1::URA3*
AS2	MH339 *tat2::URA3/TAT2*
AS2-1c	MH338-2a *tat2::URA3*
AS4	JK9-3da/α *tat2::URA3/TAT2 trp1/TRP1 his4/HIS4 leu2/LEU2*
AS5	JK9-3da/α *tat2::URA3/TAT2 trp1/TRP1 HIS4/HIS4*
AS6	JK9-3da/α *tat2::URA3/TAT2 trp1/TRP1 HIS4/HIS4 LEU2/LEU2*
AS10	**a**/α *tat1::URA3/TAT1 tyr1/TYR1 trp1/TRP1 his4/HIS4 ade2/ADE2 lys2/LYS2 ura3/ura3*
AS11-1a	MH338-2a *tat1::URA3 tat2::URA3*
AS12	JK9-3da/α *tat1::URA3/tat1::URA3 tat2::URA3/TAT2 trp1/TRP1 HIS4/HIS4 LEU2/LEU2*
AS13	JK9-3da/α *tat1::URA3/TAT1 tat2::URA3/TAT2 trp1/TRP1 HIS4/HIS4 LEU2/LEU2*
AS14	JK9-3da/α *tat1::URA3/tat1::URA3*
AS20	**a**/α *tat1::URA3/TAT1 tyr1/TYR1 leu2/leu2 ura3/ura3*
AS26	**a**/α *tat2::URA3/TAT2 arg1/ARG1 trp3/TRP3 met1/MET1 ade5/ADE5 leu1/LEU1 mal/MAL gal2/GAL2 ura3/ura3*
TK167	**a**/α *tat1::URA3/TAT1 sec18/SEC18 trp1/TRP1 leu2/LEU2 his4/HIS4 ura3/ura3*
TK201	JK9-3da/α *gcn4::URA3/gcn4::URA3*

tryptophan (20 μg/ml) and uracil (20 μg/ml). Plasmid pTAT1 is YCplac33 (Amp^r *CEN4 URA3*) (10) carrying a 4.4-kb genomic DNA insert containing the *TAT1* gene (see Fig. 1A). Plasmid pTAT2 is YCplac33 carrying a 4.2-kb genomic DNA insert containing the *TAT2* gene (see Fig. 1A). Plasmids pAS5 and pAS6 were constructed by cloning the 3.0-kb *Hin*dIII fragment of *TAT1* derived from pTAT1 and the 3.7-kb *Eco*RI fragment of *TAT2* derived from pTAT2 into the high-copy-number vector YEplac181 (Amp^r 2μm *LEU2*) (10). pGCN4 is plasmid p238 (a gift from A. Hinnebusch) containing a *GCN4* allele with the four upstream open reading frames inactivated by point mutations in the ATG codons.

Genetic techniques. Yeast mating, sporulation, and tetrad analysis were performed as described previously (15). Yeast transformation was performed by the lithium acetate procedure (23). *Escherichia coli* MH1 (*araD lac galE galK hsr rpsL*) was used for propagation and isolation of plasmid DNA as described previously (37).

DNA manipulations. Restriction enzyme digests and ligations were done by standard methods (37). All enzymes and buffers were obtained commercially (Boehringer GmbH). DNA probes for Southern analysis were labeled by using the random-prime labeling kit (Amersham International) as recommended by the manufacturer. Total yeast genomic DNA was isolated as described previously (36), fractionated by gel electrophoresis on an agarose gel in Tris-borate-EDTA, and transferred to nylon membranes. Prehybridization and hybridization were performed as recommended by the manufacturer (Amersham International). DNA was sequenced by the dideoxy-chain termination method (38) with the T7 sequencing system (Pharmacia). Overlapping deletions of the *TAT2* gene were obtained from plasmids pTAT2 and pAS2 (see below) by the exonuclease III method with the Erase-a-Base system (Promega). Custom-made oligonucleotides were used as primers for sequencing regions not accessible with deletions or subclones.

RNA manipulations. DNA probes for Northern (RNA) analysis were either labeled by using the direct nucleic acid-labeling kit (Amersham International) or the random-primed DNA-labeling kit (U.S. Biochemicals). Total RNA from exponentially growing cells was isolated as described previously (9), fractionated in morpholinepropanesulfonic acid (MOPS)-formaldehyde, and transferred to nylon membranes. Prehybridization and hybridization were performed under conditions recommended by the manufacturer (Amersham International) for enhanced chemiluminescence (ECL) detection or by the method of Sambrook et al. (37) for radioactive detection.

Disruption of the *TAT1* and *TAT2* genes. The 3.0-kb *Eco*RI fragment of *TAT1* and the 3.7-kb *Eco*RI fragment of *TAT2* were excised from the original *TAT1* and *TAT2* clones, respectively, and subcloned into the *Eco*RI site of YCplac33, from which the *Bam*HI and *Sal*I sites had previously been removed (pAS1 and pAS2). This was followed by the insertion of the *Bam*HI fragment of the *URA3* gene from pUC1318 (obtained from M. Egerton and H. Riezman, Biozentrum, University of Basel) into the unique *Bam*HI site of *TAT1* and *TAT2* (see Fig. 1B). The resulting plasmids were linearized with *Eco*RI and *Bgl*II, and the disruption fragments were transformed directly into the prototrophic strain MH339, selecting for *URA*$^+$. Disruptions were verified by Southern analysis. Diploid strains that showed the expected hybridization pattern were designated AS1 (*tat1::URA3/TAT1*) and AS2 (*tat2::URA3/TAT2*).

Disruption of *GCN4*. *GCN4* was disrupted by transforming the 3.7-kb *Bst*EII-*Mlu*I fragment of plasmid pM214 (a gift from A. Hinnebusch) into JK9-3da/α. The disruption was verified by Southern analysis. A homozygous *gcn4* diploid (TK201) was constructed by mating two haploid *gcn4::URA3* segregants from the original, diploid, heterozygous disruption strain.

Amino acid import studies. Yeast strains grown to logarithmic phase in YPD were harvested and assayed for the uptake of ^{3}H-labeled amino acids as described previously (16, 17). The radioactive amino acids were L-[5-^{3}H]tryptophan (29 Ci/mmol), L-[2,5-^{3}H]histidine (44 Ci/mmol), and L-[3,5-^{3}H]tyrosine (56 Ci/mmol) (purchased from Amersham). A typical reaction mixture contained 4.5 μCi in a volume of 5 ml.

Mapping the chromosomal site of *TAT1* and *TAT2*. The internal 3-kb *Hin*dIII fragment of plasmid pTAT1 and the 3.7-kb *Eco*RI fragment of plasmid pTAT2 were used as probes

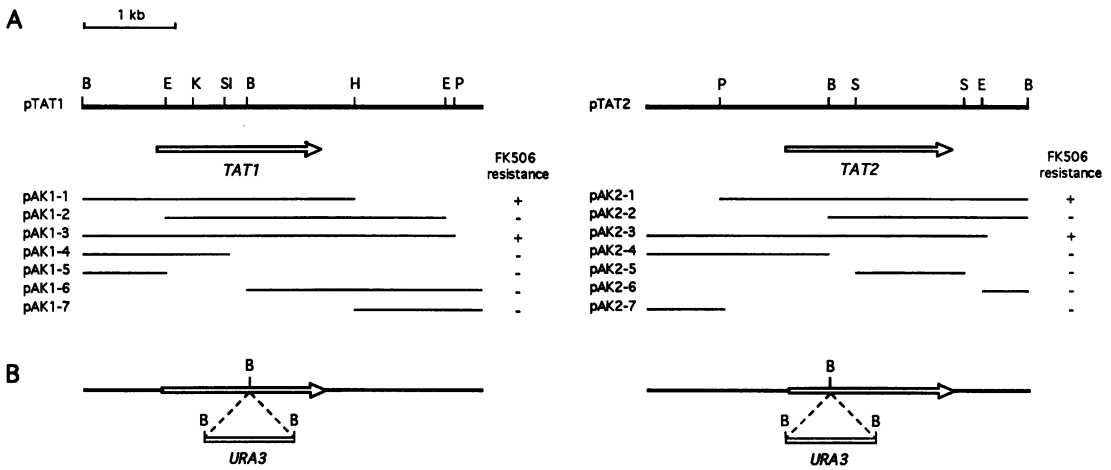

FIG. 1. Restriction maps of *TAT1* and *TAT2*, functional assay of deletion constructs, and *TAT* disruptions. (A) Plasmids pTAT1, pTAT2, and derivates containing the DNA indicated by a line were tested for ability to confer FK506 resistance when transformed into wild-type strain JK9-3da/α. Plus (+) and minus (−) signs indicate ability and inability, respectively, to confer FK506 resistance. The open arrows indicate the positions of the *TAT* open reading frames as determined by DNA sequence analysis. Restriction enzyme abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sma*I; Sl, *Sal*I. (B) *TAT* disruptions. In *tat1*::*URA3* and *tat2*::*URA3*, a *URA3* cassette was inserted as a *Bam*HI fragment into the *Bam*HI site in the coding regions of *TAT1* and *TAT2*.

for hybridization to yeast chromosomes separated by pulsed-field gel electrophoresis and for hybridization to a set of overlapping λ and cosmid clones representing ca. 99% of the *S. cerevisiae* genome (a gift of L. Riles and M. Olson, Washington University, St. Louis, Mo.). For meiotic mapping of *TAT1*, strains AS10 and TK167 were sporulated and dissected and the segregation pattern of *tat1*::*URA3*, *tyr1*, and *sec18* was observed. For meiotic mapping of *TAT2*, strains AS4 and AS26 were sporulated and dissected, and the segregation pattern of *tat2*::*URA3*, *trp1*, and *arg1* was observed.

Nucleotide sequence accession numbers. The sequences of *TAT1* and *TAT2* (Fig. 2) have been submitted to the EMBL database under the accession numbers X79151 and X79150, respectively.

RESULTS

Isolation and nucleotide sequence analysis of *TAT1* and *TAT2*. We previously showed that FK506 impairs amino acid import in the yeast *S. cerevisiae* and isolated two new genes, *TAT1* (tyrosine and tryptophan amino acid transporter) and *TAT2* (tryptophan amino acid transporter), by screening single-copy-number plasmid libraries for genes that confer resistance to FK506 (17). Restriction maps of the DNA inserts of the two isolated plasmids, pTAT1 and pTAT2, are displayed in Fig. 1A. The position of the *TAT* genes within the cloned DNA insert of pTAT1 and pTAT2 was determined by deletion analysis. Subclones of pTAT1 and pTAT2 were constructed and tested for their ability to confer FK506 resistance. *TAT1* was assigned to a *Bam*HI-*Hin*dIII fragment of pTAT1 (pAK1-1); *TAT2* was placed within a large *Eco*RI-*Pst*I fragment of pTAT2. The sequences obtained from the subclones and from exonuclease III deletions revealed a single open reading frame of 1,856 bp for *TAT1* encoding a protein of 619 amino acids with a calculated molecular mass of 68.8 kDa and a single open reading frame of 1,776 bp encoding a protein of 592 amino acids (65.4 kDa) for *TAT2* (Fig. 2).

Northern analysis with the *Eco*RI fragments of *TAT1* and *TAT2* as probes revealed mRNAs of the expected size. For both genes, a single mRNA of approximately 2 kb was over-expressed in strains containing the relevant *TAT* gene on a multicopy vector and was missing in strains disrupted at the corresponding *TAT* locus (data not shown).

The TAT1 and TAT2 proteins deduced from the nucleotide sequences show 39.7% identity, and 51 and 52% of the amino acids, respectively, are hydrophobic residues. The hydropathy profiles of TAT1 and TAT2 suggest that there are 12 membrane-spanning regions. TAT1 and TAT2 thus show characteristics typical of transmembrane transport proteins, although they do not contain an obvious cleavable N-terminal signal sequence. Searches of databases by using the FASTA program (8) revealed that the polypeptides deduced from the nucleotide sequences have 30 to 50% identity to the known yeast amino acid permeases GAP1, CAN1, LYP1, HIP1, PUT4, and YCC5 (a GAP1 homolog found by sequencing the yeast chromosome III) (35). Alignments obtained with the BESTFIT and PILEUP programs (8) revealed 49 identical amino acids, including 8 prolines and most of the tryptophan and glutamate residues. Almost 200 amino acids are conservatively replaced. All seven transport proteins exhibit 12 transmembrane domains, and, except for the N- and C-terminal regions, the Kyte-Doolittle patterns are practically superimposable. An alignment of the amino acid sequences of TAT1, TAT2, GAP1, CAN1, LYP1, PUT4, HIP1, and YCC5 is displayed in Fig. 3.

The sequences of the *TAT1* and *TAT2* genes and the homology in the deduced amino acid sequence and protein structure to the other cloned amino acid permeases in *S. cerevisiae* strongly suggested that both TAT1 and TAT2 are amino acid permeases.

Analysis of the upstream region of the *TAT2* gene reveals at least three sequence motifs, at positions −80 (TGACTG), −85 (TGACTT), and −142 (TCACTC), that resemble the consensus sequence required for GCN4 binding (TGACTC). GCN4 is a transcription factor that is responsible for the activation of a set of >30 genes required for amino acid or purine biosynthesis in response to amino acid or purine starvation (3, 19–22, 25, 35, 42). The coregulation of enzymes in different biosynthetic pathways is commonly referred to as the general control. Genes that are under this general control system have been

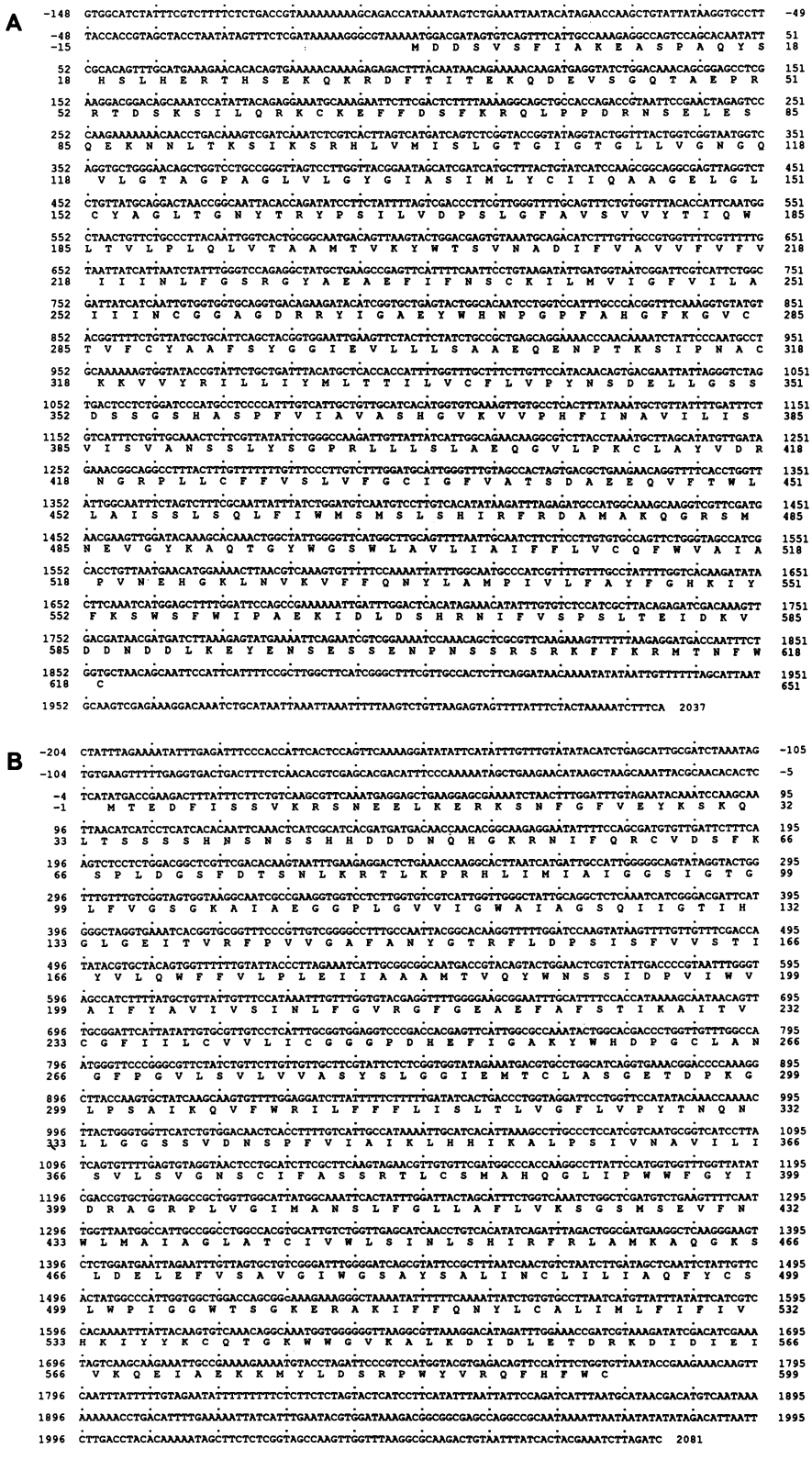

FIG. 2. (A) Nucleotide sequence of *TAT1* and deduced amino acid sequence. (B) Nucleotide sequence of *TAT2* and deduced amino acid sequence. Complete nucleotide sequences of the *TAT1* and *TAT2* open reading frames and adjacent 5′ and 3′ sequences are shown. The deduced amino acid sequences are denoted by the one-letter code.

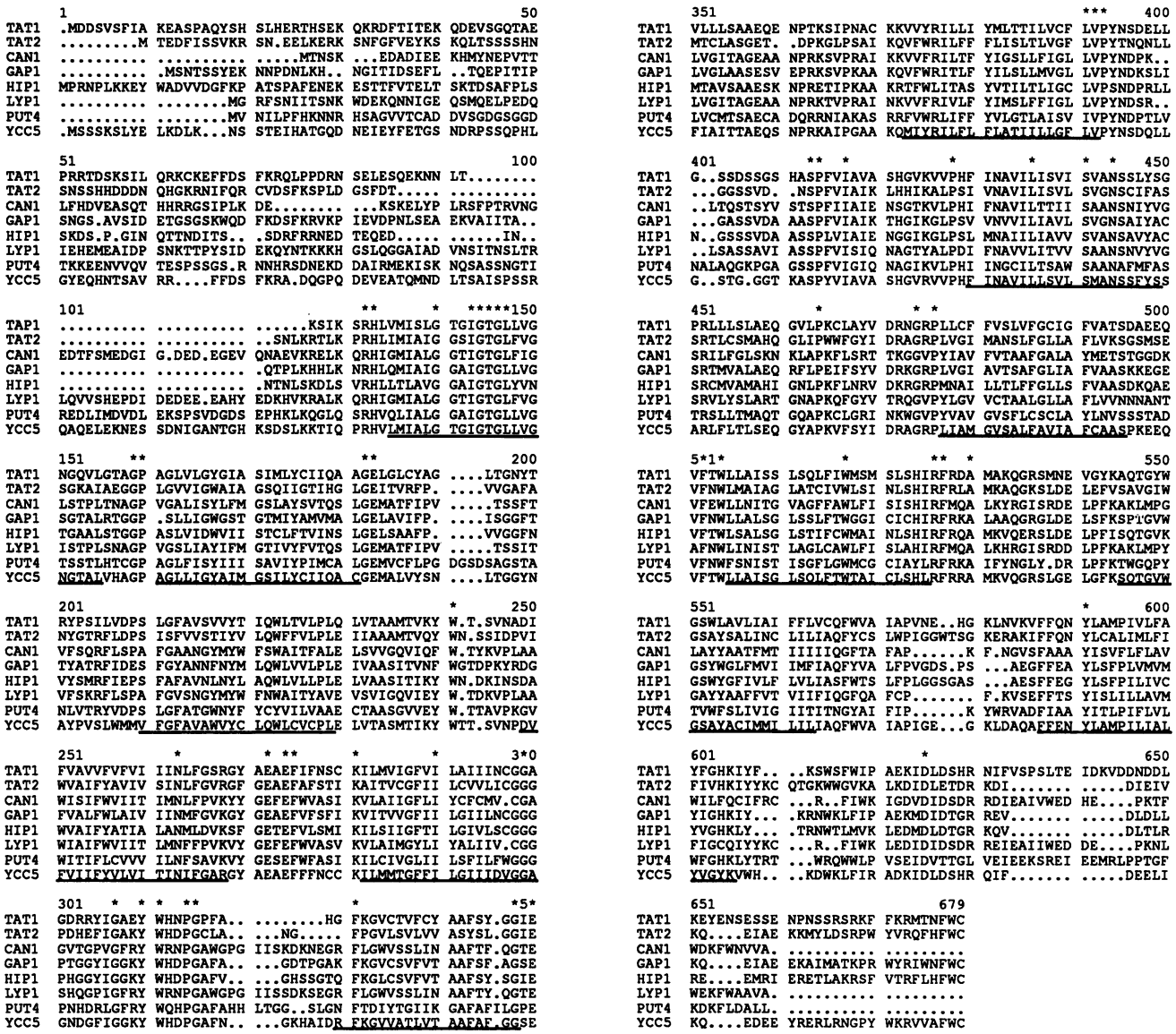

FIG. 3. TAT1 and TAT2 are homologous to known yeast amino acid permeases. The predicted amino acid sequences of TAT1, TAT2, GAP1, CAN1, LYP1, HIP1, PUT4, and YCC5 were aligned by using the PILEUP program (9). Identical amino acids are indicated by asterisks. The 12 membrane-spanning regions are underlined.

shown to possess at least one hexanucleotide sequence (TGACTC) in their 5′ upstream region which is recognized by the GCN4 protein. Potential GCN4-binding sites can also be found in the *HIP1* and *YCC5* promoter regions (35, 41, 43) but not in the *TAT1*, *CAN1*, and *LYP1* promoter regions (1, 42). Thus, the transcription of at least some constitutive amino acid permease genes might be under general control. In a model in which *TAT2* transcription is regulated by GCN4, not only overexpression of *TAT2* but also overexpression of *GCN4* should confer resistance to FK506 by increasing *TAT2* levels. However, cells constitutively expressing *GCN4* are still FK506 sensitive (Fig. 4). Furthermore, disruption of *GCN4* does not result in FK506 hypersensitivity but, surprisingly, confers resistance to the immunosuppressant (Fig. 4). Finally, we found by Northern analysis that a *GCN4* disruption does not alter the expression of *TAT2* (data not shown). Thus, the *TAT2* gene is not under general control but is constitutively expressed.

Genetic mapping of *TAT1* and *TAT2*. By physical methods (see Materials and Methods), *TAT1* and *TAT2* were assigned to the right arm of chromosome II and the left arm of chromosome XV, respectively. A more precise map position of *TAT1* was determined by crossing strain AS1-3c (*tat1*::*URA3*) to *lys2* and *sec18* strains. On the basis of parental ditype/nonparental ditype/tetratype (PD/NPD/T) ratios of 87:0:36 (*TAT1* × *lys2*) and 28:0:5 (*TAT1* × *sec18*), *TAT1* was positioned 14.6 centimorgans (cM) centromere-proximal to *lys2* and 7.6 cM from *sec18*.

A more precise map position of *TAT2* was determined by crossing strain AS2-1c (*tat2*::*URA3*) to *trp1* and *arg1* strains. On the basis of PD/NPD/T ratios of 30:14:26 (*TAT2* × *trp1*) and 34:1:31 (*TAT2* × *arg1*), TAT2 was placed 21.1 cM from centromere XV and 28.4 cM from *arg1*.

TAT2 is a tryptophan transporter. To determine whether TAT2 is essential for growth and to identify the amino acid(s)

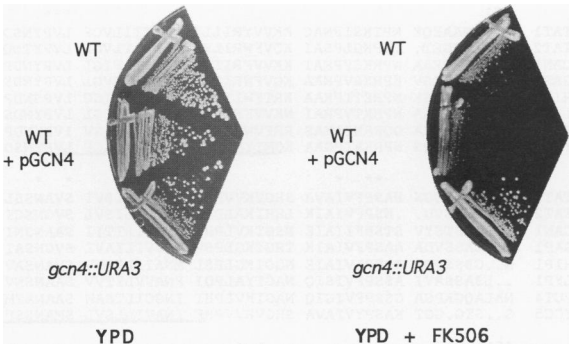

FIG. 4. Wild-type (WT) (JK9-3da/α), WT + pGCN4 (JK9-3da/α transformed with a high-copy-number plasmid containing *GCN4*), and *gcn4*::*URA3* (TK201, JK9-3da/α containing a homozygous disruption of *GCN4*) strains were streaked on YPD medium with and without 50 μg of FK506 per ml. Cells overexpressing *GCN4* are FK506 sensitive, whereas cells disrupted in *GCN4* are drug resistant. Resistance or sensitivity to FK506 was investigated on YPD medium because JK9-3da/α (WT) is resistant on minimal (SD) medium (18).

it might transport, we disrupted the genomic copy of the *TAT2* gene. The *URA3* gene was inserted into the unique *Bam*HI site within the *TAT2* gene (see Materials and Methods) (Fig. 1B). The successful disruption of the *TAT2* locus in the diploid amino acid prototroph MH339 was verified by Southern analysis (data not shown). The resulting strain heterozygous for the *TAT2* disruption, AS2, was sporulated and dissected on YPD. All tetrads yielded four viable spores with the *URA3* disruption marker segregating 2:2. It was concluded that *TAT2* is not essential for the growth of prototrophic yeast cells. The haploid strain AS2-1c (*tat2*::*URA3*) was crossed to the auxotrophic strain JK9-3da (*trp1 his4 leu2 ura3*). Dissection of the resulting diploid strain AS4 (*tat2*::*URA3*/*TAT2 trp1*/*TRP1 his4*/*HIS4 leu2*/*LEU2 ura3*/*ura3*) revealed that the disruption of the *TAT2* gene conferred a growth defect when combined with *trp1*. Of 51 dissected tetrads, only 18 yielded four visible colonies, whereas three spores were recovered from 18 tetrads and two spores were recovered from the remaining 15 tetrads after 2 days of incubation at 30°C (Fig. 5A). None of these segregants were *tat2*::*URA3 trp1*, whereas almost all of the nongrowing spores were inferred to have this genotype. Interestingly, the *tat2*::*URA3 trp1* segregants were not dead but showed a slow-growth phenotype, since they were visible as colonies after 4 days of incubation (Fig. 5A). This suggested that *TAT2* encodes a tryptophan high-affinity transporter and also that a second, low-affinity or low-capacity transporter for tryptophan exists. To test this, we dissected strain AS6 (*tat2*::*URA3*/*TAT2 trp1*/*TRP1*) on medium containing a 50-fold excess of tryptophan. Indeed, after 3 days of incubation, all four spores were growing normally, confirming the presence of a second tryptophan transporter (Fig. 5A). Furthermore, dissection of strain AS6 on medium containing proline as the sole nitrogen source, such that the general amino acid permease GAP1 is derepressed, also yielded four almost equally growing spore colonies (Fig. 5A). This suggested that tryptophan import can also occur via GAP1 and that the activation of this transport system is sufficient to overcome the slow-growth phenotype of *tat2*::*URA3 trp1* cells.

To obtain biochemical evidence that TAT2 is the tryptophan high-affinity permease, we performed amino acid import studies measuring the uptake of radiolabeled tryptophan, tyrosine, and histidine into strains AS2-1c (*TAT1 tat2*::*URA3*) and MH338-2a (*TAT1 TAT2*). Figure 6 shows that the import of

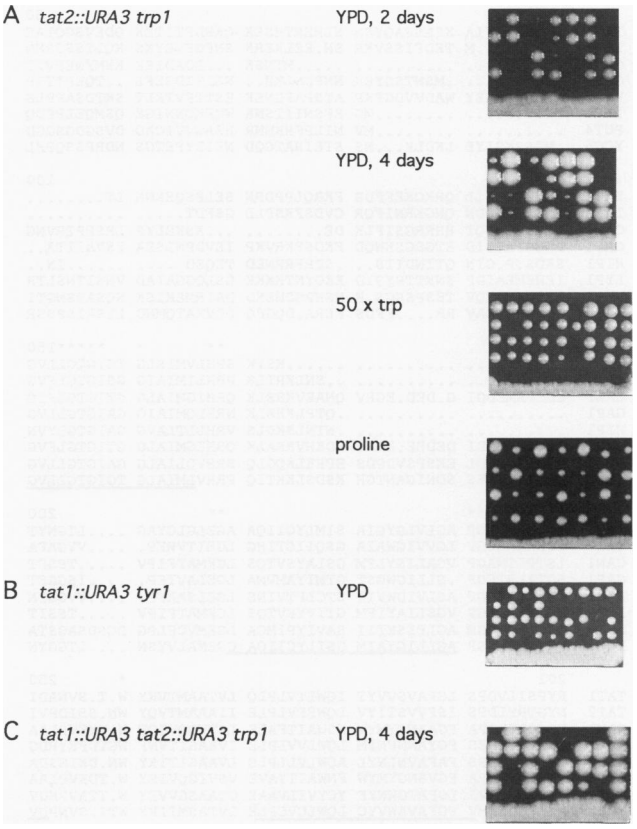

FIG. 5. (A) Growth of *tat2*::*URA3 trp1* segregants. Spores of strain AS4 (JK9-3da/α *tat2*::*URA3*/*TAT2 trp1*/*TRP1 his4*/*HIS4 leu2*/*LEU2*) were germinated on YPD for 2 or 4 days. *tat2*::*URA3 trp1* spores show a slow-growth phenotype. Spores of strain AS6 (JK9-3da/α *tat2*::*URA3*/*TAT2 trp1*/*TRP1 HIS4*/*HIS4 LEU2*/*LEU2*) were germinated on YPD medium containing a 50-fold excess of tryptophan (1 mg/ml of YPD) and on medium containing proline as the sole nitrogen source (see Materials and Methods). Under these conditions, the slow-growth phenotype of *tat2*::*URA3* trp1 cells is suppressed. (B) Growth of *tat1*::*URA3 tyr1* segregants. Spores of strain AS10 (a/α *tat1*::*URA3*/*TAT1 tyr*/*TYR1 trp1*/*TRP1 his4*/*HIS4 ade2*/*ADE2 lys2*/*LYS2 ura3*/*ura3*) were germinated on YPD. *tat1*::*URA3 tyr1* cells are inviable. (C) Growth of *tat1*::*URA3 tat2*::*URA3 trp1* segregants. Spores of strain AS12 (JK9-3da/α *tat1*::*URA3*/*tat1*::*URA3 tat2*::*URA3*/*TAT2 trp1*/*TRP1 HIS4*/*HIS4 LEU2*/*LEU2*) were germinated on YPD for 4 days. *tat1*::*URA3 tat2*::*URA3 trp1* cells are inviable. In all cases, the four spores of a tetrad are aligned vertically.

tryptophan is severely decreased when *TAT2* is disrupted. Import of the other amino acids, tyrosine and histidine, was not affected upon disruption of *TAT2*, indicating that the observed effect is specific for tryptophan. Thus, according to the above genetic and biochemical evidence, TAT2 is a tryptophan high-affinity transporter.

TAT1 is a tyrosine transporter. The *TAT1* gene was disrupted by insertion of the *URA3* marker into the unique *Bam*HI site within the *TAT1* gene (see Materials and Methods) (Fig. 1B). The disrupted gene was excised and was transformed into the diploid strain MH339. Disruption of the *TAT1* gene was confirmed by Southern analysis (data not shown). Strain AS1 (*tat1*::*URA3*/*TAT1*) was sporulated and dissected on YPD. All tetrads yielded four viable spores with the *URA3* marker segregating 2:2. The haploid strain AS1-3c (*tat1*::*URA3*) was crossed to cells auxotrophic for various amino acids, and the resulting diploid strains were dissected on

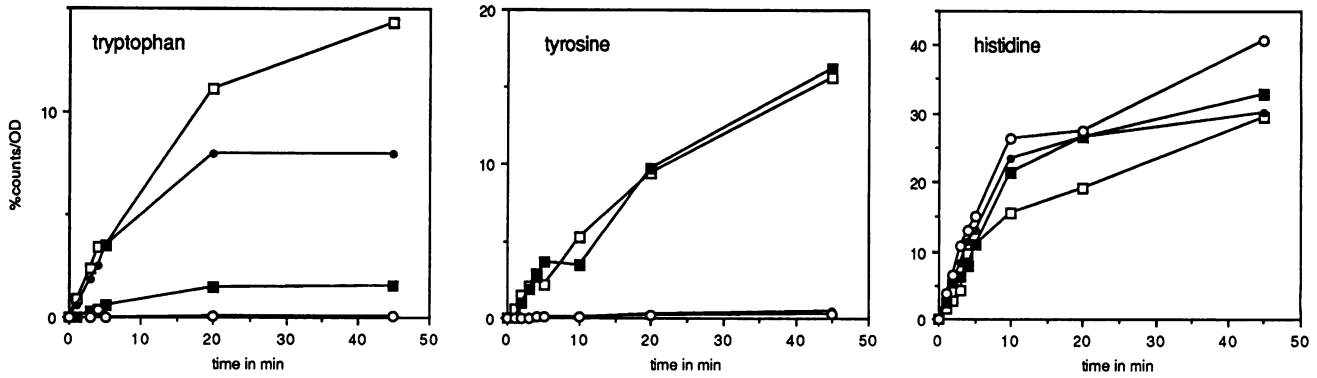

FIG. 6. The import rates of radiolabeled tryptophan, tyrosine, and histidine into strains MH338-2a (*TAT1 TAT2 TRP1 HIS4 LEU2 ura3*) (open squares), AS1-3c (MH338-2a *tat1::URA3*) (solid circles), AS2-1c (MH338-2a *tat2::URA3*) (solid squares), and AS11-1a (MH338-2a *tat1::URA3 tat2::URA3*) (open circles). Shown are representative curves. The solid circles in the tyrosine import graph are obscured by the open circles. OD, optical density.

YPD. Dissection of all strains except for strain AS10 (*tat1::URA3/TAT1 tyr1/TYR1 trp1/TRP1 his3/HIS3 ade2/ADE2 lys/LYS2 ura3/ura3*) yielded four viable spores. Of the 30 dissected tetrads of AS10, 24 yielded only three viable spores, 5 yielded four viable spores, and 1 yielded only two viable spores (Fig. 5B). None of the viable spores were *tat1::URA3 tyr1*, whereas almost all inviable cells were inferred to have this genotype. Thus, because absence of import and biosynthesis at the same time would prevent cell growth, *TAT1* most probably encodes a permease involved in tyrosine high-affinity uptake. To test this directly, amino acid import studies were performed to measure the uptake of radiolabeled tyrosine, tryptophan, and histidine into strains AS1-3c (*tat1::URA3 TAT2*) and MH338-2a (*TAT1 TAT2*). As can be seen in Fig. 6, tyrosine uptake is abolished in *tat1::URA3* cells compared with wild-type strains, confirming that TAT1 is indeed a tyrosine high-affinity transporter. Import of histidine, which served as control, was not affected upon disruption of *TAT1*. However, uptake of tryptophan was slightly decreased in cells lacking TAT1, suggesting that TAT1 might also be involved in tryptophan uptake and might be the second transporter responsible for the viability of *tat2::URA3 trp1* cells (see above).

TAT1 is also a tryptophan low-affinity or low-capacity transporter. A strain disrupted for both *TAT1* and *TAT2*, AS11-1a, was crossed to the *trp1* strain MH338-7b. Dissection of the diploid strain, AS13, revealed that when all three markers (*tat1::URA3*, *tat2::URA3*, and *trp1*) segregated together, the spores no longer exhibited a slow-growth phenotype but were inviable (Fig. 5C). Furthermore, we examined whether overexpression of *TAT1* could restore normal cell growth to *tat2::URA3 trp1* cells. High-copy-number plasmids pAS5 and pAS6 (YEplac181 based) carrying the entire *TAT1* and *TAT2* genes, respectively, were transformed into strain AS4 (*tat2::URA3/TAT2 trp1/TRP1 his4/HIS4 leu2/leu2*). Plasmid YEplac181 without insert was transformed as a control. Transformants were sporulated and dissected on YPD. Overproduction of either TAT1 or TAT2 completely complemented the slow-growth phenotype of *tat2::URA3 trp1* spores. Plasmid YEplac181 alone did not suppress the slow-growth phenotype. Thus, TAT1 is the second tryptophan transporter in addition to being a tyrosine high-affinity transporter.

We also examined whether a high dosage of *TAT2* complements a *TAT1* disruption. The high-copy-number plasmids YEplac181 (no insert), pAS5 (*TAT1*), and pAS6 (*TAT2*) were transformed into strain AS20 (*tat1::URA3/TAT1 tyr1/TYR1 leu2/leu2*). Transformants were dissected on YPD, and only the *TAT1* plasmid was able to restore normal cell growth of *tat1::URA3 tyr1* cells. The high-copy-number *TAT2* plasmid and the control failed to suppress the *TAT1* disruption. Thus, in agreement with our previous results, TAT2 does not transport tyrosine.

Import studies with the doubly disrupted strain AS11-1a (*tat1::URA3 tat2::URA3*) were performed (Fig. 6). Tryptophan uptake was most severely affected in the doubly disrupted strain, presumably as a result of an additive effect of the lack of import due to absence of both tryptophan transporters. The finding that in the *TAT* doubly disrupted strain tryptophan import is almost completely abolished is consistent with our previous finding that the double disruption is not compatible with a *trp1* genetic background.

Disruption of *TAT1* but not of *TAT2* confers resistance to 5FT. 5-Fluorotryptophan (5FT) is a toxic analog of tryptophan and acts most probably by inhibiting anthranilate synthase, which is required for tryptophan biosynthesis and is controlled by tryptophan via negative feedback (4, 33). 5FT seems to mimic tryptophan in turning off the enzyme's activity. In addition, 5FT is incorporated into proteins, causing an accumulation of defective proteins (33). 5FT is thought to enter the cells via amino acid transporters, most probably by the tryptophan high-affinity transporter TAT2. We therefore tested whether disruption of *TAT2* would confer resistance to 5FT. Strains disrupted in *TAT1*, *TAT2*, or both transporter genes were thus incubated on SD-Trp plates containing 0.5 mM 5FT (Fig. 7). Surprisingly, cells disrupted in the *TAT2* locus and cells disrupted in both transporter genes were resistant to 5FT but cells disrupted in *TAT2* alone were sensitive. This suggests that 5FT enters the cells not via the tryptophan high-affinity transporter but via the tyrosine high-affinity permease.

Disruption of *TAT1* confers resistance to FK506. Overexpression of *TAT1* and *TAT2* confers FK506 resistance on normally FK506-sensitive auxotrophic diploids (Fig. 8). (17). We therefore tested the effect of a *TAT1* disruption on growth inhibition by FK506. Surprisingly, we found that the auxotrophic strain AS14 disrupted for *TAT1* (*tat1::URA3/tat1::URA3*) is resistant to FK506, in contrast to the wild-type auxotrophic strain JK9-3da/α (*TAT1/TAT1*) (Fig. 8). We cannot explain why both disruption and overexpression of *TAT1* confer FK506 resistance. The resistance of the disruption cannot be explained by cross talk between amino acid permeases leading to increased expression or activity of some permeases because of

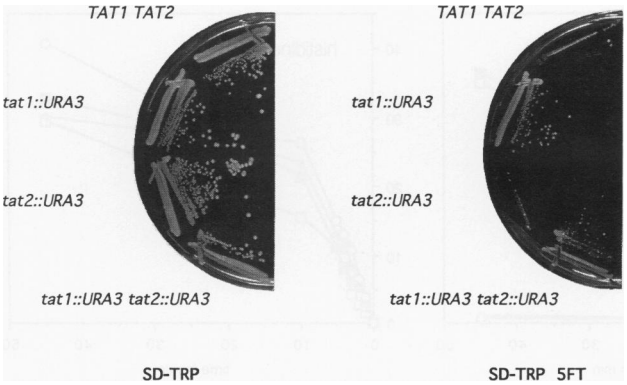

FIG. 7. Disruption of *TAT1* confers resistance to 5FT. Isogenic *TAT1 TAT2* (MH338-2a), *tat1*::*URA3 TAT2* (AS1-3c), *TAT1 tat2*::*URA3* (AS2-1c), and *tat1*::*URA3 tat2*::*URA3* (AS11-1a) strains were grown on SD-TRP with or without 5FT (0.5 mM). Only strains containing a *TAT1* disruption are resistant to the tryptophan analog 5FT.

the lack of others, thereby counteracting the action of FK506; amino acid import studies with cells lacking TAT1 have shown that the uptake of tryptophan and histidine is still inhibited by FK506. The disruption may confer resistance because TAT1 facilitates the entry of FK506 into the cell; however, we do not believe that this is likely, because FK506 is a lipophilic molecule which can diffuse across a membrane. Overexpression of *TAT1* confers FK506 resistance presumably because it enhances tryptophan import.

FK506 does not inhibit *TAT1* or *TAT2* transcription. To investigate whether FK506 inhibits amino acid import by inhibiting the transcription of the amino acid permease genes, we probed for *TAT1* and *TAT2* mRNAs in total yeast RNA from the auxotrophic strain JK9-3a/α pretreated for 5 h with FK506 (50 μg/ml) and with the vehicle (ethanol with 10% Tween 20). The Northern analysis in Fig. 9 shows that *TAT1* and *TAT2* transcripts are slightly more abundant in cells incubated with FK506. Thus, the immunosuppressive drug inhibits amino acid import posttranscriptionally.

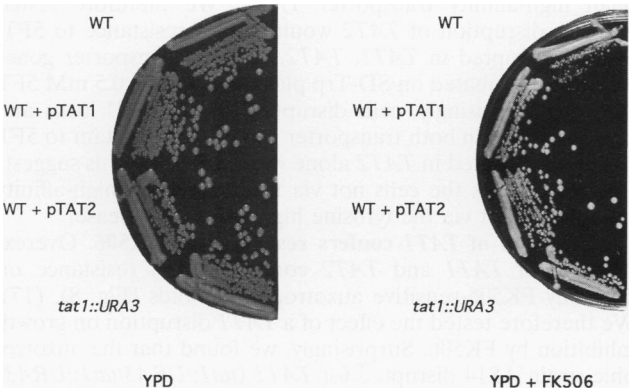

FIG. 8. Overexpression of *TAT1* and *TAT2* and disruption of *TAT1* confers resistance to FK506. Strain JK9-3da/α (wild type) transformed with either pTAT1 or pTAT2 or disrupted in *TAT1* (AS14) was streaked on YPD with and without 50 μg of FK506 per ml. Strains overexpressing *TAT1* or *TAT2* or disrupted in *TAT1* are resistant to FK506.

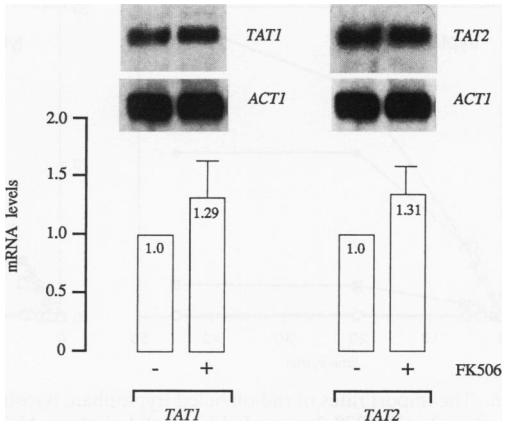

FIG. 9. *TAT1* and *TAT2* mRNA levels in JK9-3da/α (wild type) grown in YPD either treated or not treated for 5 h with 50 μg of FK506 per ml. *TAT1* and *TAT2* mRNA levels are slightly increased by treatment with FK506. Shown are arbitrary units. The bar graph results are averages of three independent experiments. The ratio of *TAT* expression in cells treated with FK506 to that in cells not treated with the drug was quantitated by scanning the autoradiogram. The ratio was internally controlled by normalizing the amount of *TAT* mRNA to levels of *ACT1* mRNA.

DISCUSSION

The immunosuppressive drug FK506 impairs the growth of the yeast *S. cerevisiae* by impeding the uptake of the amino acids tryptophan, histidine, and leucine and thus starving cells for these amino acids (17). Toxicity of the drug can be overcome by prototrophy for either one of these amino acids or by an excess of amino acids added exogenously to the growth medium. The inhibition of amino acid import by FK506 is not attributable to the binding of the drug to its known binding protein, the *cis-trans*-prolyl isomerase FKBP12, since amino acid transport is still FK506 sensitive in strains lacking this rotamase (17, 18). Thus, a second target, which is involved either directly or indirectly in the amino acid import machinery, must exist. Two novel genes, *TAT1* and *TAT2* (formerly called *TAP1* and *TAP2*), were isolated as genes conferring resistance to FK506 when present at increased levels. Here, we show that *TAT1* and *TAT2* encode novel members of the yeast amino acid permease family. TAT1 is a 619-amino-acid protein and is the tyrosine high-affinity permease that also mediates tryptophan uptake with low affinity or low capacity. TAT2 is a 593-amino-acid protein and mediates the high-affinity uptake of tryptophan. The evidence for these conclusions is as follows. First, disruption of *TAT1* is lethal only in combination with auxotrophy for tyrosine; cells that are unable to synthesize or import tyrosine are starved for this amino acid and therefore unable to grow. Disruption of *TAT2* results in only a slow-growth phenotype when combined with tryptophan auxotrophy. Thus, high-affinity uptake of tryptophan is mediated by TAT2 and a second, low-affinity or low-capacity transporter, responsible for the viability of *tat2*::*URA3 trp1* cells, must exist. Second, providing an excess of tryptophan to the growth medium restores normal cell growth of *tat2*::*URA3 trp1* cells. Third, the rate of import of labeled tyrosine and tryptophan is strongly decreased in cells lacking TAT1 and TAT2, respectively, compared with wild-type cells or with the uptake of other amino acids such as histidine. Fourth, 30 to 40% identity in the deduced amino acid sequence and the striking homology in the protein structure to the known yeast amino acid

transporters GAP1, CAN1, LYP1, PUT4, HIP1, and YCC5 strongly suggest that TAT1 and TAT2 are amino acid permeases. Fifth, the lethality of a *tat1::URA3 tat2::URA3* double disruption in combination with tryptophan auxotrophy and the finding that uptake of tryptophan is affected more severely in the doubly disrupted strain than in a strain lacking only TAT2 shows that TAT1 is involved in the low-affinity or low-capacity uptake of tryptophan. Furthermore, overexpression of *TAT1* is sufficient to overcome the slow-growth phenotype of *tat2::URA3 trp1* cells and restores normal cell growth.

The discovery of two tryptophan transporters, one mediating high-affinity uptake and the other mediating low-affinity or low-capacity uptake, conflicts with several reports stating that a specific transporter for tryptophan does not exist and that tryptophan uptake is mediated exclusively by the general amino acid permease GAP1 (11, 30, 48). Our findings that cells which lack TAT2 and are auxotrophic for tryptophan grow normally on proline medium, on which the general amino acid permease is active, support the previous reports that tryptophan is also a substrate for GAP1. However, since *gap1 trp3* double mutants are viable on YPD medium (32), tryptophan import must indeed occur by transport systems other than GAP1. Other groups have observed that a specific transport system for asparagine might also transport tryptophan, since asparagine uptake was found to be competitively inhibited by a 10-fold excess of tryptophan (12). The fact that *tat1::URA3 tat2::URA3 trp1* cells grow normally on SD complete medium containing a 50-fold excess of Trp (data not shown), although they are not viable on YPD and their uptake of tryptophan is almost completely abolished, argues that there might be a third transporter importing tryptophan with very low affinity. This transporter might be the asparagine transport system.

Very little is known regarding the uptake of tyrosine. GAP1 imports tyrosine (30). Furthermore, the low-affinity permease of histidine is competitively inhibited by tyrosine, suggesting that tyrosine is also a substrate for this transporter (7). It is unlikely that TAT1 is the histidine low-affinity permease, because the histidine low-affinity permease has been shown not to mediate transport of the other aromatic amino acids tryptophan and phenylalanine and because disruption of *TAT1* has no effect on histidine import.

The mechanism of action of FK506 on amino acid transporters is not yet known. However, we have shown that the immunosuppressant does not inhibit the transcription of the transporter genes *TAT1* and *TAT2*. Northern analysis revealed that the *TAT1* and *TAT2* mRNAs are slightly more abundant in cells treated with FK506. This suggests that the drug inhibits a posttranscriptional step required for the function of the amino acid permeases. FK506 might prevent the correct folding of amino acid transporters in the secretory pathway by binding to an unknown FKBP, whose isomerase activity is required for permease maturation. Alternatively, the drug might act on a component involved in the control of amino acid permeases. A third model suggests that FK506 affects the composition of the plasma membrane or the proton ATPases which generate the H^+ gradient providing the energy for amino acid import. All of these possibilities are discussed in greater detail in our previous paper (17), and none can be excluded yet.

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