Characterization of the Transport Mechanism and Permeant Binding Profile of the Uridine Permease Fui1p of *Saccharomyces cerevisiae* 

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The uptake of Urd into the yeast *Saccharomyces cerevisiae* is mediated by Fui1p, a Urd-specific nucleoside transporter encoded by the *FUI1* gene and a member of the yeast Fur permease family, which also includes the uracil, allantoin, and thiamine permeases. When Fui1p was produced in a double-permease knockout strain (fur4Δfui1Δ) of yeast, Urd uptake was stimulated at acidic pH and sensitive to the protonophore carbonyl cyanide m-chlorophenylhydrazone. Electrophysiological analysis of recombinant Fui1p produced in *Xenopus* oocytes demonstrated that Fui1p-mediated Urd uptake was dependent on proton cotransport with a 1:1 stoichiometry. Mutagenesis analysis of three charged amino acids (Glu\(^{259}\), Lys\(^{288}\), and Asp\(^{474}\) in putative transmembrane segments 3, 4, and 7, respectively) revealed that only Lys\(^{288}\) was required for maintaining high Urd transport efficiency. Analysis of binding energies between Fui1p and different Urd analogs indicated that Fui1p interacted with C(3')-OH, C(2')-OH, C(5)-H, and N(3)-H of Urd. Fui1p-mediated transport of Urd was inhibited by analogs with modifications at C-5', but was not inhibited significantly by analogs with modifications at C-3', C-5, and N-3 or inversions of configuration at C-2' and C-3'. This characterization of Fui1p contributes to the emerging knowledge of the structure and function of the Fur family of permeases, including the Fui1p orthologs of pathogenic fungi.

Nucleoside transporters are integral membrane proteins that mediate the uptake and release of naturally occurring nucleosides and cytotoxic nucleoside analogs (1–4). Mammalian nucleoside transporters are classified into two structurally unrelated protein families, the concentrative (CNTs)\(^6\) and equilibrative (ENTs) nucleoside transporters (1, 2, 5). Nucleoside permeation into *Saccharomyces cerevisiae* is mediated by Fui1p, a permease with high specificity for Urd and with no sequence similarities to any of the mammalian nucleoside transporters (6, 7). *S. cerevisiae* cells also salvage nucleobases through Fur4p (uracil permease) and Fyc2p (purine-cytosine permease), but they appear to lack the capacity to transport thymidine and purine nucleosides across plasma membranes (8). Although considerable information is available for the Fur4p and Fyc2p nucleobase transporters of *S. cerevisiae* (9–17), relatively little is known about Fui1p.

Fui1p belongs to the uracil/allantoin permease family (Fur family) of yeast, which also includes Fur4p, Thi10p (thiamine permease), and Dal4p (allantoin permease). Fui1p (629 amino acids, 72 kDa) shares high amino acid identity (50–60%) with the other family members. The predicted topology of Fur4p consists of 10 transmembrane (TM) segments with long N- and C-terminal tails, which have been shown to be intracellular (17). It is believed that the two-dimensional Fur4p structural model could be extended to all members of the yeast uracil/allantoin permease family (14). The similarity of amino acid sequences is greatest in the putative TM segments of the four proteins. Based on the high sequence identity of Fur4p and Fui1p, we hypothesized that these two transporters might have similar transport mechanisms and that Fui1p might operate as an electrogenic proton/permeant symporter. Charged amino acid residues in the membrane-spanning regions of transporters are known to play important roles in permeant binding (18, 19), proton coupling (20), transporter stability and activity (21), and plasma membrane targeting (22). Although Fur4p contains three charged amino acid residues in TM regions, only the one located in TM segment 4 (Lys\(^{272}\)), which is highly conserved in

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\(^{6}\)The abbreviations used are: CNTs, concentrative nucleoside transporters; ENTs, equilibrative nucleoside transporters; TM, transmembrane; h, human; ORF, open reading frame; GFP, green fluorescent protein; CMM, complete minimal medium; FurUrd, 5-fluorouridine; ChCl, choline chloride; MES, 4-morpholineethanesulfonic acid; CCCP, carbonyl cyanide m-chlorophenylhydrazone; MeUrd, methyluridine; ddUrd, dideoxyuridine.
the uracil/allantoin permease family, was identified as a critical residue involved in uracil binding and translocation (14). The functional importance of the corresponding lysine residue of Fui1p (Lys288) in TM segment 4 and of the only two other charged TM residues (in TM segments 3 and 7) was investigated in this study.

In vivo labeling of DNA using nucleosides and nucleoside analogs such as thymidine and 5-bromo-2′-deoxyuridine has long been a cornerstone of replication studies. *S. cerevisiae* has been used extensively as a model organism in defining the genetic elements required for DNA replication. In the absence of the introduction of heterogeneous nucleoside transporters (e.g., human (h) ENT1) (23), Fui1p is the dominant route that allows entry of nucleosides and nucleoside analogs into *S. cerevisiae*. Among the nucleoside transporters identified so far from bacteria to higher eukaryotes, only *S. cerevisiae* Fui1p mediates transport of 5-ductyluridine and uridine, implying a specialized function for Urd in *S. cerevisiae*. The abundance of Fur4p is determined by extracellular uracil availability by regulation of the efficiency of its ubiquitination (24). Fui1p has also been shown to be sorted for early vacuolar degradation in cells exposed to toxic levels of Urd, indicating that extracellular Urd controls Fui1p trafficking and prevents harmful Urd uptake that results in a decrease in growth rate (24). Knowledge of the transport mechanism and permeant selectivities of Fui1p will contribute to an understanding of its physiological significance in the budding yeast *S. cerevisiae*, one of the most important model organisms for DNA replication and repair studies.

Fui1p orthologs of *Candida albicans* and *Candida glabrata* with high sequence identities to Fui1p of *S. cerevisiae* (>70%) were revealed from contigs (groups of overlapping clones) of the Stanford *C. albicans* genome sequence data bank and the assembled open reading frame (ORF) data bank of the *C. glabrata* genome (GenBankTM GI:50287475) (25), respectively. One of the most commonly encountered human pathogens, *C. albicans* causes a wide variety of infections, ranging from superficial disorders in generally healthy individuals to invasive, rapidly fatal systemic infections in individuals with impaired immunity. *C. glabrata* has emerged as the second causative agent of human candidiasis worldwide and is more resistant to drug therapy than *C. albicans*. Few classes of drugs are effective against these fungal infections, and drug efficacy is limited by toxicity and side effects. Nucleoside antibiotics such as the nikkomycins and neopolyoxins have been considered as candidate inhibitors of opportunistic candidal infections in AIDS and organ transplant patients (26). Efforts to develop more effective nucleoside analogs are under way (27).

We report here the functional characterization of Fui1p in a double-permease knock-out yeast strain (fur4Δfui1Δ) that enabled us to analyze Fui1p-mediated Urd uptake in an otherwise nucleoside transport-free background. Fui1p transported Urd into yeast with high affinity and high capacity in a proton-dependent manner. The roles of three charged amino acid residues (Glu259, Lys288, and Asp474) in putative TM segments 3, 4, and 7, as well as the cellular location of the mutant transporters, were investigated using site-directed mutagenesis and green fluorescent protein (GFP) and c-Myc tags. Of the three charged residues, only Lys288 was important for the transport capacity of Fui1p. A quantitative inhibitor sensitivity assay was used to gain an understanding of the structural regions of Urd that interact with Fui1p. Because transportability is a potential determinant of the cytotoxic efficacy of nucleoside analog drugs, knowledge of the Urd binding profile of Fui1p will guide the design of novel antifungal nucleoside analogs that may selectively target Fui1p orthologs in pathogenic fungi.

**MATERIALS AND METHODS**

**Strains and Media**—BY4742-YBR021W (MATa, his3, leu2, lys2, ura3, fur4Δ), which contains a disruption in *FLU4*, the gene encoding the endogenous uracil permease, was purchased from the American Type Culture Collection (Manassas, VA) and used as the parental yeast strain to generate the double-permease knock-out strain fur4Δfui1Δ (previously named *fui1*:HIS3) by deleting *FLU1* using the PCR-mediated one-step gene disruption method as described previously (28). Other strains were generated by transformation of the yeast-*Escherichia coli* shuttle vector pYPGE15 (29) into fur4Δfui1Δ using a standard lithium acetate method (30).

Yeast strains were maintained in complete minimal medium (CMM) containing 0.67% yeast nitrogen base (Difco), amino acids (as required to maintain auxotrophic selection), and 2% glucose (CMM/Glc). Agar plates contained CMM with various supplements and 2% agar (Difco). Plasmids were propagated in *E. coli* strain TOP10F′ (Invitrogen) and maintained in Luria broth with ampicillin (100 μg/ml).

**Plasmid Construction**—All oligonucleotide primers were synthesized by Invitrogen. For *S. cerevisiae* expression, the *FLU1* ORFs were amplified from vector pYES2-FU11 (6) by PCR methodology using primers 5′-XbaI-FUI1 (5′-CTG TCT AGA ATG CCG GTA TCT GAT TCT GGA TTC-3′, with the restriction site underlined) and 3′-XhoI-FLUI1 (5′-CGA TCT GAG TTA GAT ATA TCG TAT CTT TTC ATG ACA-3′). For construction of c-Myc-tagged Fui1p, the c-Myc tag (CAG ATC TCT TCC TTA GAT GAG TTT TTT GTC) was introduced into primer 3′-XhoI-FU11. For *Xenopus* oocyte expression, primers 5′-BamHI-FU11 (5′-GTC GGA TCC ATG CCG GTA TCT GAT TCT GGA TTC-3′) and 3′-XhoI-FU11 (5′-CGA TCT AGA TTA GAT ATA TCG TAT CTT TTC ATG ACA-3′) were used. To construct GFP-tagged Fui1p, the ORF of *FLU1* without a stop codon was first amplified using forward primer 5′-XbaI-FU11 and a reverse primer containing (3′ to 5′) 21 bases with homology to *FLU1* and a unique tag sequence complementary to the first 50 nucleotides of the ORF of GFP. C-terminally GFP-tagged Fui1p was obtained by overlapping PCR using the product of the first run PCR and the pGFPuv vector (Promega, Madison, WI) as templates and 5′-XbaI-FU11 and 3′-KpnI-GFP (5′-CTG GGT ACC CTA TTT GAT GAG CTG ATC CAT GCG) as primers. GFP-ORF was also amplified by PCR using forward primer 5′-XbaI-GFP (5′-CGT TCT AGA ATG GCC AGC AAA GGA GTA TTT GAG TTT GAG GAG GAC TTC ATC CAT GCG) and reverse primer 3′-EcoRI-GFP (5′-CGT GAA TCC TTA TTT GAT GAG CCT ATC CAT GCG). The amplified ORFs were inserted into pYPGE15 (a high copy number episomal yeast vector that expresses the inserted DNA constitutively under the transcriptional control of the phosphoglycerate kinase promoter) to generate pYPFU11, pYP-
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FU11-GFP, and pYPGFp or into the Xenopus expression vector pGEM-HE to generate pGEFU1, pYPFU1-K288A, pYPFU1-K288E, pYPFU1-E259A, pYPFU1-D474A, and pYPFU1-E259A,D474A and the corresponding GFP- and c-Myc-tagged versions were generated using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The PCR products were using Pwo polymerase (Roche Applied Science), and all constructs were verified by DNA sequencing using an ABI PRISM 310 sequence detection system (PerkinElmer Life Sciences).

Nucleoside Transport in S. cerevisiae—The uptake of [3H]Urd or 5-[3H]fluorouridine (FUrd; Moravek Biochemicals Inc., Brea, CA) into logarithmically proliferating yeast cells was measured using a cell harvester as described previously (31, 32). Yeast cells containing pYPFU1, pYPFU1-GFP, or individual mutant transporters were grown in CMM/Glc to A_{600} = 0.7–0.9, washed twice with fresh medium, and resuspended to A_{600} = 4.0. Uptake assays were performed at room temperature at pH 4.5 unless specified otherwise by adding 50–μl portions of fresh suspensions to 50–μl portions of twice concentrated 3H-labeled nucleoside in CMM/Glc in 96-well microtiter plates. Yeast cells were collected on filter mats using a Micro96 cell harvester (Skatron Instruments, Lier, Norway) and rapidly washed with deionized water. The individual filter circles corresponding to wells of the microtiter plates were removed from filter mats and transferred to vials for scintillation counting.

The binding of Ur and its analogs to recombinant UFM1 was assessed by measuring their abilities to inhibit inward transport of 1 μM [3H]Urd in an “inhibitor sensitivity” assay as follows. Yeast cells producing UFM1 were incubated with 1 μM [3H]Urd for 30 s in the absence or presence of graded concentrations of Ur or Ur analogs. The 30-s exposures to [3H]Urd were shown in time course experiments to be sufficient to provide true initial rates of uptake into yeast cells, thereby providing rates of transport across plasma membranes rather than rates of intracellular metabolism (see Fig. 1, upper panel). Each experiment was repeated at least three times. Nonspecific radioactivity was determined in the presence of 10 mM nonradioactive Ur, and these values were subtracted from total uptake values. Data were subjected to nonlinear regression analysis using GraphPad Prism software (Version 3.0; GraphPad Software, Inc., San Diego, CA) to obtain IC_{50} values (concentrations that inhibited 50% Urd and Ur analogs. K_{i} (inhibitory constant) values were determined from the Cheng-Prusoff equation (33) and the K_{m} values for Ur. Gibb’s free energy (ΔG^0) was calculated from ΔG^0 = -RTln(K_{i}), where R is the gas constant and T is the absolute temperature. The thermodynamic stability of transporter-inhibitor complexes was quantitatively estimated from ΔG^0 as described (34).

Measurements of UFM1-induced H^+ Currents and H^+/Urd Coupling Ratios—pGEFU1 was linearized with NheI and transcribed with T7 polymerase using the mMESSAGE mMACHINETM transcription system (Ambion, Inc., Austin, TX). In vitro synthesized transcripts were injected into isolated mature stage VI oocytes from Xenopus laevis as described previously (35). Mock-injected oocytes were injected with water alone. Electrophysiological studies used transport medium in which choline was substituted for sodium, i.e. 100 mM choline chloride (ChCl), 2 mM KCl, 1 mM CaCl_2, 1 mM MgCl_2, and 10 mM HEPES (for pH values >6.5) or 10 mM MES (for pH values ≤6.5). Proton dependence was tested in ChCl-containing transport medium at pH 4.5–8.5.

Urd-induced membrane currents were measured in UFM1-producing oocytes at room temperature (20 °C) using a GeneClamp 500B oocyte clamp (Axon Instruments, Foster City, CA) in the two-electrode voltage-clamp mode as described previously (35) and interfaced to an IBM-compatible PC via a Digidata 1200A/D converter and controlled by pCLAMP software (Version 8.0; Axon Instruments). The microelectrodes were filled with 3 M KCl and had resistances that ranged from 0.5 to 2.5 MΩ megohms. Oocytes were penetrated with the microelectrodes, and their membrane potentials were monitored for periods of 10–15 min. Oocytes were discarded when membrane potentials were unstable or more positive than −30 mV. The oocyte membrane potential was clamped at a holding potential of −50 mV, and Urd was added in the appropriate transport medium. Current signals were filtered at 20 Hz (four-pole Bessel filter) and sampled at intervals of 20 ms. For data presentation, the signals were further filtered at 0.5 Hz by the pCLAMP program suite.

The H^+/Urd coupling ratio for UFM1 was determined by simultaneously measuring H^+ currents and uptake of [14C]Urd (200 μM, 1 μCi/ml; Amersham Biosciences) under voltage-clamp conditions. Individual UFM1-producing oocytes were placed in a perfusion chamber and voltage-clamped at a holding potential of −50 mV in sodium-free (100 mM ChCl) and nucleoside-free medium (pH 5.5) for a 10-min period to monitor baseline currents. When the base line was stable, the nucleoside-free medium was exchanged with medium of the same composition containing [14C]Urd. Current was measured for 2 min, and uptake was terminated by washing the oocyte with nucleoside-free medium until the current returned to the base line. The oocyte was then transferred to a scintillation vial and solubilized with 1% (w/v) SDS for quantitation of oocyte-associated radioactivity. Urd-induced current was calculated as the difference between base-line current and total inward current. The total charge translocated into the oocyte during the uptake period was calculated from the current-time integral and correlated with the measured radiolabeled flux for each oocyte to determine the charge/uptake ratio. Basal [14C]Urd uptake was determined in control water-injected oocytes (from the same donor frog) under equivalent conditions and used to correct for endogenous non-mediated nucleoside uptake over the same incubation period. Coupling ratios (±S.E.) were calculated from slopes of least-squares fits of Urd-dependent charge versus Urd accumulation in oocytes.

Confocal Microscopy of Yeast—Logarithmically growing yeast cells transformed with a GFP-tagged vector (10 µM, A_{600} = 0.7–0.9) were mixed with 30 µl of anti-fading mounting medium, smeared on a glass slide, and checked for green fluorescence using an excitation wavelength of 488 nm. Confocal images were collected using a Zeiss LSM510 confocal laser scanning microscope with a 63 × 1.4 objective (Plan-Apochromat) using a frame size of 512 × 512 pixels with a pixel resolution of 0.08 µm and a pixel depth of 8 bits.
Isolation of Plasma Membranes and Immunoblotting—Yeast membranes were fractionated on sucrose gradients as described (36). Briefly, 1 liter of yeast cells at $A_{600} = 1$ was collected, washed with sucrose breaking buffer (0.4 M sucrose, 1 mM EDTA, and 10 mM Tris (pH 7.4)) containing additional protease inhibitors (Complete protease inhibitor mixture, Roche Applied Science), and lysed by vortexing in the presence of glass beads (425–600 µm; Sigma) for 15 min at 4 °C. Unbroken cells and glass beads were removed from lysates by centrifugation at 500,000 $\times g$ for 20 min at 4 °C, and membrane fractions were obtained by centrifugation of lysates at 21,000 $\times g$ for 40 min at 4 °C. The resulting crude membrane pellets were resuspended in sucrose breaking buffer containing protease inhibitors. The crude membranes were layered onto a stepwise sucrose gradient (0.4, 1.1, 1.65, and 2.25 M sucrose) containing 10 mM Tris, 1 mM EDTA (pH 7.4), and protein inhibitor mixture. After centrifugation at 80,000 $\times g$ (Beckman SW 41 Ti rotor) for 14 h at 4 °C, fractions of band 3 from the top, which contained enriched plasma membranes, were collected and resuspended with sucrose breaking buffer containing protease inhibitors. After centrifugation at 21,000 $\times g$ for 90 min at 4 °C, the pellets were dissolved with sucrose breaking buffer, and the proteins present in the membranes were separated electrophoretically and analyzed by immunoblotting as described previously (31).

The primary antibodies used in immunoblotting were monoclonal antibodies against the c-Myc epitope tag (9E10; BAbCo, Richmond, CA), against Pma1 (plasma membrane marker; Abcam, Cambridge, MA), against Dpm1p (dolichol phosphate mannosyl synthase endoplasmic reticulum membrane marker; Invitrogen), and against the V-ATPase 100-kDa subunit (vacuole membrane marker; Invitrogen). The proteins were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences) and autoradiography on a film. The film was scanned, and the quantities of the proteins were evaluated using ImageQuant software (Version 5.2; GE Healthcare).

Urd Analog—The structures of Urd and its analogs were given previously (31). The Urd analogs used in this study were either obtained from R.I. Chemical, Inc. (Orange, CA) or synthesized as described previously (31). Stock solutions of test compounds were prepared in water or Me2SO (Sigma), and the final concentration of Me2SO in transport reactions was 0.1% when Me2SO was used as a solvent.

RESULTS

Transport Characteristics of Fui1p

The fur4Δfui1Δ yeast strain with or without pYPGE15 has no active uptake of Urd as described previously (28). At pH 4.5, the uptake of 1 µM [3H]Urd into fur4Δfui1Δ yeast containing pYPFU1 was rapid and linear over 90 s, with a mean rate (± S.E.) of 285 ± 5 pmol/mg of protein/s (Fig. 1, upper panel). This rate was reduced to 0.3 ± 0.1 pmol/mg of protein/s in the presence of 10 mM nonradioactive Urd, indicating the presence of functional Fui1p in yeast plasma membranes. Urd uptake rates were determined for all subsequent experiments using incubation periods of 30 s for recombinant Fui1p produced in yeast, thereby providing large signal-to-noise ratios while maintaining initial rates of uptake. FUrd is cytotoxic to S. cerevisiae (6, 7), and the evidence that its transport into yeast is mediated by Fui1p is based on the association of resistance to FUrd with the disruption of the FUI1 gene (7). The time course for uptake of 1 µM [3H]FUrd into fur4Δfui1Δ containing pYPFU1 was linear for at least 10 min (Fig. 1, lower panel), and its rate (4.0 ± 0.2 pmol/mg of protein/s) was the same as that observed during the first 120 s (inset), indicating that uptake intervals of 10 min provided initial rates, evidently because the transport step of the FUrd uptake process was rate-limiting. The uptake rate of FUrd was only 1.5% of that of Urd, indicating that FUrd was a poor permeant for Fui1p. Consistent with the results of a previous study (6), similar experiments with other [3H]-labeled nucleosides demonstrated that adenosine, inosine, guanosine, cytidine, and thymidine were not transported by Fui1p (data not shown).

Fui1p-mediated Urd uptake in yeast was strictly pH-dependent, with an optimum at pH 4–5, and the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP; 400 µM) strongly inhibited Urd uptake at every pH condition tested (Fig. 2A). Fui1p-mediated Urd uptake (pH 5.0) was sensitive to CCCP in a concentration-dependent manner, with a mean IC$_{50}$
FIGURE 2. pH and proton dependence of Urd uptake mediated by Fui1p. A, effects of pH and CCCP on Urd transport into yeast. The uptake of 1 μM [3H]Urd into fur4Δfui1Δ yeast producing Fui1p was measured over 30 s in transport medium at different pH values in the presence (□) and absence (■) of 400 μM CCCP. Each data point represents the mean ± S.E. of eight determinations; error bars are not shown where they are smaller than the symbol. B, inhibitory effects of CCCP on Urd transport mediated in yeast by Fui1p. fur4Δfui1Δ yeast cells transformed with pYFU1U were incubated with CCCP at graded concentrations for 5 min before initiation of the transport assay. The uptake of 1 μM [3H]Urd was measured over a 30-s incubation period in the presence and absence of CCCP. Uptake values in the presence of CCCP are given as the percentage of uptake values in the absence of CCCP. Each data point represents the mean ± S.E. of quadruplicate determinations; error bars are not shown where they are smaller than the symbol. Three or four independent experiments gave similar results; data from individual representative experiments are shown. C, proton currents induced in oocytes by exposure of recombinant Fui1p to Urd. Shown are averaged inward currents in Fui1p-producing oocytes perfused with 100 μM Urd in sodium-free (100 mM ChCl) transport medium at a membrane potential of −50 mV. Currents are the means ± S.E. of five different oocytes from the same batch of cells used on the same day. No currents were observed in control water-injected oocytes. D, stoichiometry of protons/Urd symport by recombinant Fui1p. A plot of charge versus [14C]Urd uptake was generated with 10 different Fui1p-producing oocytes in transport medium containing 100 μM ChCl and acidified to pH 5.5 at a membrane holding potential of −50 mV. Integration of the Urd-induced current was used to calculate the net cation influx (charge) and was fitted passed through the origin. The inset is a representative example of the current generated during application of [14C]Urd in a Fui1p-producing oocyte.

value (±S.E.) of 50 ± 4 μm (Fig. 2B), indicating that a proton gradient was required for Fui1p function.

The pH dependence of Fui1p was also studied in Xenopus oocytes producing recombinant Fui1p. Fig. 2C illustrates the effect of an imposed H+ gradient on Urd-induced proton currents mediated by Fui1p. Perfusion of Fui1p-producing oocytes with medium containing 100 μM Urd induced inward currents, the magnitude of which increased as the extracellular pH was lowered. Inward currents were not detected at pH 8.5, strongly supporting the conclusion that protons were transported with Urd. No currents were observed in control water-injected oocytes under any of the conditions tested.

The H+/nucleoside stoichiometry of FUI1 was directly determined by simultaneously measuring Urd-induced currents and [14C]Urd uptake under voltage-clamp conditions. The H+/nucleoside coupling ratio was determined in sodium-free medium (pH 5.5) at a membrane potential of −50 mV. The inset in Fig. 2D shows a representative Urd-dependent current recording in a Fui1p-producing oocyte. Each data point in Fig. 2D represents a single oocyte, and the H+/nucleoside ratio, given by the slope of the linear fit of charge (pmol) versus uptake (pmol), was 0.86 ± 0.09, indicating a proton/Urd stoichiometry of 1:1. Urd is metabolized only slowly by oocytes, mostly by phosphorylation into less permeable forms (37). It is therefore unlikely that the measured H+/nucleoside stoichiometry of Fui1p was influenced by flow of acid or base produced by addition of Urd. As evidence of this, the current recording in the inset in Fig. 2D returned to zero as soon as externally applied Urd was removed.

Mutational Analysis of Charged Amino Acid Residues in Putative TM Segments 3, 4, and 7

There are only three charged residues in the 10 TM segments in the topology model of Fui1p (38): glutamate, lysine, and aspartate at positions 259, 288, and 474, respectively. The hydrophobic locations of these charged residues suggested that they could be important in transporter structure and function. To determine the effects of mutations of these amino acids, site-directed mutagenesis was undertaken at Glu259, Lys288, and Asp474. All of the resulting mutants (Fui1p-K288A, Fui1p-K288E, Fui1p-K288R, Fui1p-D474A, and Fui1p-D474E) and their GFP- and c-Myc-tagged proteins remained functional, although some exhibited substantial changes in kinetic properties. The apparent K max and V max values for Urd transport by yeast producing wild-type or mutant Fui1p were determined by measuring initial uptake rates at increasing concentrations of [3H]Urd (Fig. 3). In all cases, Urd uptake conformed to simple Michaelis-Menten kinetics (Fig. 3, upper panel). Wild-type Fui1p showed high apparent affinity and capacity for Urd uptake (K m = 10.8 ± 0.9 μM and V max = 2500 ± 60 pmol/mg of protein/s, mean ± S.E., n = 3). The apparent K m of Fui1p reported here was lower than that reported previously (20 μM) (6), probably because the contribution to Urd uptake by the uracil permease Fur4p was eliminated in this study by using the double-permease knock-out strain fur4Δfui1Δ.

Replacement of either or both of the acidic glutamate and
aspartate residues at positions 259 and 474 of Fui1p, respectively, with a neutral alanine residue had relatively small effects on the kinetic parameters of uptake (Fig. 3 and Table 1), indicating that these residues in TM segments 3 and 7, respectively, were not critical for Urd uptake. In contrast, the replacement of lysine at position 288 with alanine produced a mutant (Fui1p-K288A) with a significantly reduced $V_{\text{max}}$ value compared with that of wild-type Fui1p, although the apparent $K_m$ values of wild-type and mutant Fui1p were similar. The resulting transport efficiency ($V_{\text{max}}/K_m$) was only 12% of that of wild-type Fui1p. This result also confirmed that the uptake kinetics represented those of the transport process rather than metabolism because the only difference between the two yeast strains was the alteration of a single amino acid residue in Fui1p. To determine whether replacement of the positive charge of the lysine residue was responsible for the reduced transportability, further site-directed mutagenesis was carried out to analyze the function of Lys$^{288}$. Replacement of this residue with positively charged arginine or negatively charged aspartate produced Fui1p-K288R and Fui1p-K288E, respectively, both of which displayed kinetic properties similar to those of Fui1p-K288A (Fig. 3 and Table 1). This result suggested that Lys$^{288}$ was evidently critical for maintaining the high transport efficiency of Fui1p but not for proton binding and/or translocation, a conclusion that was supported by the results obtained when Urd uptake was measured in buffers at different pH values in that none of the Lys$^{288}$ mutants showed altered pH dependence of uptake (data not shown). Urd uptake by Fui1p-E259A, Fui1p-D474A, or the Fui1p-E259A,D474A double mutant (with the negative charges removed from both residues 259 and 474) also exhibited strong dependence on pH, with optimal uptake into yeast occurring at pH 5.0 (data not shown), indicating that Glu$^{259}$ and Asp$^{474}$ were also not required for proton binding and/or translocation.

**Cellular Resistance to FUrd by Yeast Producing Wild-type Fui1p or Its Mutants**

Yeast cells are sensitive to FUrd, and disruption of the $FUI1$ gene results in resistance to FUrd cytotoxicity (7). To determine whether any of the $FUI1$ mutations altered sensitivity to FUrd, $fur4$ $fui1$ yeast cells producing either wild-type Fui1p or one of the Fui1p mutants were exposed to graded concentrations of FUrd. Similar growth patterns were observed for all situations in the absence of FUrd (Fig. 4). When $fur4$ $fui1$ yeast cells that contained the insert-free vector (pYPGE15) were plated on solid medium containing graded concentrations of FUrd (Fig. 4), growth was observed at the highest concentration tested (100 $\mu$M), whereas growth of $fur4$ $fui1$ yeast producing wild-type Fui1p, Fui1p-E259A, or Fui1p-D474A was inhibited at the lowest concentration tested (5 $\mu$M). No growth was observed in $fur4$ $fui1$ yeast producing the Fui1p-E259A,D474A double mutant at 5 $\mu$M FUrd (data not shown). When $fur4$ $fui1$ yeast cells were transformed with plasmids containing inserts encoding any of the three Lys$^{288}$ mutants, resistance to FUrd was

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**TABLE 1**

Kinetic properties of Urd transport by wild-type and mutant Fui1p

| Fui1p          | Apparent $K_m$ (M) | Apparent $V_{\text{max}}$ (pmol/mg/s) | $V_{\text{max}}/K_m$ (normalized to protein abundance) |
|----------------|--------------------|---------------------------------------|-------------------------------------------------------|
| Wild-type      | 10.8 ± 0.9         | 2500 ± 60                             | 232                                                   |
| E259A          | 9.2 ± 1.5          | 2100 ± 94                             | 228                                                   |
| K288A          | 7.8 ± 1.1          | 210 ± 28                              | 27                                                    |
| K288R          | 12.2 ± 1.5         | 380 ± 41                              | 31                                                    |
| K288E          | 13.5 ± 2.0         | 390 ± 37                              | 29                                                    |
| D474A          | 9.1 ± 1.3          | 2200 ± 88                             | 241                                                   |
| E259A,D474A    | 7.9 ± 1.8          | 2210 ± 92                             | 280                                                   |

*The $K_m$ and $V_{\text{max}}$ values were determined using GraphPad Prism software by nonlinear regression analysis. Representative plots for Urd transport by Fui1p, Fui1p-K288A, Fui1p-K288R, Fui1p-E259A, Fui1p-D474A, and Fui1p-E259A,D474A are shown in Fig. 3. The $K_m$ and $V_{\text{max}}$ values shown are the means ± S.E. of three separate experiments. The normalized $V_{\text{max}}/K_m$ values were obtained by dividing the $V_{\text{max}}/K_m$ values by the corresponding relative abundance values as determined by immunoblot analysis as illustrated in Fig. 5 (B and C).*

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**FIGURE 3.** Kinetic properties of Fui1p and Fui1p mutants. The mediated component of Urd transport (uptake rates of $[^3]$H]Urd at particular Urd concentrations minus uptake rates at those concentrations in the presence of 10 mM nonradioactive Urd) was plotted as a function of concentration (upper panel) and subsequently converted to $v$ versus $v/s$ plots (lower panel) to determine the kinetic constants for wild-type Fui1p (□), Fui1p-E259A (○), Fui1p-K288A (◇), Fui1p-K288E (▲), Fui1p-K288R (●), Fui1p-D474A (△), and Fui1p-E259A,D474A (▼) using GraphPad Prism software. The lower panel is the Eadie-Hofstee plot of the data presented in the upper panel. Each value is the mean ± S.E. of nine determinations, and error bars are not shown where they were smaller than the data points. Each curve represents one of three identical experiments that gave qualitatively similar results.
shown in Fig. 5, Fui1p proteins did not change their transport properties. As untagged versions (data not shown), indicating that addition of Fui1p-GFP proteins displayed kinetic parameters that were was due to changes in localization. Wild-type and mutant values for Urd in comparison with wild-type Fui1p, the GFP tag detection of GFP- and c-Myc-tagged Fui1p and Fui1p Mutants

Because some of the Fui1p mutants exhibited reduced $V_{\text{max}}$ values for Urd in comparison with wild-type Fui1p, the GFP tag was used to determine whether impaired transporter capacity was due to changes in localization. Wild-type and mutant Fui1p-GFP proteins displayed kinetic parameters that were within 98–104% of those obtained for the corresponding untagged versions (data not shown), indicating that addition of the GFP tag to the C-terminal end of the wild-type and mutant Fui1p proteins did not change their transport properties. As shown in Fig. 5A, yeast producing Fui1p mutants exhibited fluorescence patterns similar to those of wild-type Fui1p, which showed plasma membrane localization and intracellular accumulations. The intracellular patterns varied from bright fluorescent areas, which were likely due to retention of overexpressed proteins in vacuoles for degradation (24), to fluorescent circles, which were likely due to the presence of the proteins in intracellular membranes. Diffuse green fluorescence was observed in yeast transformed with pYPGFP, and no fluorescence was observed in yeast transformed with the insert-free vector (pYPGE15) (Fig. 5A, lower panels).

Considering that GFP is relatively large and therefore may itself contribute to the retention of the tagged protein in intracellular compartments, Fui1p and its mutants were also C-terminally tagged with c-Myc and quantified by immunoblotting. c-Myc-tagged Fui1p and its mutants exhibited kinetic properties that were within 96–103% of those obtained for the corresponding untagged versions (data not shown), indicating that the c-Myc tag did not change the transport properties of Fui1p or its mutants and could therefore be used for the quantification studies described below.

Yeast plasma membranes were isolated, fractionated, and analyzed by immunoblotting (Fig. 5, B and C). Four major bands were collected after equilibrium density centrifugation on sucrose gradients and subjected to immunoblotting (Fig. 5B). When the abundance of Pma1 (a plasma membrane marker) was compared with that of Dpm1p and V-ATPase (endoplasmic reticulum and vacuolar membrane markers, respectively), it was evident that band 3 contained enriched fractions of plasma membranes (Fig. 5B) with relatively small quantities of intracellular membranes (Fig. 5C). The band densities of c-Myc-tagged Fui1p and its mutants were digitized by densitometry using ImageQuant software and normalized to the band densities of the corresponding Pma1 (loading controls). The value of the ratio of the density of the c-Myc-tagged transporter to that of the corresponding Pma1 represented the abundance of c-Myc-tagged Fui1p or its mutants. The “relative abundance” of each of the Fui1p mutants was expressed as the ratio of its abundance to that of c-Myc-tagged wild-type Fui1p, which was taken as 1 (Fig. 5D). With the exception of a large increase in Fui1p-K288A, the Fui1p mutants exhibited small differences in abundance relative to that of c-Myc-tagged wild-type Fui1p. When the $V_{\text{max}}/K_m$ values of Fui1p and its mutants were normalized to their corresponding relative abundance levels (shown in Fig. 5D), all three Lys288 mutants exhibited $<10\%$ of the transport efficiency of wild-type Fui1p, whereas the other mutants exhibited transport efficiencies that were similar to that of wild-type Fui1p (Table 1), confirming a critical role of Lys288 in Fui1p function.

Interaction of Urd Analogos with Fui1p

To gain an understanding of the structural regions of Urd that interact with Fui1p, inhibitor sensitivity assays using Urd analogs with modifications of the base and/or sugar moieties were used as described previously in studies of recombinant human transporters produced in yeast (28, 31, 39). The inhibition of initial rates of Urd uptake (i.e., the transport step in the uptake process) was assumed to be competitive because (i) the inhibitors tested were close structural analogs of Urd, and (ii) Fui1p was most likely to be the only plasma membrane protein that interacted with the potential inhibitors. Representative concentration-effect curves of some of the analogs for inhibition of Urd- mediated Urd transport are shown in Fig. 6. In all cases, the Hill coefficients of the concentration-effect curves were close to $-1$ (mean $\pm$ S.E. of $-0.9 \pm 0.2$), indicating a single binding site for the inhibitors. The mean $K_i$ values ($\pm$ S.E.) and the corresponding Gibbs free energy values are listed in Table 2.

Base Modifications—There appeared to be strong interactions between Fui1p and C-5 of Urd because addition of a substituent with different sizes at C-5 resulted in $K_i$ values of $>3$ mM and decreases of $>12.9$ kJ/mol in $\Delta G^o$ values. Because 2′-dUrd is a low affinity inhibitor of Urd uptake, the inability of 5-fluoro-2′-dUrd, 5-bromo-2′-dUrd, 5-iodo-2′-dUrd, 5-ethyl-2′-dUrd, and thymidine to inhibit Urd uptake was likely due primarily to modifications at C-5. Position 3 of the base moiety...
FIGURE 5. Localization and protein abundance of GFP- or c-Myc-tagged wild-type Fui1p and its mutants produced in yeast. A, plasma membrane and intracellular locations of GFP-tagged wild-type Fui1p and its mutants. Representative fur4Δfui1Δ yeast cells producing GFP alone (control) or GFP-fused Fui1p, Fui1p-E259A, Fui1p-K288A, Fui1p-K288R, Fui1p-D474A, or Fui1p-E259A,D474A (not shown) were immobilized on glass slides using polylysine and viewed under a confocal microscope. EV, empty vector (pYPGE15). B, immunoblotting to detect enrichment of plasma membranes. Membranes from fur4Δfui1Δ yeast producing c-Myc-fused Fui1p-E259A or Fui1p-E259A,D474A were subjected to fractionation by continuous sucrose gradient centrifugation as described under "Materials and Methods," and four major fractions were isolated and subjected to immunoblotting using monoclonal antibodies against Pma1, Dpm1p, and the V-ATPase 100-kDa subunit. C, immunoblotting to detect c-Myc-tagged Fui1p and its mutants in yeast. Plasma membranes were isolated as described under "Materials and Methods" from fur4Δfui1Δ yeast producing Fui1p or c-Myc-fused Fui1p, Fui1p-E259A, Fui1p-K288A, Fui1p-K288R, Fui1p-D474A, or Fui1p-E259A,D474A. Plasma membrane preparations (10 μg of protein/sample) were subjected to SDS-PAGE, after which the proteins were transferred to polyvinylidene fluoride membranes that were subjected to immunoblotting with monoclonal antibodies against c-Myc, Pma1, Dpm1p, and the V-ATPase 100-kDa subunit. Results were similar for three independent experiments, only one of which is shown. The molecular mass of each protein is indicated. D, relative abundance of c-Myc-tagged Fui1p and its mutants in isolated yeast plasma membranes. The bands detected by immunoblotting as illustrated in B were quantified using ImageQuant software (Version 5.2). The band densities of c-Myc-tagged Fui1p mutants were normalized to those of the corresponding Pma1 proteins, and the resulting values (representing the abundance of each of the c-Myc-tagged mutants) are presented as a ratio relative to the abundance of c-Myc-tagged wild-type Fui1p. The means ± S.E. of average protein abundance (n = 3) were determined with GraphPad Prism software.
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Inhibition of recombinant Fui1p-mediated Urd uptake by some Urd analogs. The uptake of 1 μM [3H]Urd into fur4AΔfui1Δ yeast producing Fui1p was measured over a period of 30 s in the presence of graded concentrations of nonradioactive Urd or Urd analogs. IC50 values (mean ± S.E., n = 3–4) were determined using GraphPad Prism software (Version 3.0) and were converted to Ki values (33) using a Ki (mean ± S.E., n = 3) of 10.8 ± 0.9 μM for recombinant Fui1p. Gibbs free energy (ΔG′) was calculated from ΔG′ = −RTln(Ki).

| Urd analog compounds | Ki (μM) | ΔG′ (kcal/mol) | δ(ΔG′) (kcal/mol) |
|----------------------|---------|----------------|-------------------|
| Urd                  | 10.2 ± 0.4 | 26.1          | 0                 |

**Base modifications**
- 5-Bromo-Urd: >3000
- FlUrd: >3000
- 5-Iodo-Urd: >3000
- 5-MeUrd: >3000
- 3-MeUrd: >3000
- 3-Deaza-Urd: >3000

**Sugar modifications**
- 2′-dUrd: 520 ± 57, 17.2, 8.9
- 3′-dUrd: >3000, <13.2, >12.9
- 5′-dUrd: 2.7 ± 0.7, 29.1, >3.0
- 1-(β-D-Arabinofuranosyl)uracil: >3000
- 1-(β-D-Xylofuranosyl)uracil: >3000
- 2′,3′-ddUrd: >3000
- 2′,5′-ddUrd: 144 ± 36, 20.1, 6.0
- 3′,5′-ddUrd: >3000
- 2′-O-MeUrd: >3000, <15.8, >8.0
- 3′-O-MeUrd: >3000
- 5′-O-MeUrd: 13.6 ± 3.5, 25.4, 0.7
- 2′-Azido-2′-dUrd: >2000
- 3′-Azido-3′-dUrd: >2000
- 5′-Azido-5′-dUrd: 14.4 ± 0.9, 25.3, 0.8
- 5′-Chloro-5′-dUrd: 2.3 ± 0.3, 29.4, −3.3
- 2′,3′-O-Isopropylidene-Urd: >3000

**Base and sugar modifications**
- 3′-Azido-3′-deoxythymidine: >3000
- 5-Bromo-2′-dUrd: >3000
- 5-Ethyl-2′-dUrd: >3000
- 5-Fluoro-2′-dUrd: >3000
- 5-Iodo-2′-dUrd: >3000
- Thyminedine: >3000
- 5-Fluoro-5′-dUrd: 438 ± 47, 17.6, 8.5

The inhibitory effects of these Urd analogs were tested at 1 or 10 μM in our previous study (6). In the previous study (6), yeast cells were preincubated with the Urd analog (1 or 10 μM) for 20 min before the addition of [3H]Urd, whereas in this study, the Urd analogs and [3H]Urd were added simultaneously as described under "Materials and Methods." This difference in the experimental conditions explains why greater inhibition of some Urd analogs was observed in the previous study than in this study.

*Inhibition of <30% was observed.

No inhibition was observed.

(N(3)-H) also contributed a recognition determinant for Fui1p/ Urd interactions because 3-methyluridine (MeUrd) did not inhibit Urd uptake (Kf > 3 μM). There was a difference of >12.9 kJ/mol in binding energy relative to the ΔG′ of Urd, suggesting loss of hydrogen bonding. Hydrogen bonding between Fui1p and position 3 of the base was further supported by the effect of the change of N(3)-H to C(3)-H, which is not a donor for hydrogen bonding; 3-deaza-Urd yielded a Kf of >3 μM.

Sugar Modifications—The C(3′)-OH group appeared to be a critical determinant for high affinity binding of Urd by Fui1p because its removal (2′-dUrd) produced a difference of 8.9 kJ/mol in ΔG′, with a 51-fold increase in Kf, suggesting that hydrogen bonding could be important. Any further modifications at C-2′, e.g. substitution of an azido group for a hydrogen atom at C-2′ of 2′-dUrd (2′-azido-2′-dUrd), addition of a methyl group (2′-O-MeUrd), or inversion of the orientation of the hydroxyl group (1-(β-D-arabinofuranosyl)uracil), dramatically reduced interactions with Fui1p (apparent affinities for 2′-azido-2′-dUrd, 2′-O-MeUrd, and 1-(β-D-arabinofuranosyl) uracil of Kf > 3 μM).

The C(3′)-OH group also appeared to interact strongly with Fui1p because its removal (2′-dUrd) yielded Kf values of >3 μM, with losses of >12.9 kJ/mol in ΔG′. Although 2′-dUrd inhibited Fui1p-mediated Urd uptake, the additional removal of the C(3′)-OH group (2′,3′-dideoxyuridine (ddUrd)) abolished the inhibitory effects. The contribution of the C(3′)-OH group as a recognition determinant for binding to Fui1p was also apparent from the effects of substitution of an azido or O-methyl group at these positions; Fui1p-mediated Urd uptake remained unchanged in the presence of high concentrations of 3′-azido-3′-dUrd or 3′-O-MeUrd. 3′-Azido-3′-dideoxythymidine failed to inhibit Fui1p-mediated Urd uptake. Although Fui1p strongly bound Urd with the C(3′)-OH group below the
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Sugar ring plane, its affinity for 1-(β-D-xylofuranosyl)uracil, an epimer of Urd with the C(3′)-OH group oriented above the plane of the sugar ring, was markedly reduced (K_i > 3 mM). The inverted orientation of the hydroxyl group evidently produced an analog that could no longer interact with Fui1p. Similarly, 2′,3′-O-isopropylidine-Urd failed to inhibit Urd uptake, as was also observed with 2′,3′-ddUrd.

C-5′ of Urd was not required for binding to Fui1p because 5′-ddUrd and 5′-chloro-5′-ddUrd were both potent inhibitors, with somewhat lower K_i values than Urd itself (t test, p < 0.05). Fui1p displayed relatively high affinities for 5′-azido-5′-ddUrd and 5′-O-MeUrd (Fig. 5), supplying further evidence that the C(5′)-OH group was not essential for Fui1p/Urld interactions. Although Fui1p exhibited a low apparent affinity for 2′-ddUrd (K_i = 520 ± 57 μM), the additional removal of the C(40)-OH group partially restored the affinity for Fui1p (2′,5′-ddUrd, K_i = 144 ± 36 μM). The slightly higher binding energy of 2′-ddUrd (ΔG° = -2.9 kJ/mol relative to the ΔG° of 2′-ddUrd) was evidently due to energy gained by removal of the hydroxyl group at C-5′, resulting in an analog that has a better fit in the binding pocket of Fui1p. Also, inhibition of Fui1p-mediated Urd transport with FUrd was barely detectable, whereas relatively high concentrations of 5-fluoro-5′-ddUrd (K_i = 438 ± 47 μM) inhibited Urd uptake.

DISCUSSION

Fui1p is the primary permease involved in the uptake of nucleosides into the yeast S. cerevisiae (39). The uracil permease Fur4p exhibits weak transportability for Urd.7 Compared with other nucleoside transporters identified so far, Fui1p exhibits highly specific permeant selectivity. As a high affinity, high capacity transporter for Urd, Fui1p has also been suggested to mediate the uptake of the cytotoxic nucleoside analog FUrd and was initially discovered because a mutant selected for resistance to FUrd had lost the capacity to import Urd as well as FUrd (7). We undertook this study to characterize the transport mechanism and to identify structural determinants of Urd that are important for binding to Fui1p.

Results from experiments with both yeast and oocytes producing wild-type Fui1p suggested a simple proton/Urd cotransport mechanism. The optimal Urd transportability under acidic conditions and the sensitivity to proton uncoupling by CCCP suggested that Fui1p functions through a secondary active transport process. Two-electrode voltage-clamp analysis of oocytes producing recombinant Fui1p showed that proton currents induced by Urd were stimulated and inhibited by externally acidic and alkaline pH environments, respectively, and that the H+/Urd coupling ratio was 1:1. In yeast and plants, protons are likely to be the preferred coupling ions for nutrient transport, and the proton electrochemical gradient is maintained by plasma membrane H+/ATPase (41, 42). Utilization of the transmembrane proton gradient to energize active transport has been demonstrated for other members of the Fur family of transporters (15, 43), as well as for ENTs of parasitic protozoa (3) and CNTs of C. albicans and C. elegans (44, 45). In contrast, mammalian CNTs function predominantly as Na+/nucleoside symporters, although hCNT3 is also able to utilize protons (46). The H+/ATPase from S. cerevisiae is known to be essential for intracellular pH regulation, nutrient uptake, and cell growth (42). H+/ATPase activity determines cellular H+ extrusion, which might affect extracellular proton concentrations and thus regulate the rate of Urd uptake mediated by Fui1p. One of the mechanisms of growth control by H+/ATPase in yeast may be modulating the uptake of extracellular nutrients, including Urd.

Highly conserved charged residues within TM segments of transporter proteins are likely candidates for involvement in binding and translocation of ions. According to the topology model for the Fur family (17), the predicted TM segments of Fur4p contain three charged amino acid residues (Glu243, Lys272, and Glu289 in putative TM segments 3, 4, and 9, respectively) that are highly conserved in other family members, of which only the lysine residue was shown to be important for activity (14). In this study, the kinetic properties of the three conserved charged amino acid residues in putative TM segments 3, 4, and 7 of Fui1p were characterized in mutagenesis and transport assays. The two negatively charged residues (Glu259 and Asp74 in TM segments 3 and 7, respectively) were not essential for Fui1p function because mutation to alanine had no effect on transport activity. In addition, neither Glu259 nor Asp747 appeared to be involved in the sensing or binding of protons because removal of the negative charge separately or in tandem did not change the proton dependence of Urd transport. However, although mutation of the conserved lysine (Lys288 in TM segment 4 of Fui1p) to positively charged, negatively charged, or neutral residues resulted in similar affinities for Urd, it resulted in greatly decreased V_max values for Urd transport. In contrast, mutation of the corresponding lysine (Lys272) in TM segment 4 of Fur4p changed both the binding and translocation of uracil (14).

The results of the GFP and c-Myc tagging studies established that the decreases in the transport capacity of the Fui1p Lys288 mutants were not due to decreased abundance of the mutant proteins in yeast plasma membranes relative to wild-type Fui1p. The GFP-tagged mutant proteins also had similar cellular distribution patterns. Thus, Lys288 per se was important for Urd transport capacity. Kcat/Km is a measure of catalytic efficiency, and Kcat = V_max/[E]_T, where [E]_T is the amount of transporter. [E]_T of the Fui1p Lys288 mutants was not reduced compared with that of wild-type Fui1p. Replacement of lysine at position 288 with arginine, glutamate, or alanine affected Urd transport efficiency similarly, suggesting that this lysine, which lies toward the extracellular aspect of putative TM segment 4 of Fui1p, plays a key role in Urd translocation. The effects of these substitutions were further demonstrated by the FURd cytotoxicity profiles of the Lys288 mutants. Yeast producing the Lys288 mutants survived in medium containing low FUrd concentrations, which killed yeast producing either Fui1p or its Glu259 and/or Asp747 mutants. In the presence of higher concentrations of FUrd (e.g. 100 μM), the Lys288 mutants were also killed, indicating that the Lys288 mutants were functional but had reduced transport capacities.

Structural regions of the Urd molecule involved in binding to Fui1p were probed by analysis of inhibition profiles and binding energies as described previously (31, 47). These regions were identified as C(2′)-OH, C(3′)-OH, C-5′, and N(3′)-H. The loss of >12.9 kJ/mol of Gibbs free energy when the C(3′)-OH and

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7 J. Zhang and C. E. Cass, unpublished data.
N(3)-H groups were modified suggested that these groups were involved in hydrogen bonding with Fui1p. Fui1p also exhibited low affinities for Urd analogs with C-5 modifications. Removal of the C(2')-OH group resulted in a loss of 8.9 kJ/mol of binding energy. The C-5' region in the sugar moiety did not appear to be required for Urd binding because modifications at this position did not cause substantial losses in binding energy. The unchanged (or even increased with both C-2' and C-5' modifications) affinities for Urd analogs with C-5' substituents indicated that this position could be modified without seriously affecting binding to Fui1p.

The Urd binding profiles of Fui1p have both similarities and distinct differences compared with those of the human members of the ENT and CNT families of nucleoside transporters (Fig. 7). The importance of the 3'-hydroxyl group for binding interactions with hCNTs and hENTs, which are structurally unrelated proteins, is well established (31, 39, 48, 49). In this work, the 3'-hydroxyl was also identified as a critical functional group for nucleoside binding to Fui1p. Any changes at C-3', including removal of the hydroxyl group or inversion of its configuration, dramatically altered the Fui1p/Urd analog interactions. C-2', which plays a minor or moderate role in Urd interactions with hCNT and hENT proteins, was involved in strong interactions with Fui1p. Interestingly, Fui1p had no major interactions with C(5')-OH, whereas hCNTs and hENTs interact with C(5')-OH to various degrees (28, 31, 39). These results suggest that new Urd analogs might be designed to specifically target fungal nucleoside transporters.

In summary, Fui1p of *S. cerevisiae* was demonstrated to be an
electrogenic transporter with a H$^+$/Urd coupling stoichiometry of 1:1. Fui1p mediates the high affinity transport of Urd and the low but significant transport of the cytotoxic nucleoside analog FUrd. Studies of the Urd analog binding profiles of Fui1p revealed substantial differences from those of the human nucleoside transporters (hCNTs and hENTs). This raises possibilities for the design and application of Urd analog drugs with cytotoxic substituent(s) that would be transported differentially by fungal and human nucleoside transporters. Of the three charged residues in the membrane-spanning regions of Fui1p, only the lysine at position 288 was found to be important for transport of Urd. Because all of the Lys$^{288}$ mutants were found to be targeted to yeast plasma membranes with minimal changes in abundance, the severe impairment of transport capacity likely resulted from defects in the transmembrane regions. Studies of the Urd analog binding profiles of Fui1p revealed substantial differences from those of the human nucleoside transporters. The functional consequences of these differences imply that in vivo drug transport across the fungal plasma membrane will be different from drug transport across the human plasma membrane.

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