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Isolation and Characterization of a *Saccharomyces cerevisiae* Peptide Transport Gene

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We have cloned and characterized a *Saccharomyces cerevisiae* peptide transport gene (*PTR2*) isolated from a genomic DNA library by directly selecting for functional complementation of a peptide transport-deficient mutant. Deletion and frameshift mutageneses were used to localize the complementing activity to a 3.1-kbp region on the transforming plasmid. DNA sequencing of the complementing region identified an open reading frame spanning 1,803 bp. The deduced amino acid sequence predicts a hydrophobic peptide consisting of 601 amino acids, having a molecular mass of 68.1 kDa, composed in part of 12 hydrophobic segments, and sharing significant similarities with a nitrate transport protein encoded by the *CHL1* gene of *Arabidopsis thaliana*. Northern (RNA) hybridization experiments demonstrated a single transcript that was 1.8 kb in length and that was transiently induced by the addition of L-leucine to the growth medium. The *PTR2* gene was localized to the right arm of chromosome XI by contour-clamped homogeneous electric field gel chromosome blotting and by hybridization to known chromosome XI λ phage clones of *S. cerevisiae* DNA. *PTR2* was tightly linked to the *UBI2* gene, with the coding sequences being separated by a 466-bp region and oriented so that the genes were transcribed convergently. A chromosomal disruption of the *PTR2* gene in a haploid strain was not lethal under standard growth conditions. The cloning of *PTR2* represents the first example of the molecular genetic characterization of a eucaryotic peptide transport gene.

Peptide uptake is a general physiological phenomenon that is known to occur in bacteria, fungi, and mammalian cells (for reviews, see references 8, 21, 22, and 41) and that is operationally defined as the process by which individual cells are able to transport intact peptides across the plasma membrane. Peptides may be used as nutritional sources of amino acids, as carbon or nitrogen sources, and as precursors of cell wall peptides during bacterial growth (17). Peptide transport has been studied to the greatest extent in the enteric bacteria *Escherichia coli* and *Salmonella typhimurium* and to a lesser extent in the fungi *Saccharomyces cerevisiae* and *Candida albicans*.

Peptide uptake in the gram-negative bacteria *S. typhimurium* and *E. coli* is mediated by three genetically distinct yet physiologically overlapping systems. The oligopeptide permease (Opp) system of *E. coli* and *S. typhimurium* is encoded by five genes, *oppA*, *oppB*, *oppC*, *oppD*, and *oppF*, which map to a single genetic locus comprising an operon (4, 24, 27). The Opp system is capable of transporting peptides up to 5 amino acids long without specificity with regard to amino acid side-chain composition (53, 54). In contrast, the tripeptide permease (Tpp) system of *S. typhimurium* is limited to transporting di- and tripeptides and is encoded by *tppA* and *tppB*, which map at different loci on the *S. typhimurium* chromosome (16). The Tpp system has an apparent affinity for hydrophobic peptides, and *tppB* is inducible by exogenous leucine or under anaerobic conditions (16, 33). The third system is the dipeptide permease (Dpp) system of *S. typhimurium*, which preferentially transports dipeptides (1). Mutations at the *dpp* locus have been mapped to a single site on the *S. typhimurium* chromosome that differs from the known *opp* and *tpp* loci (1). The apparent lack of a high degree of amino acid side-chain specificity among the various peptide transport systems in bacteria has permitted the development of antibiotics in which an otherwise nonpermeating toxic moiety is attached to a peptide backbone which gains entrance into the cell via one of the above-mentioned transport systems (3, 14, 18, 22).

Functionally similar peptide transport systems have been reported for *C. albicans* and *S. cerevisiae*, although the number and specificity of the systems operative in these organisms are less clear (for reviews, see references 8 and 47). In *C. albicans*, a variety of peptides are transported across the plasma membrane in an energy-dependent manner (12, 38, 42), with little apparent amino acid side-chain specificity (37). As with *E. coli* and *S. typhimurium*, this broad substrate specificity immediately suggests the possibility of using peptide transporters as a means to facilitate the uptake of otherwise nonpermeating molecules that may be of medical significance as antifungal compounds. Indeed, the polyoxins and nikkomycins, two types of peptidyl nucleoside antibiotics, utilize the peptide transport pathway to enter *C. albicans* (6, 42, 44, 70). On the basis of various studies, there appear to be at least two peptide transport systems operative in *C. albicans* (5, 42, 45, 55, 70).

Peptide transport in *S. cerevisiae* is also characterized by the ability to transport small peptides across the plasma membrane in an energy-dependent manner, with little demonstrable side-chain specificity (8). Wild-type *S. cerevisiae* is highly sensitive to a variety of toxic dipeptides containing amino acid analogs, such as ethionine and DL-*m*-fluorophenylalanine (30). Furthermore, peptide transport in *S. cerevisiae* appears to be under nitrogen control (7, 46) and is inducible by a variety of amino acids (30). At the genetic

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level, the process has been shown to be mediated by three genes: *PTR1*, *PTR2*, and *PTR3* (31). *S. cerevisiae* mutants deficient in peptide transport are characterized as being resistant to the growth-inhibiting effects of toxic dipeptides while remaining sensitive to the corresponding amino acid analogs. In addition, peptide transport-deficient mutants harboring *ptr1* or *ptr2* alleles are not able to accumulate radiolabeled dipeptides and are not able to utilize a variety of defined dipeptides as sources of amino acids in satisfying auxotrophic growth requirements.

A detailed understanding of peptide transport can be gained from the molecular and biochemical characterization of the gene products controlling this phenomenon. Here we describe a one-step method for cloning peptide transport genes from a variety of organisms and also describe the isolation, nucleotide sequencing, chromosomal disruption, expression, and physical mapping of the *PTR2* gene of *S. cerevisiae*.

MATERIALS AND METHODS

Media and strains. Yeast strains were routinely maintained on YEPG medium, containing (on a weight/volume basis) yeast extract (1%), peptone (2%), and glucose (2%), or synthetic medium with amino acids and bases as specified elsewhere (65). Minimal medium (MM) was composed of 0.67% yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories, Detroit, Mich.), 1.0 mg of allantoin per ml, and 2.0% glucose. MM was supplemented with amino acids as required (65). Dipeptide medium (Leu-Leu, Lys-Leu, Lys-Lys, and His-Leu) consisted of MM supplemented with auxotrophic requirements minus the amino acid components of the added dipeptides. Solid media contained 2.0% agar. *E. coli* strains and plasmid-bearing derivatives were routinely maintained on LB medium supplemented, when necessary, with the appropriate antibiotic (61). All plasmids were routinely maintained and propagated in *E. coli* HB101 [F^- *hsd20* (r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 rpsL209* Str^r *xyl-5 mtl-s supE44* λ^-].

S. cerevisiae S288C (*MATα SUC2 mal mel gal2 CUP1*) was obtained from the Yeast Genetic Stock Center (Berkeley, Calif.). Yeast strains PB1X-9B (*MATa ura3-52 leu2-3 lys1-1 his4-38 ptr2-2*), PB1X-2A (*MATa ura3-52 leu2-3,112 lys1-1 his4-38*), and PB1X-2AΔ (*MATa ura3-52 leu2-3 lys1-1 his4-32 ptr2::LEU2*) were constructed in our laboratory by standard procedures (65). Yeast strain XL70-1B (*MATα ura3-52 leu2-3 lys1-1 his4-38 trp1-289*) was constructed and supplied by Frank W. Larimer (Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.).

Enzymes and biochemicals. Restriction endonucleases, T4 DNA ligase, the DNA polymerase I large fragment (Klenow) of *E. coli*, and T4 DNA polymerase were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), New England BioLabs (Beverly, Mass.), Stratagene (La Jolla, Calif.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used according to the manufacturer's specifications. All dipeptides used in this study were purchased from Sigma Chemical Co. (St. Louis, Mo.), except for L-alanyl-L-ethionine, oxalysine-containing peptides, and radioactive dileucine, which were synthesized by standard solution-phase techniques (48). Kits used for DNA sequencing and random primer radiolabeling of DNA probes were purchased from United States Biochemicals (Cleveland, Ohio). Sequencing primers were purchased from Genosys Biotechnologies (The Woodlands, Tex.) or the University of Tennessee Molecular Biology Resource Facility (Knoxville). GeneClean kits, used for small-scale DNA isolation preparations, were purchased from Bio 101 (La Jolla, Calif.). pZ523 columns, used in removing chromosomal DNA from plasmid preparations, were purchased from 5 Prime 3 Prime (Paoli, Pa.). Nylon membranes, used in nucleic acid hybridization experiments, and [^{32}P]dCTP and [^{35}S]dATP, used in hybridization and sequencing experiments, were purchased from Dupont, NEN Research Products (Boston, Mass.). XAR-5 film, used in autoradiography, was purchased from Eastman Kodak Co. (Rochester, N.Y.).

Growth assays. Growth assays were performed to assess the ability of yeast strains to use various dipeptides as sources of amino acids in meeting auxotrophic requirements. Strains were grown in YEPG broth at 30°C to a titer of 10^8 cells per ml. The cells were harvested by centrifugation, washed twice with sterile water, and resuspended at 1.0×10^7 cells per ml. A 5-μl aliquot was applied to solid MM containing dipeptides and amino acids as required. Four types of solid media were routinely used in assessing dipeptide utilization: MM+L-leucyl-L-leucine+L-lysine+L-histidine, MM+L-lysyl-L-lysine+L-leucine+L-histidine, MM+L-lysyl-L-leucine+L-histidine, and MM+L-histidyl-L-leucine+L-lysine. All dipeptides were present at a concentration of 80 μM, while amino acids and nucleotide bases were added as required at standard concentrations (65). The plates were incubated at 30°C for 48 h and then scored for growth.

Disk assays. Sensitivity to toxic peptides was measured by disk assays as previously described (30). Cells were grown overnight on MM with supplements and suspended in sterile water at 5.0×10^7 cells per ml. Portions (0.1 ml) were added to 1.1% Noble agar (3 ml), kept molten at 50°C, and plated on MM (10 ml) supplemented with the appropriate amino acids. A total of 0.38 μmol of toxic peptide was added to a sterile paper disk, which was then placed on the surface of a plate. The plates were incubated at 30°C for 48 h, and then zones of inhibition were measured. Each test comprised at least two independent assays, and the results represented means of the values obtained. The maximum variation between the zones of inhibition for each test was ≤2 mm.

Radiolabeled dipeptide uptake assays. The procedure for performing radiolabeled dipeptide uptake experiments has been described elsewhere (30) and was used, with minor modifications, to assess the ability of yeast strains to accumulate radiolabeled dipeptides. Yeast cells were grown in MM supplemented with the required amino acids to 0.5×10^7 to 1.0×10^7 cells per ml. The cells were collected by centrifugation, washed twice with cold sterile distilled water, and resuspended in 2% glucose at a concentration of 2.0×10^8 cells per ml. A 0.5-ml aliquot was incubated at 30°C for 10 min and then mixed with an equal volume of 2% glucose–40 mM sodium citrate-potassium phosphate buffer (pH 5.5)–0.16 mM L-leucyl-L-[^{3}H]leucine (specific activity, 9.4 mCi/mmol). Aliquots of 0.18 ml were removed at various times, and the cells were filtered immediately on GN-6 filters (pore size, 0.45 μm). The filters were washed twice with 5 ml of cold sterile distilled water. The filtered cells were then placed in scintillation vials with 5 ml of Budget-solve solution (Research Products International Corp., Mt. Prospect, Ill.) and counted.

Transformations and DNA manipulation techniques. Bacteria were transformed by the $CaCl_2$ method (19) and selected on solid LB medium supplemented with 50 μg of ampicillin per ml. Yeast strains were routinely transformed by the LiCl method (32) and selected on MM containing histidine, lysine, and leucine. The medium used in directly

selecting Ptr$^+$ transformants was composed of MM supplemented with 10 mM L-lysyl-L-leucine and 1.3 mM L-histidine. Plasmid DNA was isolated from *E. coli* by the alkaline lysis method (61). Yeast DNA was prepared by the guanidine hydrochloride method (28). Restriction endonuclease analysis, DNA ligation, and agarose gel electrophoresis were performed as previously described (61). Large-scale plasmid preparations were passed over pZ523 columns to remove contaminating chromosomal DNA (71).

The *PTR2* gene was cloned by functional complementation of the peptide transport-deficient phenotype. Yeast strain PB1X-9B was transformed with a YCp50-based *S. cerevisiae* genomic DNA library obtained from the American Type Culture Collection (59) (ATCC 37415). Bacterial cells were resuspended in 5 ml of LB broth and plated on LB plates containing 50 μg of ampicillin per ml. Approximately 10,000 clones were harvested, and the plasmid DNA was isolated by the alkaline lysis method (61). pZ523 columns were used to remove contaminating chromosomal DNA (71).

Chromosome preparations and blotting. Chromosome hybridization analysis was used to localize the *PTR2* gene to a specific *S. cerevisiae* chromosome. *S. cerevisiae* S288C cultures were grown to a titer of 1×10^8 to 2.0×10^8 cells per ml in YEPG broth for chromosome isolation by the agarose bead entrapment method as previously described (51). The chromosomes were fractionated on 1.0% agarose with a Bio-Rad Laboratories CHEF-DR II gel apparatus, Pulsewase 760 switcher, and model 200/2.0 power supply. The chromosomes were electrophoresed at 14°C for 15 h at 200 V with a switching time of 60 s and then for 9 h at 200 V with a switching time of 90 s. The chromosomes were transferred to a nylon hybridization membrane and then UV cross-linked. The chromosome blots were probed with a ^{32}P-radiolabeled 1.64-kbp *Bam*HI-*Eco*RI DNA fragment of the cloned *PTR2* gene from plasmid pJP9 (Fig. 1). The radiolabeled probe was prepared by the random primer method with a commercially available kit (United States Biochemicals). The prehybridization and hybridization procedures used were those specified by the membrane manufacturer for standard DNA-DNA hybridization experiments. In addition, the *PTR2* gene was also mapped by probing a series of three grid filters that contained 880 λ phage clones of *S. cerevisiae* genomic DNA representing approximately 82% of the *S. cerevisiae* genome (λ clone grids were supplied by L. Riles and M. Olson, Department of Genetics, Washington University School of Medicine). The λ phage clone inserts of yeast DNA were previously mapped by physical means and thereby provide an independent confirmation of chromosome assignment.

Gene disruption. Chromosomal disruption of the *PTR2* gene was performed by one-step gene disruption (50). A 2.0-kbp DNA fragment carrying the *LEU2* gene of *S. cerevisiae* was excised as a *Bam*HI-*Hin*dIII DNA fragment from plasmid pJJ283 (34). The fragment was isolated from an agarose gel, treated with T4 DNA polymerase, and then blunt-end ligated into a 653-bp deleted region of the *PTR2* protein coding region of plasmid pJP15. Plasmid pJP15 was constructed by cloning a 1.64-kbp *Bam*HI-*Eco*RI DNA fragment from plasmid pJP9 into plasmid pRS3062. This 1.64-kbp fragment contains a DNA sequence that includes 495 bp of the promoter region and 1,147 bp of the protein coding region of the *PTR2* gene. Plasmid pRS3062 is a derivative of plasmid pRS306 (66) in which a unique *Aat*II restriction endonuclease site has been removed by digestion with *Aat*II, treatment with T4 DNA polymerase, and religation. The deleted region of plasmid pJP15 was generated by restriction endonuclease digestion with *Aat*II and *Msc*I, resulting in the excision of a 653-bp region that is contained within the *PTR2* protein coding region. The ends of the deleted pJP15 molecule were made blunt with T4 DNA polymerase and ligated with the blunt-ended *LEU2* DNA fragment. The resulting plasmid, pJP23(II), was recovered in *E. coli*, and the structure was confirmed by restriction endonuclease analysis. Plasmid pJP23(II) contains the *LEU2* gene oriented so that the direction of transcription opposes that of *PTR2*. A 2.6-kbp *Nde*I-*Dra*I DNA fragment containing the inserted *LEU2* gene flanked by *PTR2* DNA sequences was excised and used as a substrate in transforming the Ptr2$^+$ strain PB1X-2A.

Leu$^+$ yeast transformants were selected on MM medium supplemented with uracil, L-lysine, and L-histidine. The resulting Leu$^+$ yeast transformants were patched onto fresh medium and then tested for the ability to use L-lysyl-L-leucine as a source of lysine in growth experiments and for sensitivity to L-alanyl-L-ethionine in disk assays. A single yeast transformant displaying the Leu$^+$ Ptr$^-$ phenotype, designated PB1X-2AΔ, was considered for further characterization. The integration event at the *PTR2* locus was confirmed by Southern transfer hybridization analysis (61). Whole-cell DNA was isolated from stationary-phase cultures of PB1X-2A and PB1X-2AΔ by the guanidine hydrochloride method (28). Aliquots of each DNA sample, containing approximately 5 μg of DNA, were digested with restriction endonuclease *Eco*RI. The digests were fractionated on a 0.9% agarose gel (2× TAE [0.04 M Tris-acetate, 0.001 M EDTA; pH 8.0] buffer) and transferred to a nylon hybridization membrane by capillary action as described previously (61). The nucleic acids were UV cross-linked, prehybridized, and hybridized according to the instructions provided by the membrane manufacturer. A ^{32}P-radiolabeled DNA probe was prepared by the random primer method. The DNA template used in the random primer reaction was prepared by polymerase chain reaction (PCR) amplification of a 1.2-kbp region of plasmid pJP9. The primers used in the PCR amplification reactions, 5′-CACCACAACTCATA TC-3′ (P1) and 5′-CCGATTGGTCTTTGAATG-3′ (P2), hybridize at nucleotide positions −270 and 902, respectively. The resulting radiolabeled probe was complementary to a region of the wild-type *PTR2* gene that was contained within a 4.4-kbp *Eco*RI-generated DNA fragment. However, the disrupted *PTR2* locus was expected to yield two *Eco*RI-generated DNA fragments capable of hybridizing with the radiolabeled probe, with complementarity extending to a 395-bp region on a 4.4-kbp DNA fragment and a 124-bp region on a 1.4-kbp DNA fragment. The PCR protocol that was used to generate the probe template has been described elsewhere (29).

DNA sequence analysis. The nucleotide sequence of the *PTR2* gene was determined by use of a Sequenase kit purchased from United States Biochemicals. The Sequenase kit is based on the dideoxynucleotide termination method (62). Sequencing primers were purchased from Genosys Biotechnologies or the University of Tennessee Molecular Biology Resource Facility. Both strands of *PTR2* were sequenced to fully confirm the order of bases. Computer-assisted searches and analyses of nucleotide sequence data and protein structure were performed with the GCG sequence analysis software package (13), the BLAST algorithm (2), and the GenBank-EMBL data base.

RNA experiments. *S. cerevisiae* S288C was grown overnight in 1 liter of MM to a titer of 2.0×10^7 cells per ml. A 100-ml portion of cells was removed, and total RNA was

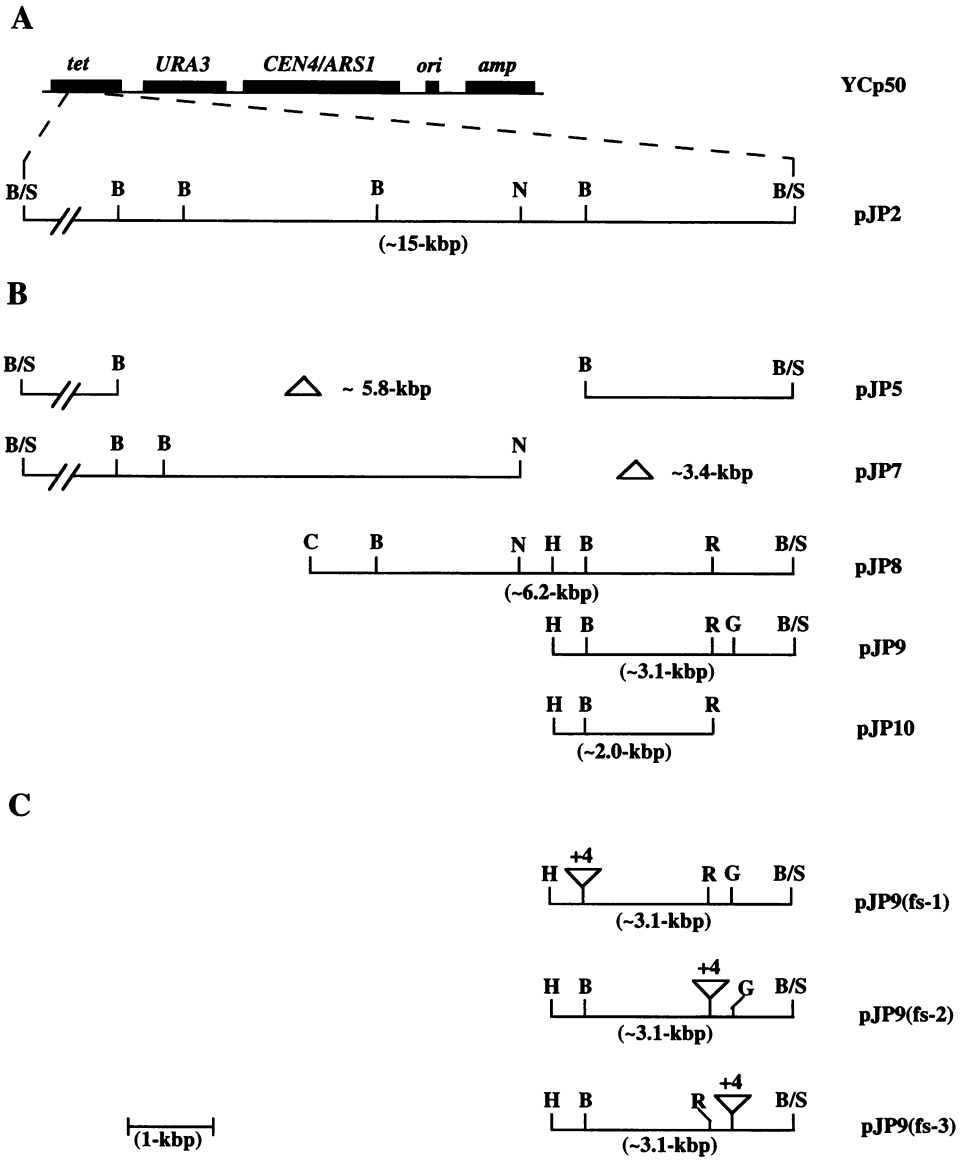

FIG. 1. Plasmid structures. Restriction endonuclease sites are indicated as follows: B, *Bam*HI; C, *Cla*I; R, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; N, *Nru*I; B/S, *Bam*HI-*Sau*3A junction site. (A) Parent plasmids YCp50 and pJP2. *tet*, *amp*, and *ori* are elements derived from plasmid pBR322 which encode tetracycline resistance, ampicillin resistance, and the origin of replication for plasmid pBR322, respectively. The *URA3* and *CEN4/ARS1* elements are derived from *S. cerevisiae*. The broken lines indicate the splice sites for the *PTR2* DNA insert, designated plasmid pJP2. (B) Deletion clones and subclones of pJP2. (C) Frameshift mutations constructed in plasmid pJP9. Plasmids pJP9(fs-1), pJP9(fs-2), and pJP9(fs-3) carry a +4 frameshift mutation at the unique *Bam*HI, *Eco*RI, and *Bgl*II sites of plasmid pJP9, respectively. All of the above-listed plasmids were maintained as YCp50-based constructions.

immediately isolated (63). L-Leucine was added at a final concentration of 150 μM to the remaining 900-ml culture, which was then returned to a 30°C shaker. Additional 100-ml aliquots were removed from the culture at time intervals of 10, 20, 30, 40, 50, 60, and 120 min and then processed in the same manner as the initial aliquot. Total RNA was isolated by the sodium dodecyl sulfate (SDS)-phenol-chloroform method as previously described (63) and quantitated by UV spectroscopy (61). Twenty-five micrograms of total RNA per sample was incubated at 70°C for 10 min in a reducing buffer composed of 20 mM morpholinepropanesulfonic acid (MOPS) (pH 7.0), 8 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde (pH 7.0), and 50% deionized formamide and then quickly cooled on dry ice-ethanol for 5 min. Samples were fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde and 1× MOPS (61) and then vacuum transferred to a nylon hybridization membrane (BioTrans; ICN Biomedicals, Inc., Irvine, Calif.). The RNA was UV cross-linked with a Stratalinker UV source as specified by the manufacturer (Stratagene). The Northern (RNA) blot was then probed with a ^{32}P-radiolabeled *PTR2* DNA probe. The *PTR2* probe was generated by the random primer method. The DNA template used in the random primer reaction was prepared by PCR amplification of a 1.2-kbp region of plasmid pJP9 as described above. Similarly, the membrane was also probed with a ^{32}P-radiolabeled *ACT1* DNA probe. The *ACT1*

gene of *S. cerevisiae* encodes actin (15), and the transcription of *ACT1* mRNA was used as an internal control in the current transcription studies. The *ACT1* DNA template used in the labeling reaction was prepared by PCR amplification of a 0.92-kbp region of the *ACT1* gene of *S. cerevisiae*. The primers used in the PCR amplification of the *ACT1* probe were 5'-GCCGGTTTTGCCGGTGACGAC-3' and 5'-GGAA GATGGAGCCAAAGCGGTG-3'. The PCR protocol that was used to generate the probe template has been described elsewhere (29). The UV-cross-linked nylon membrane was prehybridized for 3 h at 42°C in a solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM KPO_4 (pH 7.4), 250 μg of denatured salmon testis DNA (Sigma) per ml, and 5× Denhardt's solution. The prehybridization solution was removed and replaced with a hybridization solution, composed of 50% dextran sulfate and fresh prehybridization solution (1:4 [vol/vol]). The DNA probes were incubated at 100°C for 5 min and then quickly cooled on ice before addition to the blot at 10^7 cpm of total radioactivity. The blot was hybridized at 42°C for 17 h, washed twice in 1× SSC–0.1% SDS at 42°C for 15 min and twice in 0.1× SSC–0.1% SDS at 65°C for 15 min, and exposed to XAR-5 film (Kodak) for 20 h at −70°C with intensifying screens.

The resulting autoradiogram was digitally translated by use of a QuickCapture card (Data Translation, Inc., Marlboro, Mass.) and a DAGE-MTI 65 video camera fitted with a Nikon AF Micro Nikkor 55-mm lens. The digitized image was captured and analyzed with a digital image-processing program (Image 1.28; NTIS, Bethesda, Md.). Background and band intensities were measured, and peak intensities were calculated after background subtraction. Average peak heights were calculated for both the actin and the *PTR2* genes by averaging relative peak intensities over a window of 25 datum points. All actin gene peak heights were set equal to one another, and the *PTR2* gene peak heights were adjusted accordingly and plotted as the relative intensity of *PTR2*/actin gene transcripts versus time.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been entered in the GenBank data base and assigned accession number L11994.

RESULTS

Isolation of the *PTR2* gene by functional complementation. The *PTR2* gene of *S. cerevisiae* was isolated by functional complementation of the dipeptide transport-deficient ($Ptr2^-$) phenotype. Yeast strain PB1X-9B (*MATa ura3-52 leu2-3 lys1-1 his4-38 ptr2-2*) was transformed with a YCp50-based genomic DNA library harboring 10- to 15-kbp inserts. The cells were plated on solid MM supplemented with 1.3 mM L-histidine and 10 mM L-lysyl-L-leucine. The histidine served to induce the peptide transport system (30) and to meet the auxotrophic requirement conferred by the *his4-38* mutation present in the host strain. L-Lysyl-L-leucine served as a source of lysine and leucine in those yeast transformants capable of transporting the dipeptide. Two yeast transformants, designated PB1X-9B(pJP1) and PB1X-9B(pJP2), were recovered after incubation at 30°C for 5 days.

Whole-cell DNA was isolated from both primary yeast transformants, and the transforming plasmids were subsequently amplified and recovered in *E. coli* HB101. Plasmid DNA was isolated from the resulting bacterial transformants and partially characterized with restriction enzymes. Both plasmids, pJP1 and pJP2 (Fig. 1), yielded identical restriction patterns when digested with *Pst*I and *Bam*HI. Plasmid pJP2 was then reintroduced into PB1X-9B. The resulting secondary yeast transformants were tested for the ability to use L-leucyl-L-leucine, L-lysyl-L-lysine, L-lysyl-L-leucine, and L-histidyl-L-leucine as sources of amino acids and for sensitivity to the toxic dipeptide L-alanyl-L-ethionine. The secondary yeast transformants were able to utilize all dipeptides tested as sources of amino acids and were sensitive to the growth-inhibiting effects of L-alanyl-L-ethionine (Table 1). In addition, PB1X-9B(pJP2) was able to transport radiolabeled dileucine at levels approximating those of the wild type, while the control strain, PB1X-9B(YCp50), failed to accu-

TABLE 1. Phenotypes of *S. cerevisiae* transformants

Plasmid[a]	Result[b] in the presence of:				
	Leu-Leu	Lys-Lys	Lys-Leu	His-Leu	Ala-Eth
pJP2	+	+	+	+	−
pJP5	−	−	−	−	R
pJP7	−	−	−	−	R
pJP8	+	+	+	+	S
pJP9	+	+	+	+	S
pJP10	−	−	−	−	R
YCp50	−	−	−	−	R
pJP9(fs-1)	+	+	+	+	S
pJP9(fs-2)	−	−	−	−	R
pJP9(fs-3)	−	−	−	−	R

[a] All pJP plasmids are described in Fig. 1. Plasmid YCp50 is the yeast-*E. coli* shuttle vector, described in Materials and Methods.

[b] *S. cerevisiae* PB1X-9B transformed with various plasmids was assayed on dipeptide medium as described in Materials and Methods. +, presence of growth on the medium; −, absence of growth. Ala-Eth medium was MM supplemented with all auxotrophic requirements and alanyl-ethionine at 80 μM. S, the transformant was sensitive to the toxic effect of Ala-Eth; R, the transformant was resistant.

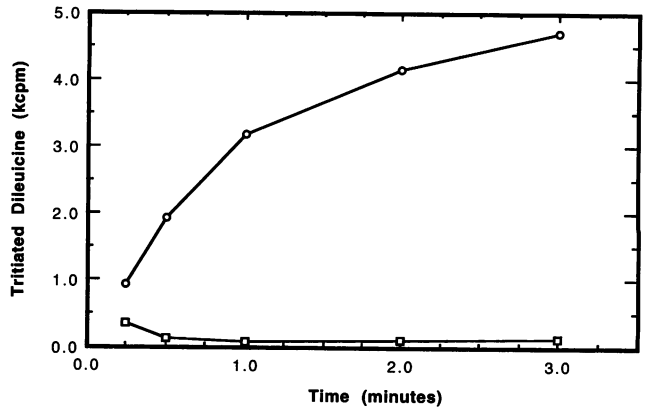

FIG. 2. Uptake of radioactive dileucine by *S. cerevisiae* transformants. *S. cerevisiae* PB1X-9B was transformed with plasmids YCp50 (□) and pJP2 (○). The host strain, PB1X-9B, carries the *ptr2-2* peptide transport mutant allele. Yeast cells were grown in MM supplemented with the required amino acids to 0.5×10^7 to 1.0×10^7 cells per ml. The cells were collected by centrifugation, washed twice with cold sterile distilled water, and resuspended in 2% glucose at a concentration of 2.0×10^8 cells per ml. A 0.5-ml aliquot was incubated at 30°C for 10 min and then mixed with an equal volume of 2% glucose–40 mM sodium citrate-potassium phosphate buffer (pH 5.5)–0.16 mM L-leucyl-L-[^{3}H]leucine (specific activity, 9.4 mCi/mmol). Aliquots of 0.18 ml were removed at various times, and the cells were filtered immediately on GN-6 filters (pore size, 0.45 μm). The filters were washed twice with 5 ml of cold sterile distilled water. The filtered cells were then placed in scintillation vials with 5 ml of scintillation solution and counted.

TABLE 2. Sensitivity of *S. cerevisiae* transformants to ethionine, oxalysine, alanyl-ethionine, and oxalysine-containing peptides[a]

S. cerevisiae transformant[b]	Zone of inhibition (mm)[c] in the presence of:						
	E	AE	O	OG	OLG	$O(L)_2G$	$O(L)_3G$
PB1X-2A(YCp50) (*PTR2*)	45	42	None	28	19	None	None
PB1X-9B(pJP9) (*ptr2-2*)	46	44	None	32	21	None	None
PB1X-9B(YCp50) (*ptr2-2*)	45	None	None	None	None	None	None

[a] Disk sensitivity assays were done as described in Materials and Methods with MM supplemented with histidine at 0.12 mM, lysine at 0.21 mM, and leucine at 0.18 mM.

[b] *S. cerevisiae* PB1X-9B (*ptr2-2*) and *S. cerevisiae* PB1X-2A (*PTR2*), which also have other auxotrophic requirements, are as described in Materials and Methods. The plasmids used to transform the strains are given in parentheses. Plasmid YCp50 is a shuttle vector which contains *S. cerevisiae URA3* as the selectable marker and is maintained at a low copy number; pJP9 is plasmid YCp50 containing the *S. cerevisiae PTR2* gene.

[c] Ethionine (E) or L-alanyl-L-ethionine (AE) (0.38 μmol) or oxalysine (O) or oxalysine-containing peptides (OG, O-Gly; OLG, O-Leu-Gly; $O(L)_2G$, $O-(Leu)_2$-Gly; $O(L)_3G$, $O-(Leu)_3$-Gly) (0.25 μmol) were spotted on a disk (diameter, 6 mm). None, no growth inhibition. Each test comprised two independent assays, and the results are means of the values obtained. The maximum variation between the zones of inhibition for each test was ≤2 mm.

mulate the radiolabeled substrate (Fig. 2). These results show that the transformant had the expected wild-type phenotype and that the *ptr2-2*-complementing activity is plasmid associated and is not due to a reversion event at the *ptr2* locus.

Localization of the *ptr2-2*-complementing region. To more precisely define the *ptr2-2*-complementing region of pJP2, a variety of internal deletions within the cloned insert DNA were constructed (Fig. 1). The deletions were constructed by digestion of plasmid pJP2 with the appropriate restriction endonuclease(s) and then ligation under conditions favoring intramolecular recircularization. The ligation products were recovered in *E. coli* HB101, characterized with regard to restriction fragment patterns, and then reintroduced into mutant yeast strain PB1X-9B. The resulting yeast transformants were examined for growth on a variety of dipeptides and for sensitivity to L-alanyl-L-ethionine (Table 1). The *ptr2-2*-complementing region was localized to a 3.1-kbp region adjacent to the *Sau*3A-*Bam*HI insert-vector junction proximal to *URA3* on plasmid pJP2. A comparison of the growth results conferred by plasmids pJP5, pJP9, and pJP10 (Table 1) allowed one functional endpoint to be localized within a 350-bp *Hin*dIII-*Bam*HI DNA fragment. The complementing region extends toward the insert-vector junction and past an *Eco*RI site proximal to the *URA3* gene.

To further aid in defining the functional limits of the *ptr2-2*-complementing region, frameshift mutations were introduced at three sites within the cloned region of plasmid pJP9 (Fig. 1). Frameshift mutations were introduced at the *Bam*HI site (fs-1), the *Eco*RI site (fs-2), and the *Bgl*II site (fs-3) of pJP9 by cleavage with the appropriate restriction endonuclease, blunt ending with the large fragment of *E. coli* DNA polymerase I (Klenow fragment), and ligation. The resulting plasmids were recovered in *E. coli*, and the frameshift mutations were confirmed by the loss of the appropriate restriction site. Each plasmid harboring a frameshift mutation was then used to transform mutant yeast strain PB1X-9B. The resulting yeast transformants were assayed for the ability to grow on dipeptides and for sensitivity to L-alanyl-L-ethionine (Table 1). Plasmid pJP9(fs-1) had no discernible effect on the Ptr^+ phenotype; however, plasmids pJP9(fs-2) and pJP9(fs-3) both abolished complementing activity. These results indicate that the *Eco*RI and *Bgl*II sites lie within the region required for complementing activity, while the *Bam*HI site probably lies within a regulatory region, given that deletions bordering the *Bam*HI site fail to complement the *ptr2-2* mutation while a frameshift mutation at the *Bam*HI site has no apparent effect.

Plasmid pJP9 was the smallest plasmid constructed that fully complemented the peptide transport-deficient phenotype conferred by the *ptr2-2* mutation. To demonstrate that full activity was restored by this plasmid, quantitative disk assays were performed to assess the sensitivity of the transformant to L-alanyl-L-ethionine and a series of oxalysine-containing peptides (Table 2). The results showed PB1X-9B(pJP9) to be as sensitive as the wild-type control, PB1X-2A(YCp50), to L-alanyl-L-ethionine and oxalysine-containing di- and tripeptides, while the negative control, PB1X-9B(YCp50), remained highly resistant to the growth-inhibiting effects of these toxic peptides. These results demonstrate that the transport-deficient phenotype of strains carrying the *ptr2-2* allele is quantitatively corrected by plasmid pJP9 and that the defect in transport due to the *ptr2-2* mutation is limited to di- and tripeptides. Furthermore, we observed little difference in sensitivity to L-alanyl-L-ethionine when the *ptr2-2*-complementing region of pJP9 was transferred as a 3.3-kbp *Hin*dIII-*Sal*I DNA fragment to a high-copy-number 2μm-based *S. cerevisiae* shuttle vector (data not shown). The growth of the strain containing the high-copy-number plasmid demonstrated that *PTR2* is not lethal when present in a high dosage.

Nucleotide and deduced amino acid sequences of the *PTR2* gene. The dideoxynucleotide chain termination method was used to determine the nucleotide sequence of both strands of the *PTR2* open reading frame from double-stranded plasmid templates. Primers were synthesized and used in stepwise sequencing of the 3.1-kbp insert of plasmid pJP9. The sequence shown (Fig. 3) contains a 1,803-bp open reading frame preceded by an 883-bp leader sequence that deletion studies showed necessary for expression. All other open reading frames are extensively blocked by termination codons. The 1,803-bp open reading frame is flanked by a *Bam*HI site, located 495 bp upstream, and spans *Eco*RI and *Bgl*II sites at positions 1,147 and 1,439 bp downstream, respectively. These findings are in agreement with the results obtained from the deletion and frameshift analyses, in which frameshift mutations at the *Eco*RI and *Bgl*II sites were shown to abolish the *ptr2-2*-complementing activity of plasmid pJP9, while a frameshift mutation at the *Bam*HI site had no discernible effect on the complementing ability of plasmid pJP9.

A search of the GenBank-EMBL DNA sequence data base demonstrated a region of 96.9% identity in a 479-bp region of overlap between the 3′ region of *PTR2* and the 3′-flanking region of the *UBI2* gene of *S. cerevisiae*, which encodes ubiquitin (52). The two genes are oriented so that they are transcribed convergently, with the termination codons being separated by a 466-bp region (Fig. 4). When

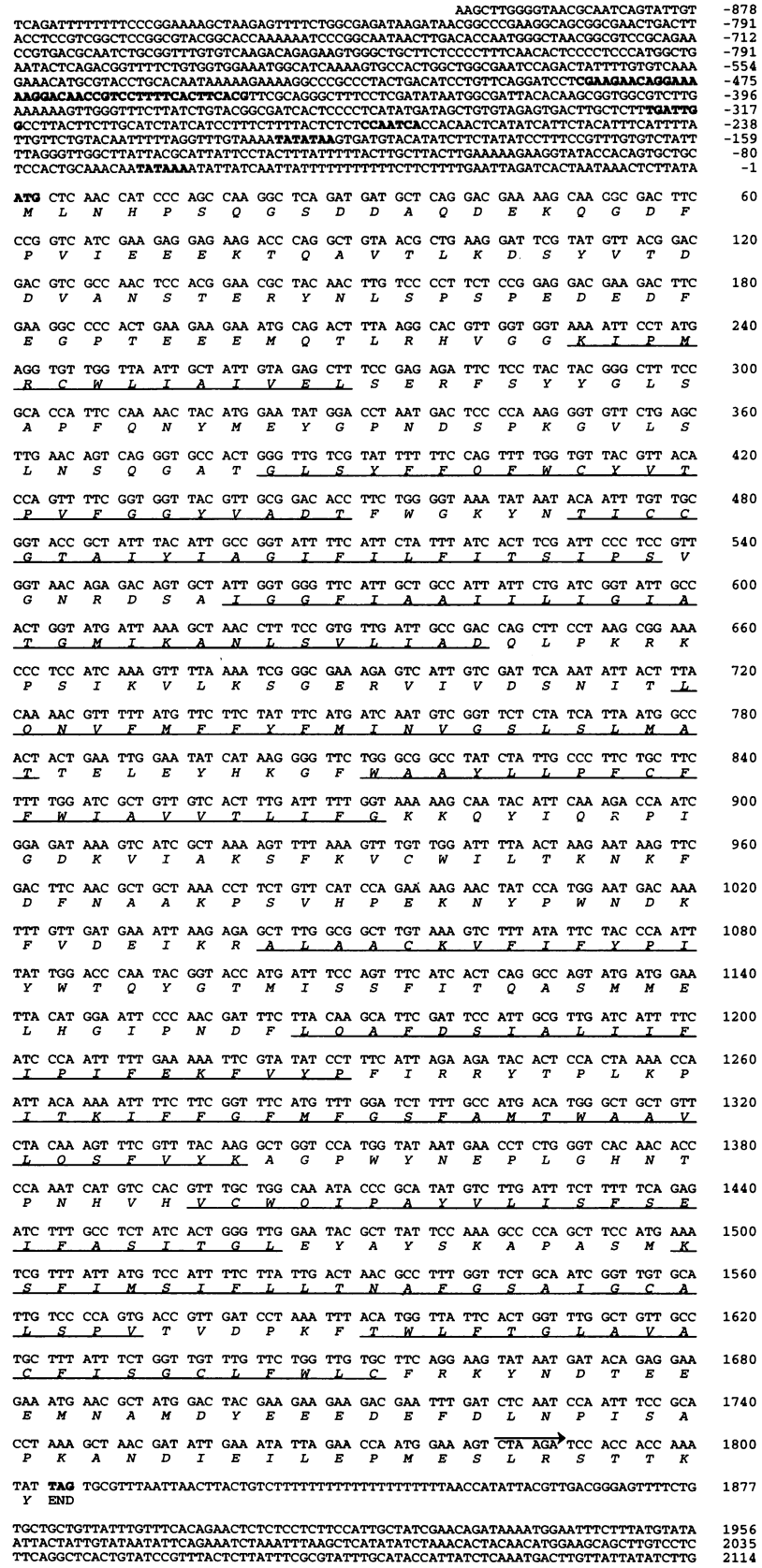

FIG. 3. Nucleotide sequence and predicted amino acid sequence of the *PTR2* gene. The open reading frame is shown, with the hydrophobic regions indicated by underlining. TATAA and CCAATCA elements and an inverted repeat element in the promoter region are shown in boldface type. The start of an overlapping region 3′ to the sequenced *UBI2* gene is indicated at nucleotide 1785.

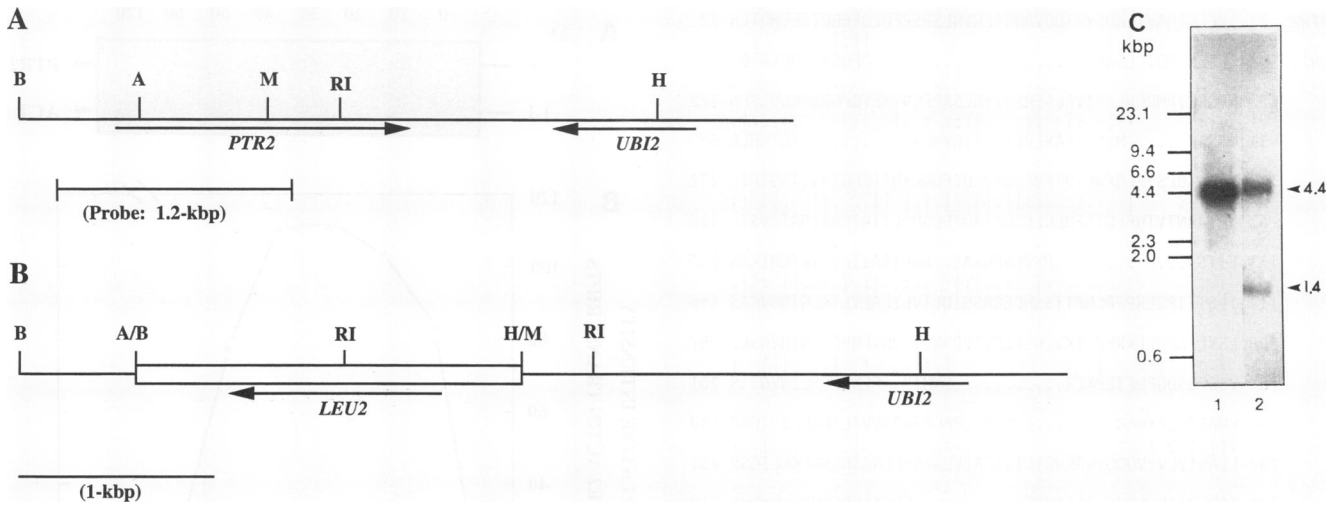

FIG. 4. Structure of the *PTR2* locus. The structure of the *PTR2* locus was deduced from plasmid maps, DNA sequence data, and Southern hybridization analysis. (A) Region of the right arm of chromosome XI, which contains the *PTR2* and *UBI2* genes. The arrows indicate the direction of transcription of *PTR2* and *UBI2*. Also shown is the region of *PTR2* that corresponds to the radiolabeled probe used in Southern hybridization experiments. (B) Predicted structure of the disrupted *PTR2* locus. The region of the *PTR2* gene extending from the *Aat*II restriction site to the *Msc*I restriction site was deleted and replaced by a 2.0-kbp DNA fragment encoding the *LEU2* gene of *S. cerevisiae* (see Materials and Methods). The structure of the disrupted locus was confirmed by Southern hybridization analysis. (C) Results of Southern blotting, in which whole-cell DNAs from wild-type strain PB1X-2A and disrupted strain PB1X-2AΔ were digested with *Eco*RI, fractionated on a 0.9% agarose gel, transferred to a nylon membrane, and then hybridized with a radiolabeled *PTR2*-specific PCR-generated probe (see Materials and Methods). Lanes: 1, 4.4-kbp *Eco*RI-generated DNA fragment from wild-type *S. cerevisiae* PB1X-2A; 2, similar 4.4-kbp *Eco*RI-generated DNA fragment and the 1.4-kbp fragment expected from the disrupted *PTR2* locus of strain PB1X-2AΔ. Restriction endonucleases: A, *Aat*II; B, *Bam*HI; H, *Hin*dIII; M, *Msc*I; RI, *Eco*RI; A/B, *Aat*II-*Bam*HI junction; H/M, *Hin*dIII-*Msc*I junction.

combined, the two sequences yield a detailed map of a 4.27-kbp region of the right arm of chromosome XI (Fig. 4A). Furthermore, a search of the GenBank data base with the BLAST algorithm (2) revealed significant similarities between the protein encoded by *PTR2* and the protein encoded by the *CHL1* gene of *Arabidopsis thaliana*, which is involved in herbicide sensitivity and nitrate-inducible transport of nitrate (68) (Fig. 5).

Chromosomal assignment. Yeast chromosomes were prepared for electrophoresis by entrapment in agarose beads (see Materials and Methods). The chromosomes were fractionated on a contour-clamped homogeneous electric field gel and then transferred and UV cross-linked to a nylon membrane. Plasmid pJP9 was radiolabeled by the random primer method and used as a probe for chromosome hybridization analysis. Plasmid pJP9 is composed, in part, of four yeast DNA sequences: *CEN IV*, *ARS1*, *URA3*, and *PTR2*. As expected, the radiolabeled probe hybridized to chromosome IV (*CEN IV* and *ARS1*) and chromosome V (*URA3*) (data not shown). In addition, hybridization to chromosome XI was also noted on autoradiograms. Sequences hybridizing to *CEN IV* and *ARS1* (39) and *URA3* (58) were included as controls, since the locations of these sequences within the yeast genome are well documented. To confirm the above-described results, yeast chromosomes were probed with a radiolabeled 1.64-kbp *Bam*HI-*Eco*RI DNA fragment from the *PTR2* cloned region and with a radiolabeled chromosome XI-specific probe composed of a 4.0-kbp *Bam*HI-*Eco*RI DNA fragment from plasmid Ep69, containing the *GCN3* gene (20). In duplicate experiments, the *PTR2*- and *GCN3*-specific probes hybridized to the same chromosome, thereby confirming the chromosome XI assignment.

To more precisely map the *PTR2* gene, a series of filters containing 880 recombinant λ phage clones (from L. Riles and M. Olson) representing 82% of the yeast genome were probed with a radiolabeled 1.64-kbp *Bam*HI-*Eco*RI DNA fragment of the *PTR2* gene. The probe hybridized to three overlapping λ phage clones containing DNA from a 5-kbp region mapping to the right arm of chromosome XI, distal to the *tif1* marker.

PTR2* gene disruption.** To obtain a stable peptide transport mutant and to address whether *PTR2* is an essential gene, a chromosomal deletion of the *PTR2* gene was generated by one-step gene disruption (60). A 653-bp sequence internal to the *PTR2* coding region, extending from the *Aat*II site at nucleotide position 124 to the *Msc*I site at nucleotide position 777 of plasmid pJP15 (see Materials and Methods), was deleted and replaced by blunt-end ligation with a 2.0-kbp DNA fragment containing the *LEU2* gene of *S. cerevisiae* (see Materials and Methods). The disrupted copy of the *PTR2* gene was excised as a 2.6-kbp *Nde*I/*Dra*I fragment, which was then used to transform the Ptr$^+$ yeast strain PB1X-2A (*MATa** *ura3-52 leu2-3 lys1-1 his4-38*). The resulting Leu$^+$ yeast transformants were tested for the ability to use L-lysyl-L-leucine as a source of lysine and for sensitivity to L-alanyl-L-ethionine. In all cases, the Leu$^+$ transformants failed to use L-lysyl-L-leucine as a source of lysine and were resistant to the growth-inhibiting effects of L-alanyl-L-ethionine. A single Leu$^+$ Ptr$^-$ colony, designated PB1X-2AΔ, was chosen for further characterization. The disrupted strain, PB1X-2AΔ, failed to accumulate L-lysyl-L-[^{3}H]leucine in radiolabeled dipeptide uptake experiments (data not shown). The alteration at the *PTR2* locus was confirmed by Southern hybridization analysis (Fig. 4) (see Materials and Methods). Whole-cell DNA isolated from the nondisrupted wild-type strain, PB1X-2A, yielded a single 4.4-kbp *Eco*RI-generated DNA fragment following Southern hybridization, whereas DNA from the disrupted strain yielded a 1.4-kbp

```
PTR2  23 IEEEKTQAVTLKDSYVTDDVANSTERYNLSPSPEDEDFEGPTEEEMQTLR 72
         :.|.|.:.: |...                        ||:|...:  .|
CHL1   3 LPETKSDDILLDAW.....................DFQGRPADRSKTGG 30

        73 HVGGKIPMRCWLIAIVELSERFSYYGLSAPFQNYMEYGPNDSPKGVLSLN 122
           .::      :| .:|  ||:. .|::. : .|:       .|.:.|.
        31 WASAA......MILCIEAVERLTTLGIGVNLVTYL.....TGTMHLG 66

       123 SQGATGLSYFFQFWCYVTPVFGGYVADTFWGKYNTICCGTAIYIAGIFIL 172
           ...|..   |   ::: .::||::||||:|:| ||.. .||  .|: ||
        67 NATAANTVTNFLGTSFMLCLLGGFIADTFLGRYLTIAIFAAIQATGVSIL 116

       173 FITSI.............PSVGNRDSAI..GGFIAAIILIGIATGMIKAN 207
           :..|             .| .:..|:|  ..:. |:.|.:::|| :||.
       117 TLSTIIPGLRPPRCNPTTSSHCEQASGIQLTVLYLALYLTALGTGGVKAS 166

       208 LSVLIADQLPKRKPSIKVLKSGERVIVDSNITLQNVFMFFYFMINVGSLS 257
           :|.: .||:.. .|. :           |.:|.  .|  |:| ||||  |
       167 VSGFGSDQFDETEPKER...........SKMTY..FFNRFFFCINVG..S 201

       258 LMATTELEYHKG.....FWAAYLLPFCFFWIAVVTLIFGKKQYIQRP 299
           |:|.| | | .:          .|: :.:.:  : ..|  :  |..|..|
       202 LLAVTVLVYVQDDVGRKWGYGICAFAIVLALSVFLAGTNRYRFKKLIGSP 251

       300 IGDKVIAKSFKVCWILTKNKF...........DFNAAKPSVHPEKNYPWN 338
           :.:  :|  :..|   | .:           |: ||..|:.....| .
       252 MTQ..VAAVIVAAWRNRKLELPADPSYLYDVDDIIAAEGSMKGKQKLPHT 299

       339 DKF.............................VDEIKRALAACKVFIFY 358
           :.|                            |:|:|. :  .::  :
       300 EQFRSLDKAAIRDQEAGVTSNVFNKWTLSTLTDVEEVKQIVRMLPIWATC 349

       359 PIYWTQYGTMISSFITQASMME....LHGIPNDFLQAFDSIALIIFIPIF 404
           ::|| .: :..  :.|.. ::      :|| . :..|   :|:: .:::
       350 ILFWTVHAQLTTLSVAQSETLDRSIGSFEIPPASMAVFYVGGLLLTTAVY 399

       405 EKFVYPFIRRY....TPLKPITKIFFGFMFGSFAMTWAAVLQSFVYKAGP 450
           ::...:.::.     .|:|: :| :|::|||:||. ||:::   .:.:.
       400 DRVAIRLCKKLFNYPHGLRPLQRIGLGLFFGSMAMAVAALVELKRLRTAH 449

       451 WYNEPLGHNTPNHVHVCWQIPAYVLISFSEIFASITGLEYAYSKAPASMK 500
           ....:   .. .: .:: ||.|::::::| :  ...|:: ....| :||
       450 AHGPTV...KTLPLGFYLLIPQYLIVGIGEALIYTGQLDFFLRECPKGMK 496

       501 SFIMSIFLLTNAFGSAIGCALSPVTVDPKFT.....WLFTGLA..... 538
           ::  :::| | |:|  :::.|  ||: .|||     |: .:|.
       497 GMSTGLLLSTLALGFFFSSVL..VTIVEKFTGKAHPWIADDLNKGRLYNF 544

       539 ...VACFIS.GCLFWLCFRKYNDTEEEMNAMDYEEEDEFDLNPISAPKAN 584
           ||.::. . |::|.|.|:   .|.. |   | : |:| :| | |.::
       545 YWLVAVLVALNFLIFLVFSKWYVYKEKRLA...EVGIELDDEP.SIPMGH 590
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FIG. 5. Alignment of the amino acid sequences of the *PTR2*- and *CHL1*-encoded proteins. A search of the GenBank data base with the BLAST algorithm revealed significant similarities between the protein encoded by the *PTR2* gene of *S. cerevisiae* and that encoded by the *CHL1* gene of *A. thaliana*. The proteins exhibit 26% identity (vertical lines) and 52.0% similarity (periods and colons) over the entire sequences, with regions of higher identity, such as those from 418 to 442 (*PTR2*) and 417 to 441 (*CHL1*), with 44% identity. The program BESTFIT was used to align the two sequences. The quality of the fit was 8 standard deviations over a random fit with a stringency of 20 randomizations of the BESTFIT algorithm.

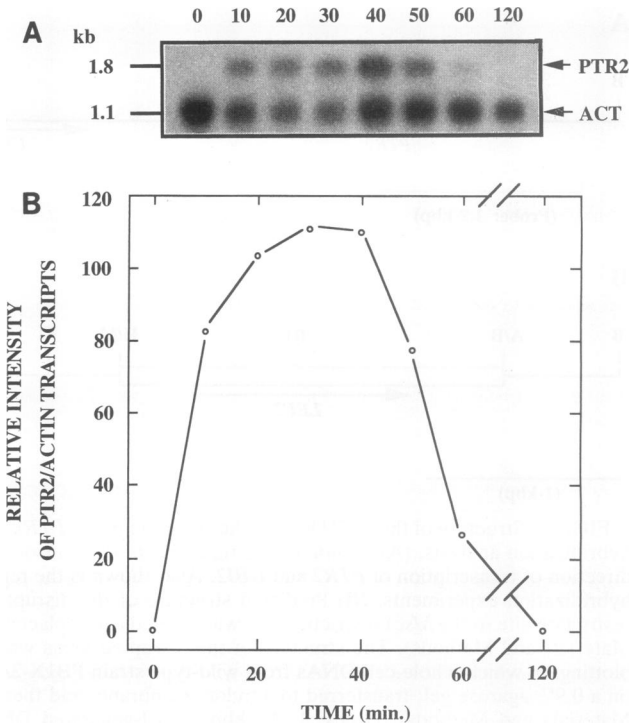

FIG. 6. Northern analysis of *PTR2*. (A) S288C cells were grown overnight to a density of 2.0×10^7 cells per ml in MM. Starting at time zero, 100-ml samples were removed every 10 min for 1 h; a final sample was removed at 2 h. Sterile 0.15 mM L-leucine was added to the culture immediately following the time-zero sampling. Total RNAs (25 μg) were subjected to Northern transfer and hybridized with ^{32}P-labeled *PTR2*- and actin (ACT)-specific probes. (B) Relative intensities of *PTR2* and actin transcripts. Data were derived from the digitally translated autoradiogram in panel A. Datum points represent arbitrary intensity values after background subtraction.

*Eco*RI-generated fragment in addition to the larger fragment, as predicted. The disrupted strain was crossed with a wild-type *S. cerevisiae* strain, XL70-1B, the diploids were allowed to sporulate, and the tetrads were dissected. The resulting haploid strains were assayed for sensitivity to L-alanyl-L-ethionine and for the Leu$^+$ phenotype. The Leu$^+$ Ptr$^-$ phenotype segregated 2:2 in 10 tetrads. These results demonstrate that the disrupted copy of the *PTR2* gene was integrated at the *PTR2* locus and, because the deletion was viable in a haploid, that *PTR2* is not an essential gene, as defined by our growth conditions. We also transformed strain PB1X-2AΔ (*ptr2::LEU2*) with plasmid pJP9, containing the *PTR2* gene. The transformant fully regained the ability to transport radiolabeled peptides and to grow on peptides containing required amino acids, and its sensitivity to toxic peptides was restored. These results prove that *PTR2* is indeed an essential gene for peptide transport.

Northern hybridizations. A previous study clearly showed the ability of a variety of amino acids to induce the uptake of toxic dipeptides in yeast cells, with L-leucine being one of the best inducers, when grown in medium containing allantoin as a nitrogen source (30). Therefore, to assess the effects of L-leucine on transcription of the *PTR2* gene, total RNA was isolated from logarithmically growing yeast cells (*S. cerevisiae* S288C) grown in MM and subsequently induced by the addition of L-leucine. Northern hybridization experiments demonstrated the presence of a 1.8-kb mRNA transcript that was induced within 10 min after the addition of L-leucine and was present for 60 min following induction (Fig. 6).

DISCUSSION

Early studies of peptide transport clearly demonstrated the ability of *S. cerevisiae* auxotrophs to use di- and tripeptides and, less frequently, tetrapeptides as sources of amino acids in growth media (40, 48, 49). These observations suggested a method by which to directly select for yeast transformants carrying a plasmid-borne copy of the *PTR2* gene. The *ptr2-2* mutation was crossed into a genetic background containing the *leu2-3*, *lys1-1*, and *his4-38* mutations. Only transformants carrying a plasmid-borne wild-type copy

of the *PTR2* gene could be expected to meet the auxotrophic requirements imposed by the *leu2*, *lys1*, and *his4* markers when the required amino acids are present in the form of dipeptides in the growth medium. To assess the feasibility of this selection system, the peptide transport-deficient yeast strain PB1X-9B (*MAT***a** *ura3-52 leu2-3 lys1-1 his4-38 ptr2-2*) was transformed with a YCp50-based *S. cerevisiae* genomic DNA library, and the cells were plated on solid MM containing L-lysyl-L-leucine and L-histidine. L-Lysyl-L-leucine served as a source of lysine and leucine in those transformants able to transport the dipeptide across the plasma membrane, while histidine served to satisfy the requirement conferred by the *his4-38* mutation and also to induce the peptide transport system. A variety of amino acids have been shown to enhance the uptake of di- and tripeptides, with L-histidine being one of the inducers (30). The uracil requirement of PB1X-9B was satisfied by the selectable *URA3* gene present on the parent vector, YCp50. The uracil requirement enhanced the stringency of the selection procedure by limiting growth to plasmid-bearing transformants able to utilize dipeptides as a source of amino acids. This double selection reduces the chances of recovering *ptr2-2* revertants. Two yeast transformants from independent transformation experiments and harboring identical plasmids, designated pJP1 and pJP2, were recovered and tested for the Ptr^+ phenotype. Yeast transformants harboring plasmid pJP2 displayed the expected wild-type phenotype when tested for the ability to accumulate radiolabeled dileucine, growth on defined dipeptides, and sensitivity to toxic dipeptides. Secondary yeast transformants harboring plasmid pJP2 demonstrated the Ptr^+ phenotype, thus showing the complementing activity to be plasmid borne.

DNA sequencing of plasmid pJP2 revealed a 1,803-bp open reading frame encoding a predicted protein with a calculated monomer molecular mass of 68.1 kDa and a pI of 5.14. The penultimate amino-terminal amino acid residue is leucine (CTC), which is found in 9.5% of known yeast proteins, following only serine (28%) and threonine (10%) in frequency at that position (26). Computer-assisted predictions showed the primary structure to consist of a hydrophilic amino terminus spanning approximately 80 amino acids, an alternating pattern of hydrophobic and hydrophilic regions, and a carboxy terminus spanning approximately 50 amino acids. The hydrophilic leader sequence and the alternating pattern of hydrophobic domains (36) flanked by hydrophilic regions observed in the *PTR2*-encoded protein are typical of membrane-associated proteins thought to be involved in transmembrane ligand transport (23, 64, 69). It is therefore not surprising to find such a predicted structure for the *PTR2*-encoded protein, since it clearly functions as a di- and tripeptide permease. A search of the GenBank-EMBL data base with the BLAST algorithm revealed significant similarities between the *PTR2*-encoded protein and the protein encoded by the *CHL1* gene of *A. thaliana* (Fig. 4). The *CHL1* gene of *A. thaliana* is involved in herbicide sensitivity and nitrate transport across the plasma membrane of plants (68). The predicted *CHL1*-encoded protein is postulated to encode an electrogenic nitrate transporter that is regulated by environmental pH and nitrate availability.

Computer-assisted predictions of secondary structures (10) suggest that the 12 hydrophobic regions are composed predominantly of amino acids which tend to form β-sheet structures rather than α-helices, as seen in the transmembrane domains of many other ligand transport proteins (23). Similar β-sheet structures were predicted in part for the transmembrane domains of the allantoin permease of *S. cerevisiae* on the basis of the deduced amino acid sequence of the *DAL5* gene (57). Furthermore, it has been shown that the transmembrane domains of the OmpF and PhoE porin proteins of *E. coli* are composed exclusively of amino acids that assume a β-sheet structure (11). The 12 hydrophobic regions of the *PTR2*-encoded protein may serve as transmembrane domains that anchor the protein and provide a structural framework for transporting small peptides.

Although not appearing to be closely related, the *PTR2*-encoded protein does have some features in common with a diverse family of membrane proteins collectively known as ABC ligand transporters (23). This family includes the bacterial peptide transport proteins of the OppABCDF system of *S. typhimurium*, the AmiABCDEF system of *Streptococcus pneumoniae*, the Opp and DciA systems of *Bacillus subtilis*, and the Dpp system of *E. coli*. The oligopeptide transporter of *E. coli* comprises two polypeptides, each consisting of a transmembrane domain and an ATP-binding domain. The transmembrane domain of each polypeptide is composed of six membrane-spanning segments, constituting the "two-times-six" paradigm of most ABC transporters. However, definitive conclusions regarding the intracellular location, structure, and biochemical function of the *PTR2*-encoded protein will depend on additional experimentation.

An analysis of the DNA sequence in the promoter region shows that TATATAA and TATAAAA are present at nucleotide positions −208 and −65, respectively, and are identical to the canonical TATA(T/A)A element usually found upstream of the point of initiation of transcription in eucaryotes (67). The TATAAAA element at position −65 is followed by an 83% A+T-rich sequence spanning the 59-bp region to the first ATG. A search of the *PTR2* promoter failed to identify any of the known *cis*-acting elements that function in the positive regulation of many genes encoding amino acid biosynthetic enzymes (25) or elements that function in nitrogen catabolite repression genes involved in allantoin utilization in *S. cerevisiae* (9). In addition, it is not likely that the *PTR2* gene has any introns, since the DNA sequence failed to reveal a TACTAAC sequence, which is required for accurate intron excision in *S. cerevisiae* mRNA transcripts (56).

The *PTR2* gene was physically mapped to the right arm of chromosome XI, distal to the *tif1* marker, by chromosome hybridization experiments. A comparison of the sequenced *PTR2* gene and flanking regions with other sequences in the GenBank-EMBL data base showed *PTR2* to be tightly linked to *UBI2* (52), which encodes ubiquitin. The *PTR2* and *UBI2* coding regions are separated by 466 bp and are encoded on opposite DNA strands so that they are transcribed convergently. What effect, if any, this structural arrangement has on the expression of *PTR2* or *UBI2* remains to be elucidated. A similar orientation pattern is seen for the *S. cerevisiae* *STE6* gene, encoding the **a**-factor export protein, and *UBA1*, encoding the ubiquitin-activating enzyme (35, 43). No other genes have been mapped to this region of chromosome XI, although *bas1* is located within approximately 15 kbp, on the basis of physical maps constructed from λ phage clones of chromosome XI.

The expression of the *PTR2* gene appears to be tightly regulated, as the message is induced by leucine addition to MM and rapidly disappears within 30 to 40 min. Earlier studies clearly demonstrated that various amino acids induce the peptide transport system of *S. cerevisiae* in the presence of allantoin, a nonrepressive nitrogen source (30). In addition, we found that *PTR2* is expressed on a continual basis in cells grown in nitrogen-rich YEPG medium (data not

shown). Collectively, these observations suggest that the peptide transport system of *S. cerevisiae* may be regulated at different levels, depending on the specific growth conditions. This regulation is likely to be highly dependent on the nitrogen source and the presence or absence of amino acids and other nitrogen-rich molecules, such as di- and tripeptides. Therefore, conclusions regarding the physiological regulation of peptide transport cannot be made with certainty on the basis of only the transcriptional regulation of the *PTR2* gene. A more complete understanding of peptide transport will depend on a more detailed study of *PTR2* transcriptional regulation and on the isolation and characterization of the *PTR1* and *PTR3* genes.

In addition to *PTR2*, using this method, we and our colleagues recently cloned the *PTR1* gene of *S. cerevisiae* (1a), a *PTR2* homolog from *A. thaliana* (66a), and a plasmid from a *C. albicans* genomic DNA library which functionally cross-complements the peptide transport-deficient phenotype conferred by both the *ptr2* and the *ptr1* mutations of *S. cerevisiae* (4a). We expect that this approach to cloning peptide transport genes will facilitate a comparative analysis which may reveal regulatory and structural features common to peptide transport genes of various organisms and provide a better understanding of peptide transport at the physiological level in eucaryotes.

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