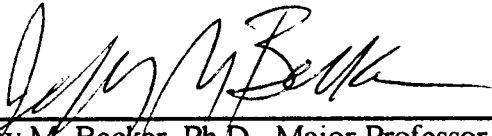


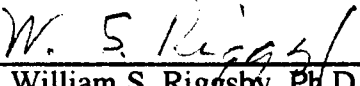
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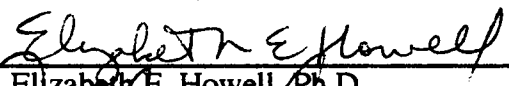


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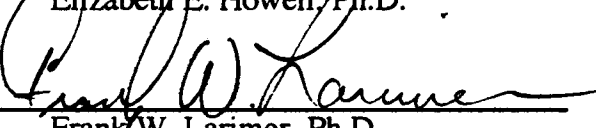
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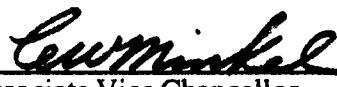


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THE GENETIC BASIS FOR PEPTIDE TRANSPORT

IN *Saccharomyces cerevisiae*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Jack R. Perry, M.D.

May, 1995

DEDICATION

This dissertation is dedicated to the memory of the late Bessie L. Perry.

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To my family, I owe everything.

ABSTRACT

The transport of intact peptides across the plasma membrane is a well characterized physiological phenomenon known to occur in a variety of organisms. However, the genetic basis underlying this process is poorly understood in eukaryotic organisms. In order to better understand the genetic basis for transmembrane peptide transport, a group of *S. cerevisiae* strains deficient in the uptake of peptides was generated by chemical mutagenesis then characterized. Here it is shown that peptide transport in *S. cerevisiae* is mediated by the action of at least three genes: *PTR1*, *PTR2*, and *PTR3*. Strains carrying mutations in these genes define two phenotypic classes, designated A and B. *S. cerevisiae* strains of phenotypic class A, represented by Ptr1^- and Ptr2^- strains, are characterized by resistance to the growth inhibiting effects of toxic dipeptides, failure to accumulate radiolabeled dileucine at wildtype levels, and the inability to use dipeptides to satisfy auxotrophic amino acid requirements. *S. cerevisiae* strains of phenotypic class B, represented by Ptr3^- strains, are also characterized by resistance to the growth inhibiting effects of toxic dipeptides, but are able to accumulate low levels of radiolabeled dileucine, and are able to use a variety of dipeptides to satisfy amino acid growth requirements. In an effort to define peptide transport at the molecular level, methodologies for cloning genes involved in the various aspects of peptide transport in *S. cerevisiae* are presented. Also presented are results from the molecular cloning and characterization of the *PTR2* gene of *S. cerevisiae*. The *PTR2* gene was isolated from a YCp50-based genomic-DNA library by

directly selecting for functional complementation of the peptide transport deficient phenotype conferred by the *ptr2-2* mutation. Deletion and frameshift mutagenesis were used to localize the complementing activity to a 3.1-kbp region on the transforming plasmid pJP2. DNA sequencing of the complementing region identified an open reading frame spanning 1803 bp. The deduced amino acid sequence predicts a hydrophobic peptide consisting of 601 amino acids with a molecular mass of 68.1-kDa. The predicted protein is composed in part of twelve hydrophobic segments similar to those found in proteins known to be plasma membrane associated and to function in transmembrane transport. Northern hybridization experiments demonstrate a single transcript of 1.8-kb in length commensurate with the size of the *PTR2* open reading frame. The *PTR2* encoded peptide was shown to share significant similarities with a nitrate inducible membrane protein, encoded by the *CHL1* gene, of *Arabidopsis thaliana* which functions in nitrate transport. A search of the DNA sequence data base showed the cloned *PTR2* gene to be tightly linked to the *UBI2* gene with the coding sequences being separated by a 466-bp region and oriented so that the genes are transcribed convergently. The *PTR2* gene was localized to the right arm chromosome XI by CHEF gel chromosome blots and by hybridization to known chromosome XI λ phage clones of *S. cerevisiae* DNA. 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 eukaryotic oligopeptide transport gene and the second member of a new class of membrane associated proteins.

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CHAPTER I

INTRODUCTION

Peptide transport is a general physiological phenomenon that has been shown to occur in bacterial, fungal, plant, and mammalian cells (for reviews see Becker and Naider, 1980; Higgins, 1984 and 1992; Matthews, 1991; Webb, *et al.*, 1992). It is operationally defined as the process by which individual cells are able to transport intact peptides across the plasma membrane. Peptide transport has been studied to the greatest extent in bacterial systems, primarily *Escherichia coli* and *Salmonella typhimurium*, and to a lesser extent in the fungi *Neurospora crassa*, *Candida albicans*, and *Saccharomyces cerevisiae*.

Peptide uptake in the gram negative bacteria *S. typhimurium* and *E. coli* is mediated by three genetically distinct, yet physiologically overlapping, systems. In these organisms, peptides may serve as sources of amino acids, as carbon or nitrogen sources (Payne, 1980), or as precursors of cell-wall peptides during bacterial growth (Goodell and Higgins, 1987). The oligopeptide permease (Opp) system of *E. coli* and *S. typhimurium* is capable of transporting peptides up to five amino acids long across the plasma membrane, with little specificity regarding amino acid side chain composition (Payne and Gilvarg, 1968; Payne, 1968), and appears dependent upon the presence of a free α -amino group on the oligopeptide substrates (Gilvarg and Katchalski, 1965; Losick and Gilvarg, 1966). In addition, the Opp system is also capable of transporting D-amino acids (Goodell and Higgins, 1987) and is induced in the presence of leucine (Andrews, *et al.*, 1986) or

alanine and by growth under anaerobic conditions (Andrews and Short, 1986). In contrast, phosphate limitation has been shown to repress the synthesis of the *Opp* encoded proteins via the actions of a repressor molecule encoded by the *phoB* gene (Smith and Payne, 1992). The genes encoding the components of the oligopeptide transport system map to a single locus, *opp*, and code for five proteins, OppA, B, C, D, and F (Hogarth and Higgins, 1983; Hiles, *et al.*, 1987). The molecular basis for the broad substrate specificity of the periplasmic oligopeptide-binding protein of *S. typhimurium* (OppA) has recently been elucidated (Tame, *et al.*, 1994). OppA was found to be composed, in part, of three domains which act in concert to bind and sequester individual peptides of various amino acid compositions. The side-chain groups of the constituent amino acids are accommodated within large hydrated cavities of the carrier-protein interior. The ability of OppA to bind peptides is conferred by amino acid residues which specifically interact with the free α -amino group and the peptidyl linkages of the peptide substrate. A similar mechanism may be employed by other proteins that function in binding peptides in other peptide transport systems. OppB and OppC appear to be hydrophobic integral membrane proteins which mediate the translocation of peptides across the plasma membrane (Pearce, *et al.*, 1992). OppD and OppF appear to be nucleotide binding proteins which are located on the cytoplasmic side of the plasma membrane (Higgins, *et al.*, 1985; Gallagher, *et al.*, 1989).

By comparison, the tripeptide permease (Tpp) system of *S. typhimurium*, previously designated the Opr I system (Naider and Becker, 1975), is functionally similar to the Opp system, but is limited to transporting di- and tripeptides. The Tpp system has

an apparent affinity for hydrophobic peptides and is inducible by the presence of leucine or by growth under anaerobic conditions (Jamieson and Higgins, 1984 and 1986). The genes encoding the components of the Tpp system map to two distantly linked loci, *tppA/ompB* and *tppB* (Gibson, *et al.*, 1984). The *tppA/ompB* locus codes for two proteins, OmpR and EnvZ, which act to positively regulate the expression of the porin genes, *ompC* and *ompF*, and *tppB*, which encodes the peptide transporter (Gibson, *et al.*, 1984 and 1987). The Tpp regulatory pathway is not well understood since the porin genes and *tppB* are not coordinately expressed. Induction of the Tpp system by anaerobiosis appears to be under the regulatory control of the *oxrC* and *tppR* gene products (Jamieson and Higgins, 1986).

The third peptide transport system of enteric bacteria is represented by the Dpp system which preferentially transports dipeptides (Payne, 1968; De Felice, *et al.*, 1973). In addition to dipeptides, the Dpp system also transports 5-aminolevulinic acid, a precursor in heme biosynthesis (Verkamp, *et al.*, 1993). Mutations affecting the Dpp system have been mapped to a single site on the *S. typhimurium* chromosome that differs from the known *opp* and *tpp* loci (Higgins and Gibson, 1986). The *dppA* gene encodes a periplasmic dipeptide-binding protein necessary for both dipeptide transport (Olson, *et al.*, 1991; Abouhamad, *et al.*, 1991) and chemotaxis (Abouhamad, *et al.*, 1991), while the other *dpp* genes mapping at the same locus are involved only in dipeptide transport. The *dppA* encoded protein shares 26.5% identity with OppA, the oligopeptide-binding protein. The similarity in structure between OppA and DppA suggests that they may play similar roles and utilize similar mechanisms in the binding and transporting of dipeptides across

the plasma membrane. As with the Opp system, the Dpp system appears to be repressed by phosphate limitation and is under the control of PhoB (Smith and Payne, 1992).

In addition to dedicated peptide transport systems, the products encoded by the porin genes, *ompF* and *ompC*, have been shown to have a profound effect on the ability of various *E. coli* strains to utilize specific peptides in growth assays (Andrews and Short, 1985). The porin proteins constitute a selective molecular sieve, located within the outer membrane of gram-negative bacteria, through which many molecules must pass prior to being transported across the plasma membrane (for reviews see Nikaido, H., 1992 and 1993). The physiochemical properties of various solutes determine the relative rates of diffusion across the outer membrane. Porin complexes are composed of three protein monomers which associate to form a water filled channel. Many porin gene mutations are found at sites which correspond to positions on the channel wall formed by the proteins. These mutations alter the charge distribution within the porin channels and thereby perturb the diffusion of charged substrates, such as small peptides (Cowan, *et al.*, 1992). In addition to the inability to utilize a variety of tripeptides as sources of amino acids, *ompF⁻* and *ompC⁻* *E. coli* strains are also resistant to a variety of toxic tripeptides (Andrews and Short, 1985).

In addition to the enteric bacteria, peptide transport systems have been described in detail for several other bacterial organisms. In *Bacillus subtilis*, oligopeptide transport is mediated by the products encoded at the *spoOK* locus (Perego, *et al.*, 1991; Rudner, *et al.*, 1991). The genetic organization of the *spoOK* locus is similar to the *opp* and *ami* loci of the enteric bacteria and *Streptococcus pneumoniae*, respectively. In addition to peptide

transport, the products encoded at the *spoOK* locus also function in sporulation, possibly by facilitating the transport of sporulation signaling molecules across the plasma membrane during periods of nutrient deprivation, and in the induction of genetic competence (Rudner, *et al.*, 1991); however, the mechanism by which this system functions in the induction of competence has not been elucidated. The recent isolation of a peptide pheromone that is required for the induction of genetic competence in *B. subtilis* has led to the suggestion that the *spoOK* encoded proteins may operate in transporting this pheromone across the plasma membrane or serve as a receptor in facilitating a transmembrane signal which stimulates the expression of genes required for genetic competence (Magnuson, *et al.*, 1994). In addition, the recent isolation of a gene encoding a pheromone-binding protein from *Enterococcus faecalis* with significant homology to the peptide binding region of *opp* encoded proteins supports the suggestion that pheromone binding may be closely linked to the transport of peptides in some bacterial systems (Ruhfel, *et al.*, 1993). A separate system for transporting dipeptides across the plasma membrane has been described for *B. subtilis* (Mathiopoulous, *et al.*, 1991). The *dciA* locus of *B. subtilis* contains five genes which encode the products that function in transporting dipeptides (Mathiopoulous, *et al.*, 1991). These genes appear to be transcriptionally repressed in the presence of glucose or amino acids and are induced in cells as they initiate sporulation (Slack, *et al.*, 1991). The *abrB* gene has been shown to encode a protein that binds to the promoter region of the *dciA* operon and, thereby, function to regulate *dciA* transcription, perhaps by acting as a repressor (Slack, *et al.*, 1991); however, this regulation appears to be independent of the negative control imposed by glucose and amino acids. The encoded proteins of the *dciA*

locus share significant protein sequence homologies with the protein components of the oligopeptide transport systems of *S. typhimurium* and *B. subtilis* (Mathiopoulos, *et al.*, 1991).

Distinct transport systems have also been described for oligopeptides and di- tripeptides in *Lactococcus lactis* (Smid, *et al.*, 1989; Smid and Konings, 1990; Kunji, *et al.*, 1993). The components of the oligopeptide transport system of *L. lactis* are encoded at the *opp* locus (Tynkkynen, *et al.*, 1993). While the *L. lactis opp* locus encodes proteins which are homologous with the *S. typhimurium opp* encoded proteins, the genetic organization of the *L. lactis opp* locus differs regarding the order in which the genes are transcribed (Tynkkynen, *et al.*, 1993). In addition, the *L. lactis opp* locus encodes an endopeptidase, PepO, that plays an uncertain role in the uptake of peptides in this organism since the disruption of this gene had no discernible effect on peptide utilization in *L. lactis* (Tynkkynen, *et al.*, 1989; Tynkkynen, *et al.*, 1993). In contrast to the oligopeptide transport system, the di- and tripeptide transport system of *L. lactis* appears to be coupled with a proton motive carrier protein and it is not a member of the ATP Binding Cassette (ABC) transporter family (Hagting, *et al.*, 1994), a superfamily of proteins that require the binding of nucleotide triphosphates in order to function. This observation suggests that procaryotic organisms have evolved a variety of biochemical strategies for transporting physiologically important peptides across the plasma membrane and that the various peptide transport systems may not be necessarily evolutionarily related.

An oligopeptide transport system similar to that of the enteric bacteria has been described for *Streptococcus pneumoniae* (Alloing, *et al.*, 1990). The *ami* locus of *S.*

pneumoniae contains an operon coding for six proteins, AmiA, B, C, D, E, and F. The organization of the *ami* locus is similar to the *opp* locus of enteric bacteria with the encoded proteins of the two systems sharing significant homologies.

In summary, many bacterial species appear to have evolved multiple peptide transport systems that function in a variety of capacities including the accumulation of nutritional substrates, the recycling of cell wall components, the induction of sporulation, and the induction of genetic competence. With the exception of the di-tripeptide transport system of *L. lactis*, all of the foregoing bacterial peptide transport systems are members of the ATP Binding Cassette (ABC) transporter superfamily of plasma membrane proteins which are dependent on ATP for functioning (for reviews see Higgins, 1992; Fath and Kolter, 1993). A highly conserved ATP-binding region is the most characteristic feature of this superfamily of proteins. In addition, 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 impermeant toxic moiety is attached to a peptide backbone which then gains entrance into the cell via one of the transport systems cited above (Ames, *et al.*, 1973; Fickel and Gilvarg, 1973; Payne, *et al.*, 1984; Payne, 1986; Hammond, *et al.*, 1987; Higgins, 1987).

Functionally similar peptide transport systems have been reported for *Neurospora crassa*, *Candida albicans*, and *Saccharomyces cerevisiae*, although the number and specificity of the systems operative in these organisms is less clear (for reviews see Wolfenbarger, Jr., 1980; Becker and Naider, 1980; Naider and Becker, 1987). In *N. crassa*, oligopeptides consisting of three to five amino acid residues are transported across

the plasma membrane of mycelial elements in an energy dependent manner (Wolfenbarger and Marzluf, 1975a; Wolfenbarger and Marzluf, 1975b). The system does not appear to have an affinity for dipeptides (Wolfenbarger and Marzluf, 1974) and is not active in conidiospores (Wolfenbarger and Marzluf, 1975b). The system appears to have a requirement for a free α -amino group, is constitutively active, and does not appear to be under carbon or nitrogen repression control (Wolfenbarger and Marzluf, 1975b). The presence of extracellular peptidases has complicated the elucidation of the peptide transport systems in *N. crassa*.

In *C. albicans* a variety of peptides are transported across the plasma membrane in a temperature, pH, and energy-dependent manner with little amino acid side chain specificity (Lichliter, *et al.*, 1976; Logan, *et al.*, 1979; Davies, 1980; McCarthy, *et al.*, 1985; Milewski, *et al.*, 1991); however, a preference for transporting peptides containing L-amino acids, rather than D-isomers, has been noted (Davies, 1980). Based on peptide uptake competition studies, there appear to be at least two functionally overlapping peptide transport systems operative in *C. albicans* with the collective capacity to transport peptides varying in size from two to five amino acid residues (Logan, *et al.*, 1979; Yadan, *et al.*, 1984; Payne and Shallow, 1985; McCarthy, *et al.*, 1985a; McCarthy, *et al.*, 1985b; Milewski, *et al.*, 1988; Milewski, *et al.*, 1991; Basrai, *et al.*, 1992). These peptide transport systems appear to be affected by the nitrogen source present in the growth medium (Logan, *et al.*, 1979; Payne, *et al.*, 1991) in that cells grown in medium containing $\text{NH}_4^+\text{NO}_3^-$ as a nitrogen source demonstrate lower transport activities than cells grown with amino acids as the nitrogen source. The addition of peptides to growth

media appears to override the nitrogen repression effect of NH_4^+ demonstrating an important regulatory effect by these substrates (Payne, *et al.*, 1991). In addition, free amino acids have also been shown to enhance peptide transport activity in *C. albicans* (Basrai, *et al.*, 1992) as demonstrated by the increased sensitivity of this organism to toxic di- and tripeptides by the addition of lysine, or several other amino acids, to growth medium. The amino acid composition of peptide substrates also has a determining effect on peptide transport in *C. albicans* (Lichliter, *et al.*, 1976); for example, lysine containing peptides usually serve as poor substrates while methionine and glycine containing peptides serve as good substrates in promoting the growth of amino acid requiring strains. Furthermore, L-lysyl-L-glycine has been shown to support the lysine growth requirements of a Met⁻, Lys⁻ double auxotrophic strain of *C. albicans* whereas L-glycyl-L-lysine is not able to do so, thereby underscoring the importance of the order of the constituent amino acid residues of peptides in determining the suitability as a substrate for the peptide transport system of *C. albicans* (Lichliter, *et al.*, 1976). Stereospecificity of the constituent amino acids appears to be important in the peptide transport process of *C. albicans* since peptides with D-isomers of amino acid residues, at positions other than the amino terminus of the peptide, do not appear to be good substrates in growth assays (Lichliter, *et al.*, 1976; Logan, *et al.*, 1979). In addition to dedicated peptide transporter molecules, long chain peptides may be taken up in bulk by fluid phase endocytosis in *C. albicans* given the ability of this system to facilitate the internalization and vacuolar accumulation of Lucifer Yellow (Basrai, *et al.*, 1990), a fluorescent marker molecule for studying fluid phase endocytosis (Swanson, *et al.*, 1985; Riezman, 1985; Riezman, *et al.*, 1986). As with bacteria, the

broad substrate specificity of the peptide transport system in *C. albicans* immediately suggests the possibility of utilizing peptide transporters as a means to facilitate the uptake of otherwise impermeant molecules that may be of significance as antifungal compounds in this, and other, medically important fungal organisms (Lichliter, *et al.*, 1976; Naider and Becker, 1987; Milewski, *et al.*, 1991). Indeed, the polyoxins and nikkomycins, two peptidyl-nucleoside antibiotics that inhibit the activity of chitin synthetase, appear to utilize the peptide transport pathway to enter *C. albicans* (Becker *et al.*, 1983; Mehta, *et al.*, 1984; Yadan, *et al.*, 1984; McCarthy, *et al.*, 1985b; McCarthy, *et al.*, 1985c; Milewski, *et al.*, 1991) based on the results from peptide uptake competition studies and the concomitant loss of peptide transport and the appearance of resistance to the antifungal compounds nikkomycin Z and polyoxin. In addition, bacilysin, a dipeptide antibiotic which inhibits the activity of glucosamine synthetase, and small peptides containing amino acid analogs, such as m-fluorophenylalanine, enter cells via the peptide transport pathway and inhibit the growth of *C. albicans* (Mehta, *et al.*, 1984; Kingsbury, *et al.*, 1983; McCarthy, *et al.*, 1985c) as evidenced by toxic peptide competition studies and by the loss of sensitivity to bacilysin in strains that had a concomitant loss of sensitivity to peptides containing toxic amino acid analogs.

Like *C. albicans*, peptide transport in *S. cerevisiae* is also characterized by its ability to transport intact di-, tri-, tetra-, and pentapeptides across the plasma membrane (Becker *et al.*, 1973; Naider, *et al.*, 1974; Marder, *et al.*, 1977; Nisbet and Payne, 1979; Parker, *et al.*, 1980; Moneton, *et al.*, 1986b) in a pH and energy-dependent manner with little demonstrable amino acid side chain specificity (Becker and Naider, 1977 and 1980) as

evidenced by the competition of di- and tripeptides, of various amino acid compositions, for the same transport pathway; however, in some instances amino acid composition and primary structure of the peptide substrates may play important roles in the transport process. Based on peptide uptake competition studies, *S. cerevisiae* appears to have a single peptide transport system capable of transporting di- and tripeptides (Nisbet and Payne, 1979; Moneton, *et al.*, 1986a and b). Specifically, di- and tripeptides have been shown to compete with each other and with sarcosine containing peptides for the same transport pathway in *S. cerevisiae* (Nisbet and Payne, 1979). Di- and tripeptides are also able to reverse the toxicity of bacilysin, nikkomycin Z, and L-leucyl-L-fluorophenylalanine presumably by competing for uptake at transport sites (Moneton, *et al.*, 1986a). A *S. cerevisiae* strain resistant to nikkomycin Z was also shown to be deficient in the accumulation of radiolabeled di- and trimethionine adding additional support to the data suggesting that di- and tri-peptides cross the plasma membrane via a common pathway (Moneton, *et al.*, 1986b). Growth assay data indicate that tetra- and pentapeptides may also be transported via the same transport pathway as di- and tripeptides in *S. cerevisiae* (Becker, *et al.*, 1973; Naider, *et al.*, 1974), but this observation may be highly dependent on the specific amino acid composition and primary structure of the peptide substrate and not a general feature of the peptide transport system in this organism. Alternately, a separate transport pathway may exist for the uptake of oligopeptides analogous to the situation in *E. coli*. In general, there is no absolute requirement for a free amino or carboxyl terminus on the peptide substrates as evidenced by the ability of a methionine requiring auxotrophic strain of *S. cerevisiae* to use peptides with chemically modified

termini as growth substrates (Naider, *et al.*, 1974); however, acetylation of the amino terminus of trimethionine did inhibit its ability to effectively compete for the uptake of radiolabeled trimethionine (Becker and Naider, 1977). Furthermore, acetylation of the amino terminus of several di- and tripeptides abolished their ability to serve as a source of amino acids in growth assays suggesting that modification of the amino terminus does have some effect on the ability of peptides to serve as a substrates for the peptide transport pathway (Marder, *et al.*, 1977). In addition to being able to use a variety of small peptides to meet amino acid requirements in growth assays (Becker, *et al.*, 1973; Naider, *et al.*, 1974; Marder, *et al.*, 1977), *S. cerevisiae* strains are also highly sensitive to a variety of dipeptides containing toxic amino acid analogs such as L-ethionine and D,L-*m*-fluorophenylalanine (Moneton, *et al.*, 1986a; Island, *et al.*, 1987). Furthermore, peptide transport in *S. cerevisiae* appears to be under nitrogen repression control (Becker and Naider, 1977; Moneton, *et al.*, 1986a) in that wildtype strains of *S. cerevisiae* display greater uptake of radiolabeled peptides and greater sensitivities to toxic dipeptides when grown on poor nitrogen sources, such as leucine, proline, and allantoin than when grown on media containing $\text{NH}_4^+\text{NO}_3^-$ as a nitrogen source. Peptide uptake in *S. cerevisiae* also appears to be inducible by a variety of amino acids (Moneton, *et al.*, 1986a; Island, *et al.*, 1987) as evidenced by increased sensitivity to toxic dipeptides when grown in the presence of amino acids on a nonrepressible nitrogen source, and it does not appear to be physiologically linked to any specific extracellular peptidase activity (Parker, *et al.*, 1980). As stated for *C. albicans*, in addition to dedicated peptide transporters, fluid phase endocytosis may also play a role in the accumulation of and utilization of larger peptides by

S. cerevisiae (for reviews see Payne, 1990; Nothwehr and Stevens, 1994).

While considerable biochemical and genetic data have accumulated regarding peptide transport systems in bacterial systems, very little is known about the molecular basis of peptide transport in fungi and higher organisms. Here I describe the isolation and characterization of two phenotypic classes of peptide transport mutants of *S. cerevisiae* and show that most, if not all, such mutants can be grouped into one of three complementation groups designated, *ptr1*, *ptr2*, and *ptr3*. I present two methods for cloning peptide transport genes from *S. cerevisiae* and demonstrate the isolation, nucleotide sequence, chromosomal disruption, and the chromosomal mapping of the *PTR2* gene of *S. cerevisiae*. I also present arguments to support the contention that the *PTR2* gene encodes the plasma membrane-associated peptide transporter of *S. cerevisiae*. It is hoped that others may benefit from the examples provided here in further elucidating this system in eucaryotes.

CHAPTER II

MATERIALS AND METHODS

Media and Strains

Saccharomyces cerevisiae strains, and plasmid bearing derivatives, were routinely maintained on YEPD medium or synthetic dextrose medium (SD) with amino acids and nucleotide bases added as specified (Sherman, *et al.*, 1986). Minimal medium (MM) was composed of 0.67% yeast nitrogen base without amino acids and ammonium sulfate, 1.0 mg/ml allantoin, and 2.0% dextrose. Where indicated, MM was supplemented with amino acids as required at established concentrations (Sherman *et al.*, 1986). Dipeptide medium consisted of minimal medium (MM) supplemented with one of four dipeptides (L-leucyl-L-leucine, L-lysyl-L-lysine, L-lysyl-L-leucine, or L-histidyl-L-leucine) and any required amino acids not included as a constituent of the dipeptide being tested. Solid media contained 2.0% agar. *Escherichia coli* strains and plasmid bearing derivatives were routinely maintained on LB medium and, when necessary, supplemented with the appropriate antibiotic (Sambrook, *et al.*, 1989). All plasmids were routinely maintained and propagated in *E. coli* HB101 (F^- *mcrB mrr hsdS20*($r_B^- m_B^-$) *recA13 supE44 ara14 galK2 lacY1 proA2 rpsL20*(Sm^r) *xyl5 λ^- leu mtl1*). *Saccharomyces cerevisiae* strains used in this study are shown in Table 1.¹

¹ All tables and figures are presented in the Appendix section, p.90.

Enzymes and Biochemicals

Restriction endonucleases, T4 DNA ligase, 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, MA), Stratagene (La Jolla, CA), or Boehringer Mannheim (Indianapolis, IN) and used according to the manufacturer's specifications. Growth media components, dipeptides, amino acids, and amino acid analogs were purchased from commercially available sources except for L-alanyl-L-ethionine, L-leucyl-L-ethionine, L-leucyl-D,L-*m*-fluorophenylalanine, oxalysine-containing peptides, and L-leucyl-L-[³H]leucine trifluoroacetic acid which were synthesized using standard solution phase techniques and supplied by Fred Naider (City University of New York, Staten Island, NY; Naider, *et al.*, 1974). Salmon testes DNA was purchased from Sigma (St. Louis, MO). Kits used for DNA sequencing and random primer radiolabeling of DNA probes were purchased from United States Biochemical (Cleveland, OH). DNA sequencing primers were purchased from GenoSys Biotechnologies (The Woodlands, TX), University of Tennessee Molecular Biology Resource Facility (Knoxville, TN), Bethesda Research Laboratories, and New England BioLabs. GeneClean kits, used in small scale DNA isolation procedures, were purchased from Bio 101 (La Jolla, CA). pZ523 columns, used in removing chromosomal DNA from plasmid preparations, were purchased from 5 Prime 3 Prime (Paoli, PA). Nylon membrane, used in DNA hybridization experiments, and ³²P-dCTP and ³⁵S-dATP, used in hybridization and sequencing experiments, were purchased from Dupont, NEN Research Products (Boston, MA). Nylon membrane used in RNA hybridization experiments was

purchased from ICN (Irvine, CA). XAR-5 film, used in autoradiography, was purchased from Eastman Kodak (Rochester, NY).

Mutagenesis

The N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) mutagenesis procedure employed in this work has been previously described (Maga and McEntee, 1985) and was used with minor modification. Yeast cells, growing logarithmically in MM medium, were treated with MNNG (25 μ g/ml) in 0.01% sodium acetate, pH 5.0, 30°C. At various time intervals, aliquots were removed and treated with an equal volume of 10% sodium thiosulfate which quenched the reaction. The cells were collected by centrifugation, then washed twice with sterile distilled water, and resuspended to 2.0×10^8 cells/ml. Appropriate dilutions of each aliquot were made and a given number of cells were plated on solid YEPD and incubated at 30°C for 48 hours to ascertain survival. The mutagenized cells (10 minutes of MNNG treatment, 5% survival) were also plated on solid MM medium supplemented with 0.15mM L-leucine and 50 μ g/ml L-alanyl-L-ethionine. The plates were allowed to incubate at 30°C for a period of 48 hours. L-alanyl-L-ethionine resistant colonies were then patched onto a fresh plate of the same medium and incubated at 30°C for an additional 48 hours. The cells were replica plated onto solid MM medium supplemented with 0.15 mM L-leucine, and 50 μ g/ml L-ethionine. The plates were then incubated at 30°C for a period of 48 hours. Only L-alanyl-L-ethionine resistant, L-ethionine sensitive isolates were chosen for further study since mutants resistant to L-ethionine were likely to be affected at the target of action of L-ethionine rather than at the

level of transport of the toxic dipeptide.

Dipeptide Growth Assays

Growth assays were performed to ascertain the ability of yeast strains to use various dipeptides as sources of amino acids in meeting auxotrophic requirements. Strains were grown in YEPD broth at 30°C to a titer of 1×10^8 cells/ml. The cells were harvested by centrifugation, washed twice with sterile water, then resuspended at 1.0×10^7 cells/ml. A 5 μ l volume of the cell suspension was applied to solid media containing dipeptides and amino acids. The medium for each assay consisted of solid MM supplemented with one of four dipeptides (L-leucyl-L-leucine, L-lysyl-L-lysine, L-lysyl-L-leucine, or L-histidyl-L-leucine) and required amino acids not present as a constituent of the dipeptide being tested. All dipeptides were present at a concentration of 80 μ M, while amino acids and nucleotide bases were added as required at standard concentrations (Sherman, *et al.*, 1986). For example, dileucine medium consisted of MM supplemented with L-leucyl-L-leucine, uracil, L-lysine, L-histidine, and L-tryptophan. The plates for each assay were incubated at 30°C for 48 hours then scored for growth.

Toxic Dipeptide Disk Assays

Sensitivity to toxic peptides was measured by using disk assays as previously described (Island, *et al.*, 1987). Cells were grown overnight on MM with supplements and suspended in sterile water at 5.0×10^7 cell/ml. Aliquots (0.1 ml) were added to 0.8% Noble agar (10 ml), kept molten at 50°C, and plated on MM (10 ml) supplemented with the appropriate amino acids. A total of 0.38 mmoles of toxic dipeptide was added to a

paper disk which was then placed on the surface of the plates. The plates were incubated at 30°C for 48 hours, and zones of inhibition were measured for each plate.

Radiolabeled Dipeptide Uptake Assay

The procedure for performing radiolabeled dileucine uptake experiments has been previously described (Island, *et al.*, 1987) 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 $0.5\text{--}1.0 \times 10^7$ cells/ml. The cells were collected by centrifugation and washed twice with cold sterile distilled water, then resuspended in 2% glucose at a concentration of 2.0×10^8 cells/ml. A 0.5 ml volume of the cell suspension was incubated at 30°C for 10 minutes then mixed with an equal volume of 2% glucose, 40 mM sodium citrate-potassium phosphate buffer (pH 5.5), and 0.16 mM L-leucyl-L-[³H]leucine (specific activity, 9.4 mCi/mmol). Aliquots of 0.18 ml were removed at defined time intervals (0.25, 0.50, 1.0, 2.0, and 3.0 minutes) and the cells filtered immediately on GN-6 filters (pore size 0.45 µm). The filters were washed twice with 5 ml cold sterile distilled water. The filtered cells were then placed in scintillation vials with 5 ml Bray's solution and counted.

Transformations and DNA Manipulation Techniques

Bacteria were transformed by the CaCl₂ method (Hanahan, 1983) and selected on solid LB medium supplemented with 50 µg/ml ampicillin. Yeast strains were routinely transformed by the LiCl method (Ito, *et al.*, 1983) and selected on MM containing L-

histidine, L-lysine, and L-leucine. 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 (Sambrook, *et al.*, 1989). Yeast whole cell DNA was prepared by the guanidine hydrochloride method (Holm, *et al.*, 1986). Restriction endonuclease analysis, DNA ligations, and agarose gel electrophoresis were performed as previously described (Sambrook, *et al.*, 1989). Large scale plasmid preparations were passed over pZ523 columns to remove contaminating chromosomal DNA (Zervos, *et al.*, 1988).

Northern Hybridization Experiment

S. cerevisiae strain S288C was grown overnight in YEPD to a titer of 2.0×10^7 cells/ml. A 10-ml sample of cells was removed and total RNA was isolated by the SDS/phenol/chloroform method as previously described (Schmitt, *et al.*, 1990) and quantitated by U.V. spectroscopy (Sambrook, *et al.*, 1989). Total RNA, 10 μ g per sample, was incubated at 70°C for 10 minutes in a reducing buffer composed of 20 mM MOPS (pH 7.0), 8 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde (pH 7.0), and 50% deionized formamide, then quick cooled on ice for 5 min. The sample was fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde and 20 mM MOPS (pH 7.0), 8 mM sodium acetate, 1 mM EDTA (Sambrook, *et al.*, 1989) then vacuum-transferred to a nylon hybridization membrane. The RNA was UV-crosslinked with a UV light source, Stratalinker, as specified by the manufacturer (Stratagene). The Northern blot was then probed with a ^{32}P -radiolabeled *PTR2* specific 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 below (See Gene Disruption section of Material and Methods). The UV-crosslinked nylon membrane was prehybridized for 3 hours at 42°C in a solution containing 50% formamide, 5x SSC, 50 mM K₂HPO₄/KH₂PO₄ (pH 7.4), 250 µg/ml denatured salmon testes DNA, and 5x Denhardt's solution. Afterward, the prehybridization solution was removed and replaced with hybridization solution composed of 50% dextran sulfate and fresh prehybridization solution (1:4 v/v). The DNA probe was denatured by incubating at 100°C for 5 minutes, quick cooled on ice, then added to the hybridization bag at 1.0 x 10⁷ CPM total radioactivity. The membrane was hybridized at 42°C for 17 hours. Afterward, the membrane was washed twice with 1x SSC, 0.1% SDS at 42°C for 15 minutes, twice with 0.1x SSC, 0.1% SDS at 65°C for 15 minutes, then exposed to XAR film for 20 hours at -70°C with intensifying screens.

Genomic-DNA Library

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 ATCC (Rose, *et al.*, 1987, ATCC Collection Number 37415). The bacterial cells harboring the library plasmids were resuspended in 5 ml LB broth and plated on LB solid medium containing 50 µg/ml ampicillin. Approximately 10,000 clones were harvested and the plasmid DNA isolated by the alkaline lysis method (Sambrook, *et al.*, 1989). pZ523 columns were used to remove

contaminating chromosomal DNA.

Chromosome Preparations and Blotting

Chromosome hybridization analysis was used to localize the *PTR2* gene to a specific *S. cerevisiae* chromosome. *S. cerevisiae* cultures were grown to a titer of $1-2.0 \times 10^8$ cells/ml in YEPD for chromosome isolation using the agarose bead entrapment method as previously described (Overhauser, *et al.*, 1987). The chromosomes were fractionated on 1.0% agarose using a Biorad CHEF-DR II gel apparatus, Pulsewave 760 switcher and Model 200/2.0 power supply. The chromosomes were electrophoresed at 14°C for 15 hours at 200 volts with a switching time of 60 seconds followed by 9 hours at 200 volts with a switching time of 90 seconds. The chromosomes were transferred to a nylon hybridization membrane then UV-crosslinked (Stratalinker, Stratagene). The chromosome blots were then probed with a ^{32}P -radiolabeled 1.64-kbp *Bam* HI - *Eco* RI DNA fragment from plasmid pJP9. The radiolabeled probe was prepared by the random primer method using a commercially available kit (United States Biochemical). 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 contain 880 λ phage clones of *S. cerevisiae* genomic DNA representing approximately 82% of the *S. cerevisiae* genome (Riles, L., and M. Olson, Washington University School of Medicine, Department of Genetics). The λ phage clone inserts of yeast DNA have been previously mapped by physical means and thereby provide an independent confirmation of chromosome assignment.

Gene Disruption

A chromosomal disruption of the *PTR2* gene was performed by one step gene disruption (Orr-Weaver, *et al.*, 1983; Figure 10). A 2.0-kbp DNA fragment carrying the *LEU2* gene of *S. cerevisiae* was excised as a *Bam* HI - *Hind* III DNA fragment from plasmid pJJ283 (Jones and Prakash, 1990). The fragment was isolated from an agarose gel, treated with T4 DNA polymerase, then blunt end ligated in place of a 653-bp deleted region of the *PTR2* protein coding sequence 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 DNA sequence that includes 495-bp of the promoter region and 1147-bp of the protein coding region of the *PTR2* gene. Plasmid pRS3062 is a derivative of plasmid pRS306 (Sikorski and Hieter, 1989) in which a unique *Aat* II restriction endonuclease site has been removed by digestion with *Aat* II, treatment with T4 DNA polymerase, then ligation. The deleted region of plasmid pJP15 was generated by restriction endonuclease digestion with *Aat* II and *Msc* I resulting in the excision of 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 plasmids, pJP23(I) and pJP23(II), were recovered in *E. coli* and the structures confirmed by restriction endonuclease analysis (Figure 5). Plasmid pJP23(I) contains the *LEU2* gene oriented so the direction of transcription parallels that of *PTR2*, whereas pJP23(II) contains the *LEU2* gene oriented so that the direction of transcription opposes that of *PTR2*. 2.6-kbp *Nde* I-

Dra I DNA fragments from both pJP23 plasmid constructs, containing the inserted *LEU2* gene flanked at both ends by *PTR2* DNA sequence, were excised and used as substrates in separate transformation experiments with the *Ptr*²⁺ strain PB1X-2A.

Leu⁺ yeast transformants were selected on SD medium supplemented with uracil, L-lysine, and L-histidine. The resulting *Leu*⁺ yeast transformants were then patched onto fresh medium 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 isolate from each transformation experiment, displaying the *Leu*⁺*Ptr*⁻ phenotype, designated PB1X-2A(Δ I) and PB1X-2A(Δ II), was considered for further characterization. The integration event at the *PTR2* locus was confirmed by Southern transfer hybridization analysis (Sambrook, *et al.*, 1989; Figure 11). Whole cell DNA was isolated from stationary cultures of PB1X-2A, PB1X-2A(Δ I), and PB1X-2A(Δ II) by the guanidine hydrochloride method (Holm, *et al.*, 1986). Aliquots of each DNA sample, containing approximately 5 μ g of DNA, were digested with *Eco* RI restriction endonuclease. The digestions were fractionated on 0.9% agarose gel (2X TAE buffer) then transferred to nylon hybridization membranes by capillary action as described (Sambrook, *et al.*, 1989). The nucleic acids were UV-crosslinked (Stratalinker, Stratagene) then prehybridized and hybridized according to the instructions provided by the membrane manufacturer. A ³²P-radiolabeled DNA probe was prepared by the random primer method. The DNA probe was denatured by incubating at 100°C for 5 minutes, quick cooled on ice, then added to the hybridization bag at 1.0 x 10⁷ CPM total radioactivity. The DNA template used in the random primer reaction was prepared by PCR amplification of a 1.2-kbp region of plasmid

pJP9. The primers used in the PCR amplification reactions, 5'-CACCACTCATATC-3' (P1) and 5'-CCGATTGGTCTTTGAATG-3' (P2), hybridize at nucleotide positions -270 and 902, respectively. The resulting radiolabeled probe is complementary to a region of the wildtype *PTR2* gene that is contained within a 4.4-kbp *Eco* RI generated DNA fragment. However, the disrupted *PTR2* locus is expected to yield two *Eco* RI generated DNA fragments capable of hybridizing with the radiolabeled probe. The PCR protocol used to generate the probe template has been previously described (Innis and Gelfand, 1990).

DNA Sequence Analysis

The nucleotide sequence of the *PTR2* gene was determined with a Sequenase kit purchased from United States Biochemical. The Sequenase kit is based on the dideoxynucleotide termination method (Sanger, *et al.*, 1977). Sequencing primers were purchased from GenoSys Biotechnologies, University of Tennessee Molecular Biology Resource Facility, Bethesda Research Laboratories, and New England BioLabs (Table 9). Computer assisted searches and analysis of nucleotide sequence data and protein structure were performed with the GCG Sequence Analysis Software Package (Devereux, *et al.*, 1984), the BLAST algorithm (Altschul, *et al.*, 1990), and the GenEMBL Data Base.

CHAPTER III

RESULTS

Isolation of *Saccharomyces cerevisiae* Strains Resistant to L-alanyl-L-ethionine

A logarithmically growing culture of *S. cerevisiae* haploid strain S288C was mutagenized with the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), as described in Materials and Methods, for the purpose of generating a large number of mutant strains deficient in the uptake of dipeptides. A cell survival frequency of five percent was chosen to assure mutagenic saturation of the genome. Following treatment with MNNG, the reaction was quenched and the cells were plated on solid MM containing L-alanyl-L-ethionine and L-histidine. The plates were then incubated at 30°C for a period of 48 hours to confirm the toxic dipeptide resistant phenotype. Afterward, a total of fifty colonies measuring more than 1mm in diameter were chosen and patched onto MM containing L-alanyl-L-ethionine and L-histidine and placed back at 30°C for a period of 48 hours. Each isolate was also plated on MM supplemented with L-ethionine and L-histidine and incubated at 30°C for 48 hours. Thirty-two of the fifty isolates resistant to the growth-inhibiting effects of L-alanyl-L-ethionine were sensitive to L-ethionine. A single isolate failed to grow at a rate comparable to the others and was, therefore, eliminated from further study. Therefore, thirty-one L-alanyl-L-ethionine resistant, L-ethionine sensitive isolates were retained for this study.

Complementation Groups

The thirty-one remaining *S. cerevisiae* isolates were placed into complementation groups in order to estimate the minimal number of genes involved in conferring resistance to toxic dipeptides. *S. cerevisiae* isolate PB1 was backcrossed with strain X2180-1A in order to ascertain whether the L-alanyl-L-ethionine resistant phenotype was due to a single defective gene or attributable to a multifactorial genetic phenomenon. The ascospore derived colonies from the cross were assayed on solid MM supplemented with L-alanyl-L-ethionine and L-histidine. The L-alanyl-L-ethionine resistant phenotype of the ascospore derived colonies segregated 2:2 in seven of seven tetrads, thereby confirming that isolate PB1 harbored a single defective locus involved in conferring resistance to toxic dipeptides. Two additional backcrosses were performed with *S. cerevisiae* tester strains X2180-1A or X2180-1B, both isogenic with the parental strain S288C, to further reduce the background mutagenic burden of the original isolate, PB1 (Figure 1). Segregation analysis of all ascospore-derived colonies from these subsequent backcrosses demonstrated 2:2 segregation patterns, with regard to resistance to L-alanyl-L-ethionine, further confirming the results obtained from the first backcross. After three rounds of backcrossing, two *S. cerevisiae* strains of opposite mating type were chosen for further experimentation, PB13X-1B (MAT α) and PB13X-1D (MATa). Both strains were shown to be resistant to a variety of toxic dipeptides while remaining highly sensitive to the corresponding toxic amino acid analogs (Table 3), had no demonstrable need for amino acid or nucleic acid supplementation in growth media, were easily cultured on routine growth media under standard laboratory conditions, and had no identifiable microscopic or macroscopic

morphologic abnormalities. The diploid strain constructed by mating PB13X-1B and PB13X-1D displayed the L-alanyl-L-ethionine resistant phenotype characteristic of the haploid strains, thereby demonstrating a failure to genetically complement. Furthermore, when PB13X-1D was mated with the parental wildtype strain of the opposite mating type, the resulting diploid displayed a toxic dipeptide-sensitive phenotype demonstrating that the mutant strain harbors a recessive genetic lesion (Table 3).

Strain PB13X-1D was mated with the remaining original isolates, PB2 through PB31, in order to ascertain genetic complementation. Zygotes from each mating were isolated by micromanipulation and incubated at 30°C for a period of 72 to 96 hours to permit colony formation. The resulting diploid colonies were then assayed for sensitivity to L-alanyl-L-ethionine and L-ethionine. All strains assayed were resistant to the growth-inhibiting effect of L-alanyl-L-ethionine with the exception of the diploid strain constructed by mating PB13X-1D and PB5. This diploid strain designated PB13X-1D/PB5 proved to be sensitive to L-alanyl-L-ethionine. This genetic complementation suggests the existence of a mutation in isolate PB5 that differs from the mutation recovered in isolate PB1. Isolate PB5 was then subjected to three rounds of backcrossing in a manner identical to that of PB1 (Figure 1). As with PB1, the L-alanyl-L-ethionine resistant phenotype conferred by the mutation recovered in PB5 segregated 2:2 in all crosses, thereby demonstrating that the resistance to L-alanyl-L-ethionine seen in this isolate is due to a defect at a single genetic locus. A single strain derived from the third round of backcrossing PB5, designated PB53X-1A, was chosen for further study. Like PB13X-1D, strain PB53X-1A was shown to be resistant to a variety of toxic dipeptides while

remaining highly sensitive to the corresponding amino acid analogs (Table 3), had no demonstrable need for amino acid or nucleic acid supplementation in growth media, was easily cultured on routine growth media under standard laboratory conditions, and had no identifiable microscopic or macroscopic morphologic abnormalities. Furthermore, the diploid strain obtained from mating PB53X-1A with tester strain X2180-1B was sensitive to L-alanyl-L-ethionine, thereby demonstrating that the mutation harbored by PB53X-1A is recessive (Table 3). In addition, the diploid strain arising from the mating of PB13X-1B and PB53X-1A was shown to be as sensitive as wildtype diploids to a variety of toxic dipeptides (Table 3). In summary, these results demonstrate that the backcrossed mutant strains obtained from the mutagenesis procedure harbor recessive mutations and that genetic complementation was retained throughout the backcrossing procedures. Thus, the L-alanyl-L-ethionine resistant isolates obtained from the mutagenesis experiment define two genetic complementation groups affecting the transport of dipeptides across the plasma membrane.

To better define the number of genetic loci involved in conferring resistance to toxic dipeptides, complementation analysis was performed between *S. cerevisiae* strains PB13X-1D, PB53X-1A, and a group of previously isolated *S. cerevisiae* strains known to be resistant to the growth inhibiting effects of toxic dipeptides (Island, 1987). The strains, LeuAE-1, LeuLE-1, and HisLE-1, were isolated as spontaneously occurring toxic dipeptide resistant colonies. The diploids resulting from pairwise matings between the mutants obtained from mutagenesis experiments and the spontaneously occurring mutants were tested for sensitivity to toxic dipeptides using disk assays (Materials and Methods;

Table 8). Strain LeuLE-1 complemented the toxic peptide resistant phenotypes of both PB13X-1D and PB53X-1A yielding diploid strains sensitive to the toxic dipeptides tested. Thus, strain LeuLE-1 defines a complementation group not represented by PB13X-1D or PB53X-1A. Similar experiments demonstrated that strain LeuAE-1 complemented PB53X-1A yielding a diploid sensitive to toxic dipeptides, but failed to complement the toxic peptide resistant phenotype of PB13X-1D. These results demonstrate that LeuAE-1 and PB13X-1D represent strains harboring genetic lesions that define a second complementation group. In addition, strain HisLE-1 was shown to complement PB13X-1D but not to complement PB53X-1A, thereby defining a third complementation group. Collectively, all existing *S. cerevisiae* strains resistant to the growth-inhibiting effects of toxic dipeptides fall into one of three complementation groups designated as *ptr1*, *ptr2*, or *ptr3*. The genetic nomenclature, designated *ptr* for peptide transport deficient, was chosen to reflect the physiological function encoded by the three genes defined by the complementation groups. In summary, *S. cerevisiae* strain LeuLE-1 carries a mutant allele designated *ptr1-1*, LeuAE-1 and PB13X-1B carry mutant alleles designated *ptr2-1* and *ptr2-2*, respectively, and HisLE-1 and PB53X-1A carry mutant alleles designated *ptr3-1* and *ptr3-2*, respectively. The allele numbers are assigned to distinguish between the mutant alleles carried in the various strains. The allele numbers do not necessarily imply the existence of different genetic lesions in the mutations described but rather reflect differences in the origins of the mutations.

The complementing diploids constructed between haploid strains obtained from mutagenesis experiments and spontaneously occurring mutants displayed significantly less

sensitivity to L-alanyl-L-ethionine than that observed in wildtype diploids. The level of sensitivity in these diploid strains was also significantly less than that observed in diploids heterozygous at a single genetic locus involved in toxic dipeptide resistance. The level of sensitivity to L-alanyl-L-ethionine of complementing diploids heterozygous at a single genetic locus did not differ significantly from that observed in wildtype diploids. These results suggest that while complementation may confer sensitivity in diploids heterozygous at two genetic loci involved in resistance to toxic dipeptides, the degree of complementation is not complete.

Radiolabeled Dileucine Uptake

The cellular accumulation of radiolabeled dileucine was used as a direct measure of peptide transport in wildtype and L-alanyl-L-ethionine resistant strains of *S. cerevisiae*. It has been previously shown that strains resistant to toxic dipeptides fall into two categories relative to cellular radiolabeled dileucine accumulation. One category, characterized by strains LeuLE-1 (*ptr1-1*) and LeuAE-1 (*ptr2-1*), is completely deficient in the accumulation of radiolabeled dileucine, while the second category, characterized by strain HisLE-1 (*ptr3-1*), is able to accumulate radiolabeled dileucine, but at levels much reduced from that of wildtype strains (Island, 1987). In similar radiolabeled dileucine uptake experiments, strains PB13X-1D (*ptr2-2*) and PB53X-1A (*ptr3-2*) were shown to be deficient in the accumulation of radioactive dileucine (Figure 2). Strain PB13X-1D (*ptr2-2*) demonstrated a complete failure to accumulate the radioactive substrate as seen with strains LeuLE-1 (*ptr1-1*) and LeuLA-1 (*ptr2-1*), whereas PB53X-1A (*ptr3-2*) accumulated the substrate at

an intermediate level similar to that reported for HisLE-1 (*ptr3-1*). These results are in good agreement with those obtained from complementation analysis and demonstrate that resistance to toxic dipeptides and failure to accumulate radiolabeled dileucine are related phenomena attributable to the inability of cells that harbor *ptr* mutant alleles to transport dipeptides across the plasma membrane at wildtype levels.

Utilization of Dipeptides as Substrates in Growth Assays

Yeast strains carrying *ptr1*, *ptr2*, or *ptr3* mutations were tested for the ability to utilize a variety of dipeptides in meeting the amino acid requirements imposed by the *leu2-3*, *lys1-1*, and *his4-38* mutations. *S. cerevisiae* strains LeuLE-1 (*MAT α ptr1-1*), PB13X-1D (*MAT α ptr2-2*), and PB53X-1A (*MAT α ptr3-2*) were crossed with strain XL70-5C (*MAT α ura3-52 leu2-3 lys1-1 his4-38*) or XL70-1B (*MAT α ura3-52 leu2-3 lys1-1 his4-38 trp1-289*). Zygotes were isolated by micromanipulation and the resulting diploids sporulated. Four-spored asci were dissected, and the resulting spore colonies tested for amino acid requirements and sensitivity to L-alanyl-L-ethionine. Three strains, PB4X-2B (*ptr1-1*), PB1X-9B (*ptr2-2*), and PB2X-4D (*ptr3-2*), carrying multiple amino acid auxotrophic mutations and their wildtype counterparts were chosen from each cross for further analysis. Each strain was tested for its ability to use dipeptides as a source of amino acids in growth assays (Table 4). Strain PB4X-2B (*ptr1-1*) and PB1X-9B (*ptr2-2*) failed to grow when placed on solid media containing dipeptides in the place of required amino acids, whereas the wildtype counterparts were able to use dipeptides as sources of amino acids in satisfying growth requirements. However, strain PB2X-4D (*ptr3-2*) was

able to use all dipeptides tested in satisfying the auxotrophic growth requirements imposed by the *leu2-3*, *lys1-1*, and *his4-38* mutations. These results demonstrate that the peptide transport deficient phenotype conferred by the mutant *ptr1* and *ptr2* genes impairs the ability of *S. cerevisiae* to utilize a wide variety of dipeptides in growth assays. However, while the *ptr3-2* allele confers resistance to L-alanyl-L-ethionine, it fails to eliminate completely the transport and utilization of dipeptides in growth assays. These results are in good agreement with the radiolabeled dileucine uptake data which show strains harboring *ptr1* and *ptr2* mutations to be completely deficient in the uptake of dipeptides, while strains carrying the *ptr3-2* mutation display an intermediate phenotype.

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 parent vector, YCp50, is an *E. coli* / *S. cerevisiae* shuttle vector. It contains the *S. cerevisiae* *CENIV* element which confers mitotic stability and low copy number (Mann and Davis, 1986) and the *URA3* gene which is selectable in *S. cerevisiae* *Ura3⁻* strains (Rose, *et al.*, 1984). The parent plasmid also contains the *colE1* ori and the *amp^R* gene which function in plasmid replication and selection, respectively, in *E. coli*. The low copy number and mitotic stability conferred by the *CENIV* element reduces the chances of obtaining spurious results due to gene dosage effects. In addition, the YCp50-based library plasmids contain insert-DNA fragments are

derived from the same genetic background as the original peptide transport mutants. The likelihood of cloning a suppressor when both the mutants and the DNA library are derived from the same genetic background is diminished since the presence of such a suppressor would have masked the mutant phenotype, thereby making the isolation of the original mutant strains difficult. 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 pathway (Island, *et al.*, 1987) 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 five days.

Whole-cell DNA was isolated from both primary yeast transformants and the transforming plasmids 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, yielded identical restriction patterns when digested with *Pst* I and *Bam* HI. Plasmid pJP2 was then reintroduced into strain 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 7). In addition, PB1X-9B[pJP2] was able to transport radiolabeled dileucine at levels

approximating wildtype while the control strain, PB1X-9B[YCp50], failed to accumulate the radiolabeled substrate (Figure 3). These results show the Ptr^+ transformant to have the expected wildtype phenotype and that the *ptr2-2* complementing activity is plasmid associated and not due to a reversion event at the *ptr2* locus.

Localization of the *ptr2-2* Complementing Region

To define more precisely the *ptr2-2* complementing region of pJP2, a variety of internal deletions within the cloned insert DNA were constructed (Figure 4). The deletions were constructed by digesting plasmid pJP2 with the appropriate restriction endonuclease(s) then ligating under conditions favoring intramolecular recircularization. The ligation products were recovered in *E. coli* HB101, characterized with regard to endonuclease restriction fragment patterns, then reintroduced into mutant yeast strain PB1X-9B. The resulting yeast transformants were then examined for growth on a variety of dipeptides and for sensitivity to L-alanyl-L-ethionine (Table 7). 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 allowed one functional endpoint to be localized within a 0.4-kbp *Hind* III-*Bam* HI DNA fragment. The complementing region extends toward the insert-vector junction past an *Eco* RI site proximal to the *URA3* gene (Figure 4).

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 (Figure 4). 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 followed by end-filling the linear DNA molecules with the large fragment of *E. coli* DNA polymerase I (Klenow fragment) and deoxynucleotide triphosphates, and ligation. The resulting plasmids were recovered in *E. coli* and the frameshift mutations confirmed by the loss of the appropriate restriction site (Figure 5). Each plasmid harboring a frameshift mutation was then used to transform the mutant yeast strain PB1X-9B. The resulting yeast transformants were assayed for their ability to grow on dipeptides and for sensitivity to L-alanyl-L-ethionine (Table 7). 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 since 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 (Figure 4, Table 7). To demonstrate that full activity was restored by this plasmid, quantitative disk assays were performed to assess sensitivity of the transformant to L-alanyl-L-ethionine and a series of oxalysine-containing peptides (Table 5). The results showed PB1X-9B[pJP9] to be as sensitive as the the wildtype control, PB1X-2A[YCp50], to L-alanyl-L-ethionine, while the negative control, PB1X-9B[YCp50], remained highly resistant to the growth-inhibiting effects of the toxic dipeptide. The transformant, PB1X-9B[pJP9], and the wildtype

control produced similar disk assay halo diameters in response to oxalysine-containing di- and tripeptides (Table 6). 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, little difference in sensitivity to L-alanyl-L-ethionine was observed when the *ptr2-2* complementing region of pJP9 was transferred as a 3.3-kbp *Hind* III - *Sal* I DNA fragment to a high copy, 2 μ based, *S. cerevisiae* shuttle vector (data not shown). The growth of the strain containing the high copy plasmid demonstrates that *PTR2* is not lethal when present in high dosage.

Nucleotide and Deduced Amino Acid Sequence 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 3.15-kbp insert of plasmid pJP9 (Figures 6, 7, 8, and 9). The DNA sequence of the 3.1-kbp insert of pJP9 contains a 1803-bp open reading frame preceded by a 896-bp leader sequence that deletion studies showed necessary for expression. All other open reading frames are extensively blocked by termination codons. The 1803-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 1147-bp and 1439-bp downstream, respectively. These findings are in agreement with the results obtained from the deletion and frameshift analysis 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.

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 (Figure 5; Struhl, 1989). The TATAAAA element at position -65 is followed by an 83% AT-rich sequence spanning the 59-bp region to the first ATG. The immediate upstream region contains the poly(dA-dT) sequence AATTATTTTTTTTTTCTTCTTTT beginning at nucleotide position -51. Poly(dA-dT) sequences greater than 10-bp in length are common to many *S. cerevisiae* promoters and have been implicated in constitutive gene expression (Struhl, 1989). The sequence CCAATCA, which is identical to the CCAAT element found in many eucaryotic gene promoters (Johnson and McKnight, 1989), is present on opposite DNA strands at nucleotide positions -278 and -319 in the promoter region of the *PTR2* gene. The significance of this promoter element is not known. The nucleotide at position -3 is A in *PTR2* as is usually found in eucaryotic mRNAs (Cigan, *et al.*, 1987). The sequence surrounding the start codon is CTTATAATG which strongly resembles the consensus sequence(A/Y)A(A/Y)A(A/Y)AATG (Hinnebusch, 1991) surrounding other *S. cerevisiae* initiation codons, differing only at the -5 position. In addition to the promoter elements mentioned above, there is a large, inverted DNA sequence repeat near the *Bam* HI site beginning at nucleotide position -488, that reads as follows:

CGaaGAACaggaAAAAGGACaaccGTCCTTTTcacTTCaCG.

This element may be of regulatory significance since it lies immediately adjacent to a region shown by deletion studies to be required for expression of the plasmid borne *PTR2* gene. 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 under general control (Hinnebusch, 1986) or elements that function in the nitrogen catabolite repression of genes involved in the allantoin utilization pathway in *S. cerevisiae* (Bysani, *et al.*, 1991). 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 (Langford and Gallwitz; 1983; Pikielny, *et al.*, 1983).

The 1803-bp open reading frame encodes a predicted protein with a calculated monomer molecular mass of 68.1-kDa and a pI of 5.14. The predicted amino acid sequence suggests a highly hydrophobic protein consisting of 48.4% nonpolar, 32.4% polar, 9.15% basic, and 10.0% acidic amino acid residues. The penultimate amino terminus 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 (Hinnebusch, *et al.*, 1991).

The *S. cerevisiae* consensus tripartite transcription termination and polyadenylation signal, TAG...TA(T)G...TTT (Zaret and Sherman 1982), is not found in the immediate 3' flanking region of the *PTR2* gene. However, the sequence TTG...(28-bp)...TATGTA...(33-bp)...TTT, which strongly resembles the tripartite element, is present beginning at 142-bp down stream of the stop codon (Figure 9). In addition, the tripartite element,

TAG...(18-bp)...TAGT...(24-bp)...TTT, is found 381-bp down stream of the termination codon (Figure 9). While these elements might suggest that two mRNA species are transcribed from the *PTR2* gene, Northern blots consistently demonstrate a single transcript of approximately 1.8-kb in length (Figure 15), supporting the first element as the probable transcription termination site. The polyadenylation motif, AATAAA, frequently found associated with eucaryotic genes (Proudfoot, 1991) is not present in the *PTR2* 3' flanking region. Also absent is the sequence TTTTAT which has been shown to play a role in the unidirectional polyadenylation of several yeast genes (Irniger, *et al.*, 1991).

A search of the GenEMBL 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 (Figure 9; Ozkaynak, *et al.*, 1987). The two genes are oriented so that they are transcribed convergently with the termination codons being separated by a 466-bp region (Figure 11). When combined, the two sequences yield a detailed map of a 4.27-kbp region of the right arm of chromosome XI. Furthermore, a search of the GenBank database using the BLAST algorithm (Altshul, *et al.*, 1990) revealed similarities in the amino acid sequences between the proteins encoded by the *PTR2* gene of *S. cerevisiae* and the *CHL1* gene of *Arabidopsis thaliana*. The *CHL1* gene is involved in herbicide sensitivity and nitrate-inducible transport of nitrate (Figure 12).

Chromosomal Assignment of the *PTR2* Gene

S. cerevisiae chromosomes were prepared for electrophoresis by entrapment in

agarose beads (Materials and Methods). The chromosomes were fractionated on a contour-clamped homogeneous electric field gel then transferred and UV crosslinked to a nylon membrane. Plasmid pJP9 was radiolabeled by the random primer method and used as a probe for chromosome hybridization (Figure 13, panels A and B). Plasmid pJP9 is composed, in part, of four *S. cerevisiae* DNA sequences: *CEN IV*, *ARS1*, *URA3*, and *PTR2*. As expected, the radiolabeled probe hybridized to Chromosome IV(*CEN IV* and *ARS1*) and chromosome V. In addition to Chromosomes IV and V, hybridization to Chromosome XI was also noted on autoradiograms. Sequences hybridizing to *CEN IV/ARS1* (Mann and Davis, 1986) and *URA3* (Rose, *et al.*, 1984) were included as controls since the locations of these sequences within the *S. cerevisiae* genome are well documented. To confirm the conclusions drawn above, 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 (Figure 13, panels C, D, and E; Hannig and Hinnebusch, 1988). In duplicate experiments, the *PTR2* and *GCN3* probes hybridized to the same chromosome, thereby confirming the Chromosome XI assignment.

To map the *PTR2* gene more precisely, a series of filters containing 880 recombinant λ phage clones (Riles, L., and M. Olson, Washington University School of Medicine, Dept. of Genetics), representing 82% of the yeast genome, were probed with the same radiolabeled 1.64-kbp *Bam HI* - *Eco RI* DNA fragment of the *PTR2* gene. The probe hybridized to three overlapping λ phage clones: #3536, #3836, and #5620. The

phage clones have a 7.6-kbp region of DNA in common and all map to the right arm of Chromosome XI approximately 592-kbp from the centromere and distal to the *tif1* marker. The three λ phage clones have DNA from a 2.05-kbp *Hind* III - *Eco* RI fragment in common that is similar to a 2.04-kbp *Hind* III - *Eco* RI fragment produced from plasmid pJP9. No other genes have been mapped to these phage clones or to this region of Chromosome XI.

***PTR2* Gene Disruption**

In order to obtain a stable peptide transport mutant and to address whether or not *PTR2* is an essential gene, a chromosomal deletion of the *PTR2* gene was generated by one-step gene disruption (Rothstein, 1983). 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 was deleted then replaced by blunt end ligation with a 2.0-kbp DNA fragment containing the *LEU2* gene of *S. cerevisiae* (Figure 10; Materials and Methods). Plasmids pJP23(I) and pJP23(II) were recovered from the ligation pool in *E. coli*. The disruption plasmids differ only in the orientation of the *LEU2* gene. The disrupted copies of the *PTR2* gene were excised from both plasmid constructions as 2.6-kbp *Nde* I/*Dra* I fragments which were 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 then 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. Two $\text{Leu}^+ \text{Ptr}^-$ transformants, designated PB1X-2A(Δ I) and PB1X-2A(Δ II), were chosen for further characterization. The alteration at the *PTR2* locus in the two transformants was confirmed by Southern hybridization analysis (Figure 11; Materials and Methods). Whole cell DNA isolated from the nondisrupted wildtype strain, PB1X-2A, yielded a single 4.4-kbp *Eco* RI generated DNA fragment detected by Southern hybridization. Transformants PB1X-2A(Δ I) and PB1X-2A(Δ II) yielded 1.4-kbp and 1.2-kbp *Eco* RI DNA fragments, respectively, in addition to a 4.4-kbp DNA fragment similar to that seen in the wildtype strain. The Southern hybridization results are in agreement with the predicted structures of the *PTR2* loci of the two $\text{Leu}^+ \text{Ptr}^-$ transformants. The disruption strain, PB1X-2A(Δ II), failed to accumulate L-lysyl-L-[^3H]leucine in radiolabeled dipeptide uptake experiments (data not shown). The disruption strain was then crossed with a wildtype *S. cerevisiae* strain, XL70-1B, the diploids sporulated, and the tetrads dissected. The resulting haploid strains were then assayed for sensitivity to L-alanyl-L-ethionine and for the Leu^+ phenotype. The $\text{Leu}^+ \text{Ptr}^-$ phenotype segregated 2:2 in ten tetrads. These results demonstrate that the disrupted copy of the *PTR2* gene integrated at the *PTR2* locus and, because the deletion is viable in a haploid, that *PTR2* is not an essential gene as defined by standard laboratory growth conditions.

CHAPTER IV

DISCUSSION

To facilitate the genetic study of peptide transport in *Saccharomyces cerevisiae*, a wildtype haploid strain, S288C (Table 1), was mutagenized with the methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in order to generate a large number of mutant strains deficient in the transport of dipeptides across the plasma membrane. MNNG induces predominantly GC to AT transition mutations with the principle mutagenic lesions being O⁶-methylguanine, 3-methyladenine, 3-methylguanine, and 7-methylguanine. To select for mutants deficient in peptide transport, the mutagenized cells were plated onto solid medium containing L-leucine and L-alanyl-L-ethionine, a dipeptide containing the toxic amino acid analog L-ethionine. The mode of action of L-ethionine in fungi is not certain; however, given the close structural similarity with L-methionine, it may function as an antimetabolite by interfering with amino-acid utilization or protein synthesis and function (Gehrmann, et al., 1989) or by interfering with biochemical methylation reactions involving S-adenosyl-L-methionine (Kajander, et al., 1986; Hancock, 1987). L-alanyl-L-ethionine inhibits the growth of wildtype *S. cerevisiae* strains which are capable of transporting the dipeptide across the outer cellular membrane. Leucine is one of many amino acids that has been shown to induce the uptake of peptides in *S. cerevisiae* (Island, et al., 1987). Thus, those mutagenized cells with biochemical defects in the transport of dipeptides are able to grow in the presence of L-alanyl-L-

ethionine, yet remain highly sensitive to the analog L-ethionine. Sensitivity to the toxic amino acid analog is a requirement for studying peptide specific uptake. This selection method resulted in the isolation of fifty L-alanyl-L-ethionine resistant strains from the mutagenized population of yeast cells. Only the largest yeast colonies were chosen from the selection plates. This selection bias was introduced in order to eliminate from study isolates with poor growth characteristics due to heavy mutation burdens. Of the fifty original isolates, thirty-one were found to be resistant to L-alanyl-L-ethionine, while remaining sensitive to L-ethionine, and were therefore retained for further study. Those yeast isolates resistant to L-ethionine were excluded from this study.

The thirty-one toxic-dipeptide resistant yeast isolates, designated PB1 through PB31, were grouped with three previously isolated yeast strains known to be resistant to toxic dipeptides (Island, *et al.*, 1991). These three additional haploid strains, LeuLE-1, LeuAE-1, and HisLE-1, were originally selected from a nonmutagenized population and, therefore, had the potential to expand the mutation spectrum of the mutant population. Furthermore, all isolates were derived from a common ancestral strain, thereby making phenotypic variability due to genetic background differences less likely a problem in a comparative genetic analysis. The three naturally-occurring mutant strains were chosen for inclusion in this study due to the complementing nature of their genetic lesions, which suggests that each of the three strains harbors a mutation in a different gene involved in dipeptide transport. Genetic complementation among the thirty-one isolates from the mutagenized population was ascertained by screening diploids, constructed by pairwise mutant matings, for resistance to L-alanyl-L-ethionine. A single mutant isolate, PB1, was

backcrossed three times, using wildtype isogenic strains X2180-1A (*MATa*) and X2180-1B (*MATα*) as maters (Figure 1), into a genetic background of opposite mating type. A minimum of seven tetrads were dissected in each backcross, and in each case the L-alanyl-L-ethionine phenotype segregated 2:2 suggesting that the original mutant strain harbored a monogenic mutation affecting the transport of dipeptides. The mutation present in PB13X-1D was shown to be recessive by the loss of L-alanyl-L-ethionine resistance in the heterozygous diploid strain PB13X-1D/X2180-1B (Table 3). Pairwise mating were then performed between this backcrossed mutant strain, PB13X-1D, and all other remaining mutants. The resulting diploids were assayed for L-alanyl-L-ethionine sensitivity using a quantitative disk assay as previously described (Island, *et al.*, 1987). Genetic complementation was established in only one of the pairwise matings. Diploid strain PB13X-1D/PB5 was shown to be sensitive to L-alanyl-L-ethionine while all other diploids remained resistant. Therefore, it was concluded that haploid strains PB5 and PB13X-1D harbored nonallelic mutations that affect the transport of dipeptides across the plasma membrane. Furthermore, it was concluded that all of the remaining mutant isolates harbored at least a single mutation affecting the dipeptide transport system allelic to the lesion present in strain PB13X-1D. Yeast isolate PB5 was subjected to three rounds of backcrossing as previously performed for PB1 (Figure 1). In each backcross the L-alanyl-L-ethionine resistant phenotype segregated 2:2 demonstrating that the defect in dipeptide transport in this mutant is due to a mutation at a single genetic locus. In a similar manner, the mutation in PB53X-1A was shown to be recessive by the loss of resistance to L-alanyl-L-ethionine in the heterozygous diploid strain PB53X-1A/X2180-1B (Table 3). Therefore,

it was concluded that the mutants derived from the mutagenized population define two complementation groups affecting the transport of dipeptides across the plasma membrane of *S. cerevisiae*.

To better characterize the genetic defects present in the naturally-occurring isolates, LeuLE-1, LeuAE-1, and HisLE-1 were crossed with the isogenic strain X2180-1A. The resulting diploids were sporulated and the L-alanyl-L-ethionine resistant phenotype shown to segregate 2:2 in the resulting spore colonies, thereby demonstrating that each strain harbored a recessive genetic lesion affecting dipeptide transport. Furthermore, complementation analysis between the naturally occurring and MNNG induced mutants (Table 8) showed strain LeuLE-1 to harbor a unique mutation, designated *ptr1-1*, that complemented the lesions present in strains PB13X-1D and PB53X-1A. Strain LeuAE-1 failed to complement the defect present in PB13X-1D suggesting that these two strains harbor allelic mutations, designated *ptr2-1* and *ptr2-2*, respectively. Likewise, the mutation present in HisLE-1 failed to complement the lesion present in PB53X-1A suggesting that these two strains also harbor allelic mutations, designated *ptr3-1* and *ptr3-2*, respectively. *S. cerevisiae* strains harboring *ptr1* and *ptr3* mutant alleles tend to produce smaller colonies than those strains carrying *ptr2* mutations. This observation may account for the failure to select any *Ptr1*⁻ isolates and a single *Ptr3*⁻ isolate from the mutagenized population since only isolates from large, well formed colonies were chosen for inclusion in this study. In contrast, the previously isolated spontaneously occurring *Ptr*⁻ yeast strains were selected without regard to colony size or morphology, and this method of selection likely accounts for the difference in the allelic profile between the two groups of

mutants. Genetic complementation between the mutagen induced and naturally occurring mutants was not complete in any of the crosses examined (Table 8). The L-alanyl-L-ethionine sensitive diploid strains produced from these crosses consistently displayed smaller zones of inhibition than those of the wildtype diploid controls. These observations may reflect differences in the genetic backgrounds of the strains. In summary, complementation analysis of naturally occurring and MNNG induced L-alanyl-L-ethionine resistant isolates of *S. cerevisiae* showed dipeptide transport in *S. cerevisiae* to be mediated by products encoded by at least three genes: *PTR1*, *PTR2*, and *PTR3*.

Wildtype yeast strains have the ability to accumulate radiolabeled dileucine when grown in the presence of a nonrepressive nitrogen source and amino acids (Island, *et al.*, 1987). The inability of strains LeuLE-1 (*ptr1-1*), LeuAE-1 (*ptr2-1*), and HisLE-1 (*ptr3-1*) to accumulate radiolabeled dileucine at wildtype levels is a manifestation of the peptide transport deficient phenotype. Similarly, strains PB13X-1D (*ptr2-2*) and PB53X-1A (*ptr3-2*) were shown to be deficient in the uptake of radiolabeled dileucine (Figure 2). Strain PB13X-1D was not able to accumulate any radioactive dipeptide above background levels, whereas PB53X-1A was able to accumulate the radioactive substrate, but at levels much reduced from that seen in wildtype strain S288C. The radiolabeled dileucine uptake profiles appear comparable for strains LeuLE-1, LeuAE-1, and PB13X-1D. Likewise, strains HisLE-1 and PB53X-1A have similar uptake profiles. These results are in agreement with the complementation analysis which show LeuAE-1 and PB13X-1D to be members of the same complementation group, *ptr2*. Likewise, the similar radiolabeled dileucine uptake profiles of HisLE-1 and PB53X-1A agree with the conclusion that they

belong to the same complementation group, *ptr3*. Strain LeuLE-1 has a radiolabeled dileucine uptake profile that is identical to that of LeuAE-1 and PB13X-1D. Yet, LeuLE-1 defines a complementation group, *ptr1*, that is unique and not represented by any of the isolates from the mutagenized population. The failure to recover any Ptr1^- strains from the mutagenized population is most likely due to the previously mentioned selection bias, rather than any mutagen specific effect.

Previous studies suggest that wildtype *S. cerevisiae* strains have the ability to utilize di-, tri-, and to a lesser extent, tetrapeptides as sources of amino acids (Naider, *et al.*, 1974; Marder, *et al.*, 1977). In order to ascertain the ability of the *ptr* mutants to utilize dipeptides as a source of amino acids, the *ptr1-1*, *ptr2-2*, and *ptr3-2* mutations were crossed into genetic backgrounds containing multiple amino acid auxotrophic mutations. Each resulting strain was then tested for its ability to use four dipeptides as sources of amino acids in growth assays. Strains harboring *ptr1* or *ptr2* mutations were found to be unable to use any of the dipeptides tested as a source of amino acids in meeting growth requirements (Table 4). However, a strain carrying the *ptr3-2* mutation was shown to utilize dipeptides to satisfy the amino acid requirements imposed by the *leu2-3* and *lys1-1* mutations (Table 4). The inability to use native dipeptides to meet amino acid requirements is an additional characteristic conferred by *ptr1* and *ptr2* mutations. The results from growth assays are in good agreement with previous experimental results which show strains carrying a *ptr1-1*, or *ptr2-2*, mutation to be completely deficient in the transport of dipeptides while strains carrying the *ptr3-2* mutation are able to transport dipeptides, but at a reduced level.

In summary, mutations in the *PTR1* and *PTR2* genes appear to completely abolish dipeptide transport in *S. cerevisiae*. Strains harboring *ptr1* or *ptr2* mutant alleles appear to display identical phenotypes. In addition to being resistant to toxic dipeptides, *Ptr1*⁻ and *Ptr2*⁻ strains fail to accumulate radiolabeled dileucine in uptake experiments and they are not able to use dipeptides to satisfy the amino acid requirements imposed by the *leu2-3*, *lys1-1*, and *his4-38* mutations. In contrast, mutations in the *PTR3* gene confer an intermediate phenotype characterized by resistance to toxic dipeptides, low levels of radiolabeled dileucine accumulation in uptake experiments, and the ability to use dipeptides as sources of amino acids in growth assays. These observations suggest that while resistance to L-alanyl-L-ethionine is useful in characterizing the degree of impaired dipeptide transport in mutant *S. cerevisiae* strains, the ability to utilize dipeptides as a source of amino acids in growth assays is more sensitive in distinguishing between the two phenotypic classes of mutants. A low level of dipeptide transport in *Ptr3*⁻ mutants may permit these cells to transport enough native dipeptide to accumulate adequate levels of intracellular amino acids for growth while being sufficiently impaired to prohibit high levels of toxic dipeptides from accumulating. Alternately, a difference in affinity for toxic dipeptides and native dipeptides could also account for the intermediate phenotype displayed by *Ptr3*⁻ strains, but this explanation implies a degree of substrate specificity that is not typical of peptide transporters of any organism thus far examined. Given the identical phenotypes conferred by the *ptr1-1* and *ptr2-2* mutations, it seems likely that both wildtype counterpart genes encode products that play essential roles in the process of transporting dipeptides across the plasma membrane; whereas, the *PTR3* gene may encode

a regulatory product given the pleiotropic phenotype, the concomitant reduction in sensitivity to toxic dipeptides and their amino acid analogs, conferred by the *ptr3-2* mutation. However, conclusions regarding the roles played by the respective *PTR* genes in the transport of dipeptides across the plasma membrane is dependent on their isolation and characterization.

The phenotypic characteristics conferred by these two classes of peptide transport mutants suggest two possible means by which to clone the respective *PTR* genes. Both methods involve plasmid-mediated complementation of the Ptr^- phenotype of the mutant strain under study. The first method requires the selection of plasmid-bearing cells followed by a simple screen for assessing restoration of sensitivity to toxic dipeptides. This method has the advantage of permitting a precise count of plasmid-bearing clones examined, so that DNA library equivalents may be quantitated and appropriate endpoints instituted for limiting the phenotypic screen. However, it has the disadvantage of requiring large amounts of toxic dipeptides, which are limited and difficult to obtain commercially, and is time consuming. The second approach involves a one-step, double selection procedure that does not require a phenotypic screen for identifying yeast transformants harboring plasmids carrying wildtype *PTR* genes. This method is limited to *ptr* mutations that completely abolish peptide transport activity and requires that the *ptr* mutation of interest be crossed into a genetic background containing a genetic marker for plasmid selection in *S. cerevisiae*, usually *ura3-52* or *leu2-3*, and several auxotrophic amino acid mutations, such as *leu2-3*, *lys1-1*, *his4-38*, and *trp1-289*. For example, *S. cerevisiae* strains PB4X-2B (*ura3-52 leu2-3 lys1-1 his4-38 ptr1-1*) or PB1X-9B (*ura3-52 leu2-3*

lys1-1 his4-38 ptr2-2) could be transformed with a DNA-library carrying a vector borne copy of the selectable *URA3* gene and then plated on minimal medium supplemented with L-lysyl-L-leucine and L-histidine. The L-lysyl-L-leucine would serve as a source of L-lysine and L-leucine in those transformants harboring a plasmid borne copy of the *PTR* gene of interest. The addition of L-histidine to the selective medium serves to satisfy the requirement imposed by the *his4-38* mutation and also to induce the peptide transport system. The lack of uracil in the medium ensures that only cells harboring a plasmid borne copy of the selectable *URA3* gene will have the potential for growth. This double selection scheme increases the stringency of the selection system by ensuring that only cells harboring a plasmid-borne copy of the *PTR1* or *PTR2* gene will grow on the dipeptide medium. This approach has the potential of reducing the chances of recovering spontaneously occurring Ptr^+ revertants. The molecular cloning of the *PTR3* gene may require the use of the former method of screening individual yeast transformants for restoration of sensitivity to toxic dipeptides. The intermediate phenotype conferred by the *ptr3-2* mutation prohibits a direct selection for the ability to use dipeptides. Further characterization of the strains harboring *ptr3* alleles will be necessary in order to define what growth requirements, if any, will permit the formulation of a method for directly selecting for plasmid borne copies of the *PTR3* gene.

To ascertain the feasibility of the one-step, double selection system, the peptide transport deficient strain PB1X-9B (*MATa ura3-52 leu2-3 lys1-1 his4-38 ptr2-2*) was transformed with a YCp50 based *S. cerevisiae* genomic-DNA library and the cells plated on solid minimal medium containing L-lysyl-L-leucine and L-histidine. The YCp50 library

plasmids are mitotically stable and low copy, which closely mimics the *in vivo* situation and improves the chances of recovering genes that may be lethal in high copy plasmids (Rose, *et al.*, 1987). L-lysyl-L-leucine served as a source of L-lysine and L-leucine in those transformants able to transport the dipeptide across the plasma membrane while L-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 (Island, *et al.*, 1987). The uracil requirement of PB1X-9B was satisfied by the selectable *URA3* gene present on the library vector, YCp50. Two yeast transformants from independent transformation experiments, harboring identical plasmids designated pJP1 and pJP2, were recovered and tested for the *Ptr*⁺ phenotype. Yeast transformants harboring plasmid pJP2 displayed the expected wildtype characteristics when tested for the ability to accumulate radiolabeled dileucine, growth on defined dipeptides, and for sensitivity to L-alanyl-L-ethionine (Figure 4, Table 7). Secondary yeast transformants harboring plasmid pJP2 also demonstrated the *Ptr*⁺ phenotype, demonstrating that the complementing activity was plasmid-borne.

To better localize the *ptr2-2* complementing region of plasmid pJP2, a series of plasmid deletions and frameshift mutations were generated and tested for the ability to restore dipeptide utilization in growth assays (Figure 4, Table 7). The results showed the *ptr2-2* complementing activity of pJP2 to span a 3.1-kbp region near the insert-vector junction site proximal to the vector-borne *URA3* gene. DNA sequencing of this region revealed a 1803-bp open reading frame encoding a predicted protein of 601 amino acids

with a secondary structure typical of many membrane associated transport proteins (Figures 6, 7, 8, and 9; Weber *et al.*, 1988; Serrano, 1991; Higgins, 1992; Fath and Kolter, 1994). Computer-assisted predictions show the primary structure to consist of a hydrophilic amino terminus spanning approximately 80 amino acids followed by an alternating pattern of hydrophobic and hydrophilic regions then terminating with a hydrophilic carboxy region spanning approximately 50 amino acids (Figure 8). Ten of the hydrophobic regions, designated hydrophobic domains II, III, IV, V, VI, VIII, IX, X, XI, and XII, beginning at amino acid positions 128, 157, 187, 240, 271, 389, 421, 466, 500, and 531 respectively, are twenty one amino acids in length, or longer (Figure 8). All of them have a hydrophobicity index of greater than 1.1 (Kyte and Doolittle, 1982) with the exception of domain II which has a hydrophobicity index of 0.71. Hydrophobic domains I and VII are fourteen and thirteen amino acids in length, respectively, and have hydrophobicity indices of 1.1 and 1.3. The hydrophilic leader sequence and the alternating pattern of hydrophobic domains flanked by hydrophilic regions observed in the *PTR2* encoded protein is typical of membrane associated proteins thought to be involved in transmembrane ligand transport (Figure 12; Weber, *et al.*, 1988; Serrano, 1991; Higgins, 1992). It is, therefore, not surprising to find such a predicted structure for the *PTR2* encoded protein since it is clearly implicated in the transport of dipeptides across the plasma membrane. A search of the GenEMBL Data Base using the BLAST algorithm revealed significant similarities between the *PTR2* encoded protein and that encoded by the *CHL1* gene of *Arabidopsis thaliana*. (Figure 12). The *CHL1* gene of *A. thaliana* is involved in herbicide sensitivity and nitrate transport across the plasma membrane of plants

(Tsay, *et al.*, 1993). The predicted *CHL1* protein is postulated to encode a plasma membrane associated, electrogenic nitrate transporter that is regulated by environmental pH and nitrate availability.

Computer assisted predictions of secondary structures (Chou and Fasman, 1974, 1978) suggest that the twelve hydrophobic regions are composed predominately 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 (Higgins, 1992; Fath and Kolter, 1993). Similar β -sheet structures are predicted, in part, for the plasma membrane associated domains of the allantoin permease of *S. cerevisiae* based on the deduced amino acid sequence of the *DAL5* gene (Rai, *et al.*, 1988). Likewise, 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 (Cowan, *et al.*, 1992). Therefore, the twelve hydrophobic regions of the *PTR2* encoded protein may serve as membrane-associated domains that anchor the protein and provide a structural framework for transporting small peptides.

Each of the twelve hydrophobic regions of the *PTR2* encoded protein is flanked by an amino acid sequence that is predicted to be able to assume a turn conformation. Such structural conformational turns may serve to facilitate the insertion of transmembrane domains into the plasma membrane. Based on structural similarities with other membrane proteins (Higgins, 1992; Fath and Kolter, 1993), the hydrophilic regions are predicted to constitute the extracellular and intracellular domains of the *PTR2* encoded protein. The sequence, GGYVADTFWGKY, beginning at amino acid position 144, resembles the

glycine rich Walker A ATP-binding site motif, (A,G)x4GK(S,T) (Walker, *et al.*, 1982;), with a mismatch at position 155 where tyrosine is present in place of the predicted serine or threonine. A mismatch at this position has also been observed in the Walker A element of porcine adenylate kinase where Glycine is substituted for the usual Serine or Threonine (Walker, *et al.*, 1982). Among many nucleotide binding proteins, the Walker A element lies within a region of the protein that bridges an area of β -sheet secondary structure to an area of α -helical secondary structure. The sequence (A,G)XXXXGK provides a structurally and energetically favorable strand design for binding the pyrophosphate group of mononucleotides (Moller and Amons, 1985). This element is predicted to be located between hydrophobic domains II and III of the *PTR2* encoded peptide and is predicted to be tightly associated with the cytoplasmic side of the plasma membrane (Figure 14). While the bulky aromatic side chain of Tyrosine differs markedly from that of Serine or Threonine, all three residues possess hydroxyl groups that are protonated under usual physiological conditions. An amino acid sequence, **G GFIADTFLGRY**², similar to the above shown element of the *PTR2* encoded protein is present beginning at amino acid position 89 in the *CHL1* encoded protein of *A. thaliana*. Like *S. cerevisiae*, the *A. thaliana* element is flanked by hydrophobic domains II and III of the predicted *CHL1* protein; however, the *CHL1* encoded element differs considerably from the consensus Walker A sequence. A comparative analysis of several recently described peptide transport proteins (Steiner, *et al.*, 1994), *S. cerevisiae* Ptr2p, *A. thaliana* Ptr2p, *A. thaliana* Chl1p, and mammalian PepT1, shows a conserved motif,

² Invariant amino acid residues in this region of both proteins are shown in bold type.

(G₃,A₁)(G₂,A₂)X(I₃,V₁)**ADX**(F₃,W₁)(L₃,W₁)G(R₂,K₂)(Y₃,F₁)X**TI**³,

at the site of the Walker A-like element in the *S. cerevisiae* *PTR2* encoded protein. These elements may represent variants of the Walker A motif usually found in the ABC transporter proteins (for review see Higgins, et al., 1992). Whether or not these putative Walker A elements are functional as nucleotide binding sites in the various peptide transporters awaits the biochemical characterization of the respective proteins.

Present at amino acid position 210 of the *PTR2* encoded protein is the sequence VLIAD, which is similar to the Walker B motif (Figure 8; Walker, *et al.*, 1982). The Walker B element is composed of a short sequence of hydrophobic amino acids and a highly conserved aspartic acid residue which is believed to interact with a Mg⁺ ion in binding mononucleotides. An amino acid sequence similar to the Walker B element is located at a position where hydrophobic region IV makes a transition to a hydrophilic region. This hydrophilic region is located between hydrophobic domains IV and V and is also predicted to be present on the cytoplasmic face of the plasma membrane (Figure 14). The Walker B element has been implicated in binding ATP in concert with the Walker A element. The Walker B element is a characteristic feature of many plasma membrane associated transporters that bind ATP (Higgins, 1992). Alternately, the sequence VDE present at amino acid position 342, which is located between hydrophobic regions VI and VII, has also been implicated in binding ATP in concert with the Walker A element in a family of replicative proteins (Figure 8; Hodgman, 1988). Likewise, this element is

³ Invariant amino acid residues are shown in bold type. X represents any amino acid residue. Subscripts represent the relative number at which the amino acid residue at that position is found in the four peptides examined (Steiner, *et al.*, 1994).

predicted to be present on the cytoplasmic side to the plasma membrane (Figure 14). Both elements, VLIAD and VDE, contain an aspartic acid residue which may bind a phosphate of either ATP or GTP, via a magnesium ion, and thereby facilitate hydrolysis (Walker, *et al.*, 1982; Hodgman, 1988). However, a nucleotide binding site does not necessarily imply that hydrolysis is required for protein function. Alternative roles for nucleotide binding sites may include structural and regulatory functions (Higgins, 1992). Peptide sequence homology has also been noted between Ptr2p and PepT1, a proton-coupled oligopeptide transport protein of mammals (Fei, *et al.*, 1994; Steiner, *et al.*, 1994). The PepT1 protein is larger and the structure has several regions with no apparent counterparts in the other transporters (Steiner, *et al.*, 1994); however, given the structural similarity with *Arabidopsis thaliana* Chl1p, which functions in the depolarization of the plasma membrane upon exposure to nitrate (Tsay, *et al.*, 1993), this class of membrane proteins may utilize membrane potentials as a source of energy in driving the transport process. Thus, the primary structure of Ptr2p has failed to define the precise mode of energy utilization in the peptide transport system of *S. cerevisiae*.

The phenotype displayed by *S. cerevisiae* strains carrying *ptr1* mutant alleles is indistinguishable from those carrying *ptr2* mutant alleles. This phenotypic similarity may suggest a role for *PTR1* that is similar to that proposed for *PTR2*. Hence, suggesting a dimeric structure, similar to that of the *E. coli* oligopeptide transporter, may exist in *S. cerevisiae*. This model predicts that the *PTR1* and *PTR2* encoded peptides associate at the membrane to form a functional transport molecule. Alternately, the function of the *PTR2* transport molecule may be strictly regulated by the function of the *PTR1* gene product. A

plasmid complementing the *ptr1-1* mutation has recently been isolated and shown to code the *UBR1* gene of *S. cerevisiae* (K. Alagramam, *et al.*, 1994). The *UBR1* gene encodes the recognition component of a ubiquitin-protein ligase that has been implicated in the protein degradation pathway of *S. cerevisiae* (Bartel, *et al.*, 1990). These findings strongly suggest a regulatory role for the *PTR1(UBR1)* gene in the peptide transport pathway. It has recently been shown that the *PTR2* mRNA is completely absent in *Ptr1*⁻ strains (Alagraman, *et al.*, 1994). These finding suggest that the *PTR1* encoded protein functions at some point prior to the transcription of the *PTR2* gene. One mechanistic model proposes that the *PTR1* encoded protein functions in the derepression of *PTR2* transcription, perhaps by participating in the degradation of a *PTR2* specific repressor protein (Alagramam, *et al.*, 1994). The derepression may be triggered by environmental stimuli such as the presence of amino acids, peptides, and other nitrogen containing molecules. If such a model is correct, it presupposes the existence of a gene encoding a repressor molecule. The *PTR3* gene may be such a candidate given the pleiotropic phenotype typical of *Ptr3*⁻ strains. Such a model accounts for the indistinguishable phenotype conferred by *ptr1* and *ptr2* mutant alleles. In support of this hypothesis is the observation that a *PTR2* homolog from *C. albicans* is able to complement the phenotype conferred by the *ptr1-1* and *ptr2-2* mutations of *S. cerevisiae* (Basrai, *et al.*, 1994). This observation suggests that the *C. albicans* *PTR2* encoded transport protein is able to bypass the defect imposed by the *ptr1-1* mutation of *S. cerevisiae* presumably by not requiring the function of the *PTR1(UBR1)* encoded protein to signal the degradation a transcriptional repressor. These observations do not contradict the suggestion that the degradation of a

putative repressor of the *S. cerevisiae* *PTR2* encoded protein is an essential step in the transport of di- and tripeptides. Alternately, the *PTR1* encoded protein may be required for directly regulating the level of the plasma membrane associated *PTR2* transporter. It has recently been shown that the plasma membrane associated **a**-factor transporter, encoded by the *STE6* gene, accumulates in the ubiquitinated form in the plasma membrane of *S. cerevisiae* strains deficient in endocytosis (Kolling and Hollenberg, 1994), presumably as a result of blocking the major degradative pathway for Ste6p. If a similar situation exists for Ptr2p, then Ptr1p may function to ubiquitinate and signal for degradation Ptr2p while some other pathway functions to regulate the transcription of the *PTR2* gene. Such a regulatory system may depend on ubiquitinated Ptr2p intermediates as a signal for activity; in the absence of degradative intermediates, no transcription would occur. However, both models leave open the question of what proteins function to directly regulate the activity of the peptide transport system. How do amino acids and other nitrogen sources regulate the activity of the peptide transport pathway? What are the molecular intermediates in the signaling pathway? Why is the *PTR2* transcript produced transiently upon the introduction of leucine to growth media rather than at a continuous level in the presence of the inducer? The answers to these questions and others regarding the intracellular location, structure, and biochemical function of the *PTR* encoded proteins will depend on additional genetic and biochemical experimentation.

The *PTR2* gene was physically mapped to the right arm of chromosome XI, distal to the *tif1* marker, by chromosome hybridization experiments (Figure 13; Materials and Methods). A comparison of the sequenced *PTR2* gene and flanking regions with other

sequences in the GenEMBL Data Base showed *PTR2* to be tightly linked with a ubiquitin encoding gene, *UBI2* (Figure 9; Ozkaynak, *et al.*, 1987). The *PTR2* and *UBI2* coding regions are separated by a 466-bp region and are encoded on opposite DNA strands (Figure 11). What effect, if any, this structural arrangement has on the expression and activity of *PTR2*, or *UBI2*, remains to be elucidated. A similar orientation pattern is seen with the *S. cerevisiae* *STE6* gene, encoding the α -factor export protein, and *UBA1*, encoding a ubiquitin activating enzyme (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989). No other genes have been mapped to this region of chromosome XI, although *bas1* is located within approximately 15-kbp based on physical maps constructed from λ phage clones of chromosome XI.

Transcription of the *PTR2* gene appears to be induced by the addition of L-leucine to yeast cultures grown in minimal medium (Perry, *et al.*, 1994). Earlier studies clearly demonstrated that various amino acids act to induce the peptide transport system of *S. cerevisiae* in the presence of allantoin, a non-repressive nitrogen source (Island, *et al.*, 1987). Transcription of the *PTR2* mRNA is negligible in yeast cells grown in minimal medium which contains allantoin as the sole nitrogen source. However, immediately upon addition of L-leucine to the growth medium, the *PTR2* transcript accumulates rapidly to a maximum then plateaus at approximately 30 to 40 minutes post-induction. The mRNA levels then begin to fall rapidly and return to the basal level by 120 minutes post-induction. This data is in good agreement with earlier physiological experiments which showed that various amino acids act to potentiate the growth inhibiting effects of toxic dipeptides when yeast cells are grown in medium containing allantoin as the nitrogen source (Island, *et al.*,

1987). However, the *PTR2* transcript can be detected in cells grown overnight in YEPD medium (Figure 15). 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 quality and quantity of the nitrogen source and the presence, or absence, of amino acids and other nitrogen containing molecules, such as di- and tripeptides. Therefore, conclusions regarding the physiological regulation of peptide transport cannot be made with certainty based only on 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 and their encoded products.

While the approach to cloning peptide transport genes described here is extremely efficient in that no primary screen is necessary for first identifying plasmid-bearing clones, it does require that mutations in genes involved in peptide transport be present in a genetic background containing multiple auxotrophic amino acid markers. In addition to *PTR2*, the *PTR1* gene of *S. cerevisiae* (Alagramam, *et al.*, 1994), a *PTR2* homolog from *Arabidopsis thaliana* (Steiner, *et al.*, 1994), and a *PTR2* homolog from *C. albicans* (Basrai, *et al.*, 1994) have been cloned using the *S. cerevisiae* strains and methodology described above. In addition, a plasmid carrying a DNA insert which functionally complements the *ptr3-2* mutation of *S. cerevisiae* has recently been isolated (Perry, J., manuscript in preparation). The deduced protein of the *A. thaliana* *PTR2* gene shows a high degree of amino acid sequence conservation when compared with the *S. cerevisiae*

PTR2 encoded protein (Steiner, *et al.*, 1994). In *A. thaliana*, 52% of the amino acids in the secondary structure of the predicted protein sequence are identical with the predicted *S. cerevisiae* protein sequence and the two proteins have an overall similarity of 68 % with regard to the amino acid sequences in the secondary structures. The *PTR2* homolog from *C. albicans* shows an overall similarity of approximately 56% when compared with the *S. cerevisiae* protein with 31% of the amino acids in the protein sequence being identical (Basrai, *et al.*, 1994). It has also been shown that the *A. thaliana* and the *C. albicans* *PTR2* homologs cross complement the *ptr1-1* and *ptr2-2* mutations of *S. cerevisiae* (Basrai, *et al.*, 1994). This is further suggestive that the *PTR1* gene product functions to regulate the *PTR2* encoded transporter and that the homologous genes from *A. thaliana* and *C. albicans* are able to bypass this regulatory mechanism and function in mutant *S. cerevisiae* strains carrying *ptr1* or *ptr2* alleles. Conversely, it has also been shown that the cloned *PTR1(UBR1)* gene does not complement the *ptr2-2* mutation (K. Alagramam, personal communication).

The overall similarity among the various peptide transporters further suggests that peptide uptake may be a highly conserved process in the evolution of these organisms and while peptide transport in *A. thaliana* and *C. albicans* may be regulated in a different manner than that of *S. cerevisiae*, it should be possible to dissect the peptide transport pathway of *C. albicans* and *A. thaliana*, and perhaps that of other fungi and plants, in a *S. cerevisiae* genetic background. This approach to cloning peptide transport genes has proven its utility in providing a methodology for permitting the comparative genetic analysis of proteins thought to be directly involved in the transport of peptides across the

plasma membrane. Future work will likely reveal the 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. The insights gained from this approach to cloning and characterizing genes involved in the transport of peptides across the plasma membrane may give direction to the development and application of new peptidyl-based antifungal compounds of medical significance.

CHAPTER V

SUMMARY OF KEY FINDINGS AND CONCLUSIONS

- 1 . The transport of intact peptides across the plasma membrane of *Saccharomyces cerevisiae* is mediated by the products encoded by at least three genes: *PTR1*, *PTR2*, and *PTR3*.
- 2 . *S. cerevisiae* strains carrying *ptr1* or *ptr2* mutations display identical phenotypes characterized by resistance to the growth inhibiting effects of toxic dipeptides, the failure to accumulate radiolabeled dileucine, and the inability to use dipeptides to satisfy auxotrophic amino acid requirements. These strains remain as sensitive as wildtype to toxic amino acid analogs. Strains carrying *ptr1* or *ptr2* mutations are defined as members of Phenotype Class A.
- 3 . *S. cerevisiae* strains carrying *ptr3* mutations are also resistant to the growth inhibiting effects of toxic dipeptides, but are able to accumulate low levels of radiolabeled dileucine, and are able to use dipeptides to satisfy auxotrophic amino acid requirements. These strains are less sensitive than wildtype to toxic amino acid analogs. Strains carrying *ptr3* mutations are defined as members of Phenotype Class B.
- 4 . A one-step, double selection cloning method which permits the cloning of peptide transport genes was developed. Using this method the *PTR2* gene of *S. cerevisiae*, the *PTR1* gene of *S. cerevisiae*, a *PTR2* homolog of *C. albicans*, and a *PTR2* homolog of *A. thaliana* have been successfully cloned and characterized.

5. The *PTR2* encoded protein, Ptr2p, is able to transport di- and tripeptides as shown by toxic peptide sensitivity assays.
6. The *PTR2* gene of *S. cerevisiae* was isolated then defined at the molecular level by employing deletion mutagenesis, frameshift mutagenesis, and DNA sequencing. The *PTR2* open reading frame is 1803-bp long and encodes a putative peptide composed of 601 amino acids. The peptide has a predominantly hydrophobic character with alternating regions of relative hydrophobicity and hydrophilicity which is typical of many plasma membrane-associated transport proteins.
7. The *PTR2* transcript is approximately 1800 nucleotides in length which is in good agreement with the size of the proposed *PTR2* open reading frame found on plasmid pJP9. The DNA sequence down stream from the translation termination codon contains two tripartite DNA elements which have the potential to serve as transcription termination sites.
8. The *PTR2* gene was localized to the right arm of Chromosome XI. DNA sequencing shows *PTR2* to be adjacent to the *UBI2* gene. The translation termination codons of the two genes are separated by 466-bp and the two genes are oriented so that they are transcribed convergently.

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APPENDIX

TABLE 1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Source ^a
S288C	<i>MATα SUC2 mal mel gal2 CUP1</i>	YGSC
X2180-1A	<i>MATa SUC2 mal mel gal2 CUP1</i>	YGSC
X2180-1B	<i>MATα SUC2 mal mel gal2 CUP1</i>	YGSC
XL70-1B	<i>MATα ura3-52 leu2-3 lys1-1 trp1-289 his4-38</i>	FWL
XL70-5C	<i>MATa ura3-52 leu2-3 lys1-1 his4-38</i>	FWL
PB1	<i>MATα ptr2-2</i>	JRP
PB5	<i>MATα ptr3-2</i>	JRP
PB13X-1B	<i>MATα ptr2-2</i>	JRP
PB13X-1D	<i>MATa ptr2-2</i>	JRP
PB53X-1A	<i>MATa ptr3-2</i>	JRP
PB1X-9B	<i>MATa ura3-52 leu2-3 lys1-1 his4-38 ptr2-2</i>	JRP
PB1X-2A	<i>MATa ura3-52 leu2-3 lys1-1 his4-38</i>	JRP
PB1X-2A(Δ I)	<i>MATa ura3-52 leu2-3 lys1-1 his4-38 ptr2::LEU2</i>	JRP
PB1X-2A(Δ II)	<i>MATa ura3-52 leu2-3 lys1-1 his4-38 ptr2::LEU2</i>	JRP
PB2X-4D	<i>MATa ura3-52 leu2-3 lys1-1 ptr3-2</i>	JRP
PB2X-1B	<i>MATa ura3-52 leu2-3 lys1-1 trp1-289</i>	JRP
PB4X-2B	<i>MATa ura3-52 leu2-3 lys1-1 his4-38 ptr1-1</i>	JRP
PB4X-1B	<i>MATa ura3-52 leu2-3 lys1-1 his4-38</i>	JRP
LeuLE-1	<i>MATα ptr1-1</i>	MDI
LeuAE-1	<i>MATα ptr2-1</i>	MDI
HisLE-1	<i>MATα ptr3-1</i>	MDI

^a YGSC, Yeast Genetics Stock Center, Berkeley, CA; FWL, Frank W. Larimer, Oak Ridge National Laboratory, Oak Ridge, TN; JRP, Jack R. Perry; MDI, Michael D. Island. Mutations *ptr2-2* and *ptr3-2* were formally designated as *aet1-1* and *aet2-1*, respectively.

TABLE 2. Plasmids used in this study^a

Plasmid	Description	Source/Reference
YCp50	Genomic-DNA library vector	Rose, <i>et al.</i> , 1987
pRS306	Yeast integrating plasmid	Sikorski and Hieter, 1989
pRS3062	pRS306 with unique <i>Aat</i> II site removed	This study
pJJ252	<i>LEU2</i> gene fragment source	Jones and Prakash, 1990
Ep69	<i>GCN3</i> gene fragment source	Hannig and Hinnebusch, 1988
pJP2	Functional <i>PTR2</i> parent plasmid	This study
pJP5	Nonfunctional <i>ptr2</i> deletion plasmid	This study
pJP7	Nonfunctional <i>ptr2</i> deletion plasmid	This study
pJP8	Nonfunctional <i>ptr2</i> deletion plasmid	This study
pJP9	Functional <i>PTR2</i> deletion plasmid	This study
pJP10	Nonfunctional <i>ptr2</i> subclone	This study
pJP9(fs-1)	Functional <i>PTR2 Bam</i> HI frameshift plasmid	This study
pJP9(fs-2)	Nonfunctional <i>ptr2</i> Eco RI frameshift plasmid	This study
pJP9(fs-3)	Nonfunctional <i>ptr2</i> Bgl II frameshift plasmid	This study
pJP15	Nonfunctional <i>ptr2</i> disruption plasmid	This study
pJP23(I)	Nonfunctional <i>ptr2</i> disruption plasmid	This study
pJP23(II)	Nonfunctional <i>ptr2</i> disruption plasmid	This study

^a See text and references for detailed descriptions of all plasmids.

TABLE 3. Sensitivity of *S. cerevisiae* strains to toxic dipeptides and the corresponding amino acid analogs ^a

<i>S. cerevisiae</i> strain ^b	<u>Zone of inhibition (cm)</u> ^c				
	Eth	f-Phe	Ala-Eth	Leu-Eth	Leu-f-Phe
S288C (PTR1, PTR2, PTR3)	4.4	2.5	4.4	4.4	3.7
PB13X-1B (PTR1, ptr2, PTR3)	4.4	2.8	0	0	0
PB13X-1D (PTR1, ptr2, PTR3)	4.4	2.9	0	0	0
PB53X-1A (PTR1, PTR2, ptr3)	3.6	1.9	0	0	0
<u>X2180-1A</u> X2180-1B (PTR ⁺ /PTR ⁺)	4.2	N/D	3.7	N/D	N/D
<u>PB13X-1D</u> X2180-1B (ptr2/PTR2)	4.2	N/D	3.6	N/D	N/D
<u>PB53X-1A</u> X2180-1B (ptr3/PTR3)	4.3	N/D	3.7	N/D	N/D
<u>PB13X-1B</u> PB53X-1A (PTR1, ptr2, PTR3/PTR1, PTR2, ptr3)	4.1	N/D	3.5	N/D	N/D

- ^a Disk sensitivity assays were performed as described in Materials and Methods.
ND, Not done.
- ^b *S. cerevisiae* strains are described in Materials and Methods and Table 1.
- ^c Eth, L-ethionine; f-Phe, D,L-m-fluorophenylalanine; Ala-Eth, L-alanyl-L-ethionine; Leu-Eth, L-leucyl-L-ethionine; Leu-f-Phe, L-leucyl-D,L-m-fluorophenylalanine. All amino acid analogs and toxic dipeptides are 0.38 μ moles/disk.

TABLE 4. Dipeptide utilization in auxotrophic *S. cerevisiae* strains harboring *ptr* mutations^a

<i>S. cerevisiae</i> strain ^b	<u>Growth on Dipeptides^c</u>				
	Dileu	Dilys	Lys-Leu	His-Leu	Ala-Eth
PB4X-1B (<i>PTR</i> ⁺)	+	+/-	+	+	S
PB4X-2B (<i>ptr1-1</i>)	NG	NG	NG	NG	R
PB1X-2A (<i>PTR</i> ⁺)	+	+/-	+	+	S
PB1X-9B (<i>ptr2-2</i>)	NG	NG	NG	NG	R
PB2X-1B (<i>PTR</i> ⁺)	+	+/-	+	+	S
PB2X-4D (<i>ptr3-2</i>)	+	+/-	+	+	R

^a Growth on dipeptides was ascertained as described in Materials and Methods.

^b *S. cerevisiae* strains are described in Materials and Methods and Table 1.

^c +, Growth at 48 hours; +/-, Poor growth at 48 hours; NG, No growth; S, sensitive; R, resistant.

TABLE 5. Sensitivity of *S. cerevisiae* transformants to L-ethionine and L-alanyl-L-ethionine^a

<i>S. cerevisiae</i> strain ^b	Plasmid ^c	<u>Zone of inhibition (cm)</u> ^d	
		Eth	Ala-Eth
PB1X-2A (<i>PTR2</i>)	YCp50	4.5	4.2
PB1X-9B (<i>ptr2-2</i>)	pJP9	4.6	4.4
PB1X-9B (<i>ptr2-2</i>)	YCp50	4.5	0

^a Disk sensitivity assays were performed using minimal medium supplemented with histidine (0.12 mM), lysine (0.21 mM), leucine (0.18 mM).

^b *S. cerevisiae* PB1X-9B (*MATa ura3-52 leu2-3 lys1-1 his4-38 ptr2-2*).
S. cerevisiae PB1X-2A (*MATa ura3-52 leu2-3 lys1-1 his4-38*).

^c Plasmid YCp50 is a shuttle vector which contains the *S. cerevisiae URA3* as the selectable marker and is maintained at a low copy number. Plasmid pJP9 is YCp50 containing the *S. cerevisiae PTR2* gene.

^d Eth, L-ethionine; Ala-Eth, L-alanyl-L-ethionine. Eth or Ala-Eth (0.38 μ moles/disk).

TABLE 6. Sensitivity of *S. cerevisiae* transformants to oxalysine and oxalysine-containing peptides^a

<i>S. cerevisiae</i> strain ^b	Plasmid ^c	<u>Zone of inhibition (cm)^d</u>				
		O	OG	OLG	O(L) ₂ G	O(L) ₃ G
PB1X-2A (<i>PTR2</i>)	YCp50	0	2.8	1.9	0	0
PB1X-9B (<i>ptr2-2</i>)	pJP9	0	3.2	2.1	0	0
PB1X-9B (<i>ptr2-2</i>)	YCp50	0	0	0	0	0

^a Disk sensitivity assays were performed as described in Materials and Methods.

^b *S. cerevisiae* PB1X-9B (*MATa ura3-52 leu2-3 lys1-1 his 4-38 ptr2-2*).
S. cerevisiae PB1X-2A (*MATa ura3-52 leu2-3 lys1-1 his 4-38*).

^c Plasmid YCp50 is a shuttle vector which contains the *S. cerevisiae URA3* as the selectable marker and is maintained at a low copy number. Plasmid pJP9 is YCp50 containing the *S. cerevisiae PTR2* gene.

^d Oxalysine or oxalysine-containing peptides (0.25 μ moles/disk). O, Oxalysine ; OG, Oxa-Gly; OLG, Oxa-Leu-Gly; O(L)₂G, Oxa-(Leu)₂-Gly; O(L)₃G, Oxa-(Leu)₃-Gly.

TABLE 7. Growth of *S. cerevisiae* transformants on dipeptide medium^a

Plasmid ^b	<u>Growth on Dipeptides^c</u>				
	Leu-Leu	Lys-Lys	Lys-Leu	His-Leu	Ala-Eth
pJP2	+	+	+	+	S
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 The ability of *S. cerevisiae* PB1X-9B transformants, harboring the above plasmids, was assayed on MM with dipeptides as described in Materials and Methods. The "+" symbol indicates growth on the dipeptide containing medium and a "-" symbol indicates no growth. "S" indicates that the transformant is sensitive to the toxic effect of L-alanyl-L-ethionine and "R" indicates that the transformant is resistant.

^b Plasmids pJP2, pJP5, pJP7, pJP8, pJP9 and pJP10 are described in Figure 4. Plasmids pJP9 (fs-1), pJP9 (fs-2) and pJP9 (fs-3) are described in Figure 4. Plasmid YCp50 is the *S. cerevisiae* / *E. coli* shuttle vector as described in Materials and Methods.

^c Leu-Leu, L-leucyl-L-leucine; Lys-Lys, L-lysyl-L-lysine; Lys-Leu, L-lysyl-L-leucine; His-Leu, L-histidyl-L-leucine; Ala-Eth, L-alanyl-L-ethionine.

TABLE 8. Sensitivity of *S. cerevisiae* diploid strains to L-alanyl-L-ethionine and L-ethionine^a

<i>S. cerevisiae</i> strain ^b	<u>Zone of inhibition (cm)^c</u>	
	Eth	Ala-Eth
<u>X2180-1A</u>	4.2	3.7
X2180-1B (<i>PTR</i> +/ <i>PTR</i> +)		
<u>PB13X-1D</u>	4.2	3.6
X2180-1B (<i>ptr2-2/PTR2</i>)		
<u>PB53X-1A</u>	4.3	3.7
X2180-1B (<i>ptr3-2/PTR3</i>)		
<u>PB13X-1B</u>	4.1	3.5
PB53X-1A (<i>PTR1, ptr2-2, PTR3/PTR1, PTR2, ptr3-2</i>)		
<u>LeuLE-1</u>	3.7	2.1
PB13X-1D (<i>ptr1-1/ptr2-2</i>)		
<u>LeuAE-1</u>	3.6	0.0
PB13X-1D (<i>ptr2-1/ptr2-2</i>)		
<u>HisLE-1</u>	3.8	2.3
PB13X-1D (<i>ptr3-1/ptr2-2</i>)		
<u>LeuLE-1</u>	3.7	2.1
PB53X-1A (<i>ptr1-1/ptr3-2</i>)		
<u>LeuAE-1</u>	3.7	2.4
PB53X-1A (<i>ptr2-1/ptr3-2</i>)		
<u>HisLE-1</u>	3.6	0.0
PB53X-1A (<i>ptr3-1/ptr3-2</i>)		

- ^a Disk sensitivity assays were performed as described in Materials and Methods.
- ^b *S. cerevisiae* strains are described in Materials and Methods and Table 1.
- ^c Eth, L-ethionine; Ala-Eth, L-alanyl-L-ethionine.
All amino acid analogs and toxic dipeptides are 0.38 μ moles/disk.

TABLE 9. Primers used in DNA sequencing of the *PTR2* gene

Designation ^a	Oligonucleotide Sequence	Position ^b	DNA Strand ^c
GS01	5'-CAAGACGCCACCGC-3'	-396	B
GS02	5'-GTGCTATTGGTGGG-3'	553	A
GS03	5'-GGGGTTCTGGGCGG-3'	803	A
GS04	5'-GAGAGCTTTGGCGG-3'	1037	A
GS05	5'-GGGAGGGGAGTGTTG-3'	-640	B
GS08	5'-GAGCCTTGGCTGGG-3'	25	B
GS09	5'-GGGTCACAACACCC-3'	1366	A
GS10	5'-CGGATACAGTGAGC-3'	2052	B
GS11	5'-TCGCGTATTTGC-3'	2065	A
P01	5'-CACCACAACATCATC-3'	-271	A
P02	5'-CCGATTGGTCTTTGAATG-3'	901	B
P03	5'-CACGGAAGGTTAGC-3'	629	B
P04	5'-CCCGGTCATCGAAGAGG-3'	59	A
P05	5'-ATCACTCAGGCCAG-3'	1115	A
P06	5'-CCAAACTACATGG-3'	308	A
P07	5'-GGGAGTCATTAGG-3'	342	B
P09	5'-CTCTATCACTGGGTTGG-3'	1449	A
P10	5'-GGTTTTAGTGGAGTG-3'	1258	B
P11	5'-CAAAGAAAACCTCC-3'	1877	B
P13	5'-GTTTATTATGTCC-3'	1503	A
P14	5'-CAGTGCTGCTCCAC-3'	-88	A
P15	5'-GCGGTTTGTGTCAAG-3'	-728	A
P17	5'-CTTCCTGAAGCACAACC-3'	1661	B
P18	5'-CATGGAAGCTGGGGC-3'	1497	B
T3	5'-ATTAACCTCACTAAAGGGA-3'		B
T7	5'-ATCGAAATTAATACG-3'		A
CCW	5'-ATACCAAACGACGAG-3'		B

^a Primers designated GS were obtained from GenoSys Biotechnologies, Inc.; primers designated P were obtained from the University of Tennessee Molecular Biology Resource Facility; primers designated T3 and T7 were obtained from New England BioLabs, Inc.; the primer designated CCW was obtained from BRL Life Technologies, Inc.

^b The position given reflects the position of the 5'-residue of each oligonucleotide relative to the first nucleotide of the initiation codon of the *PTR2* gene on plasmid pJP9. Please see Figure 6 legend for annealing sites of primers T3, T7, and CCW.

^c The "A" DNA strand represents the strand extending 5' to 3' from the *Hind* III site to the *Sau* 3A / *Bam* HI site of plasmid pJP9. The "B" DNA strand represents the strand extending 5' to 3' from the *Sau* 3A / *Bam* HI site to the *Hind* III site of plasmid pJP9.

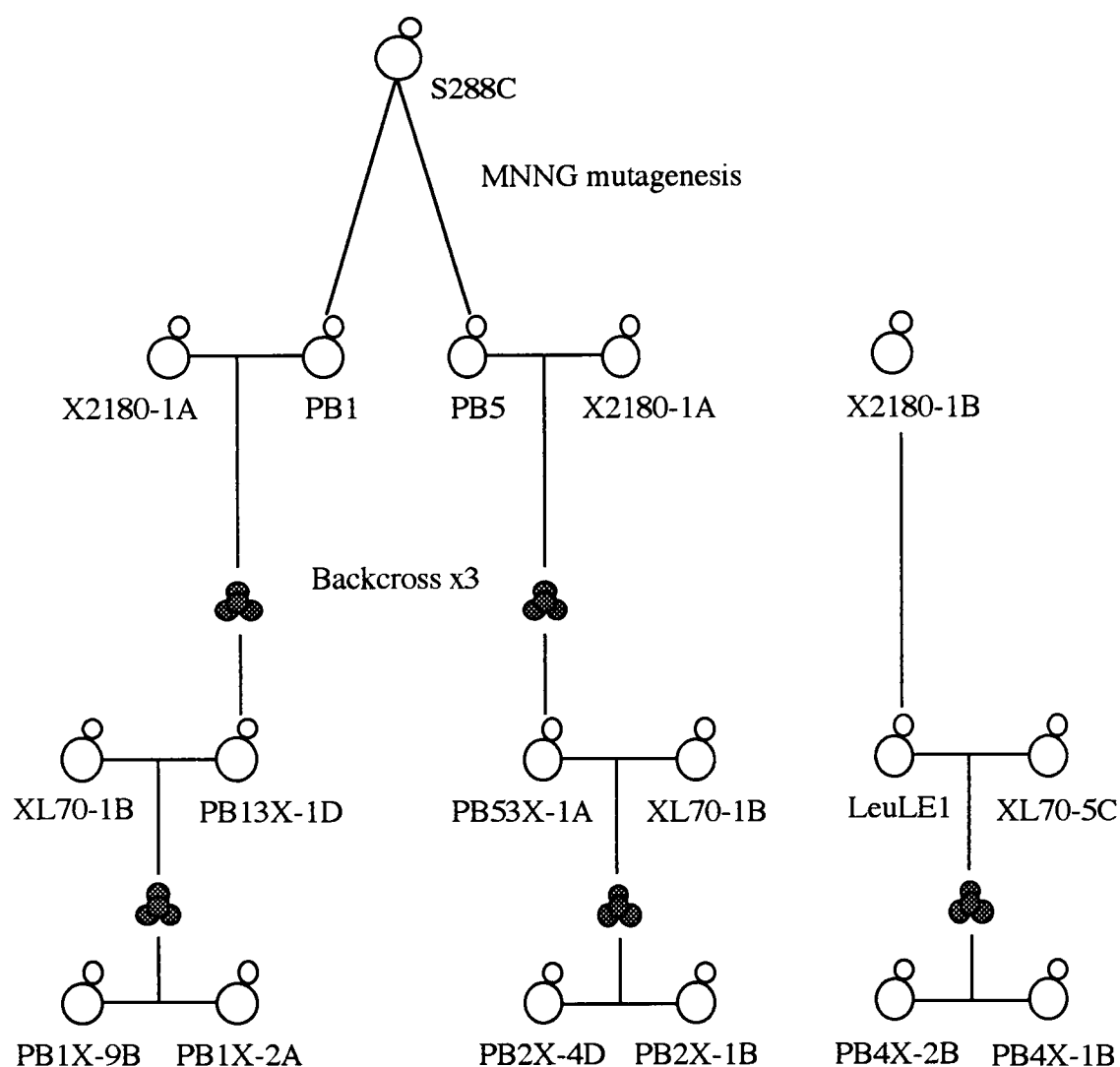


Figure 1.

Pedigree chart of *S. cerevisiae* strains. Shown is a pedigree demonstrating the genetic relationships among many of the yeast strains used or developed in this study. All yeast strains shown are haploid. Diploid strains resulting from matings are not shown. Horizontal bars represent matings between the haploid strains indicated. Vertical bars represent the process of sporulation. The genotypes and sources of all yeast strains shown are presented in Table 1. The point in this pedigree where MNNG mutagenesis was employed to generate dipeptide transport deficient mutants is shown. Strains X2180-1A and X2180-1B are derived from strain S288C. Strains PB13X-1B and PB13X-1D are derived from the same ascus and are of opposite mating type.

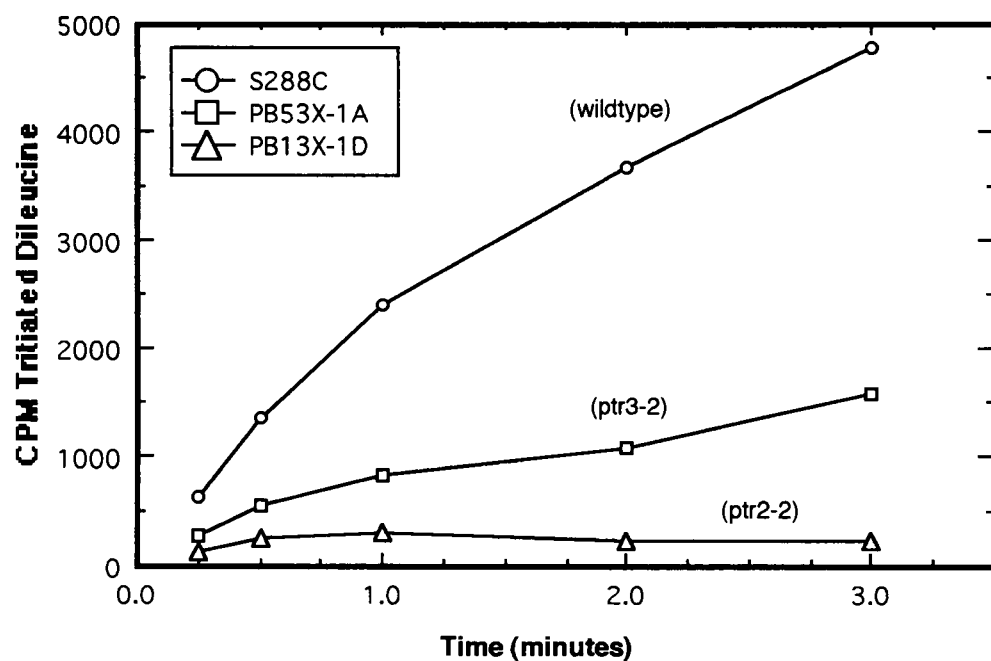


Figure 2.
Uptake of radioactive dileucine by *S. cerevisiae* peptide transport mutants (Materials and Methods). S288C is a wildtype parental strain. PB53X-1A and PB13X-1D are dipeptide transport deficient strains.

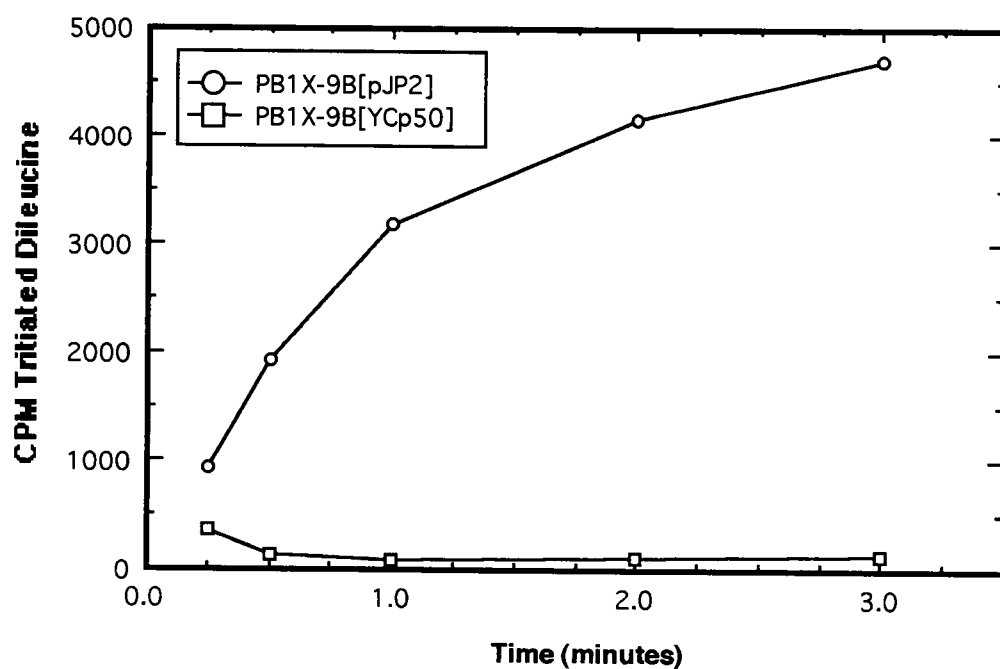


Figure 3.

Uptake of radioactive dileucine by *S. cerevisiae* transformants (Materials and Methods). *S. cerevisiae* strain PB1X-9B transformed with plasmids pJP2 (O) and YCp50 (□). The host strain, PB1X-9B, carries the *ptr2-2* peptide transport mutant allele.

Figure 4.

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. The *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 *CENIV/ARS1* elements are derived from *S. cerevisiae*. The dashed lines indicate the splice sites for the PTR2⁺ DNA insert, designated plasmid pJP2. (B) Deletions and subclones of pJP2. (C) Frameshift mutations constructed in plasmid pJP9. Plasmids pJP9(fs-1), pJP9(fs-2), and pJP9(fs-3) carry +4 frameshift mutations at the unique *Bam* HI, *Eco* RI, or *Bgl* II sites of plasmid pJP9, respectively. All of these plasmids were maintained as YCp50-based constructions except for pJP10 which was maintained as a pRS306-based construction.

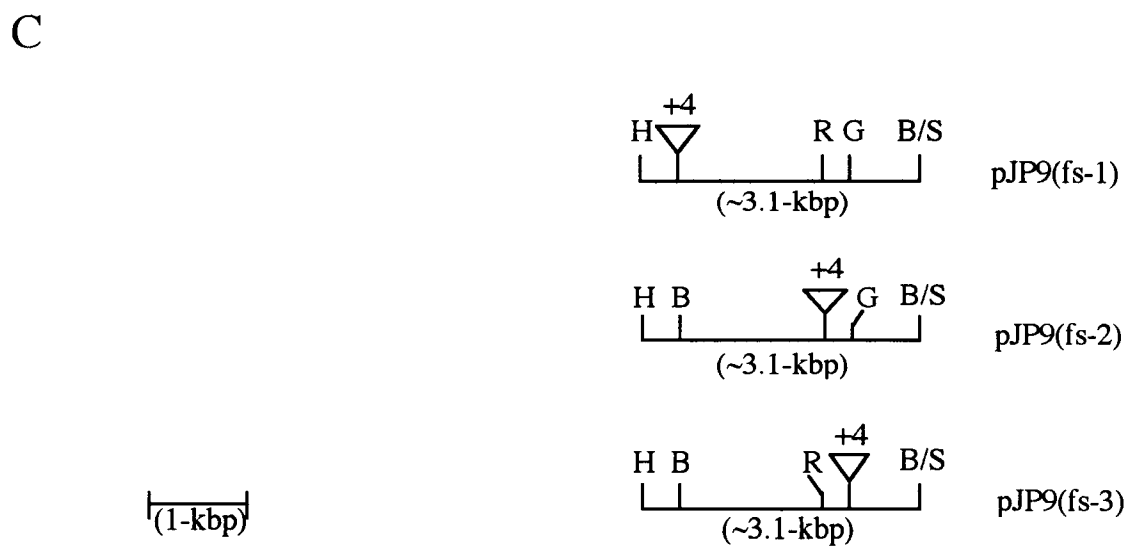
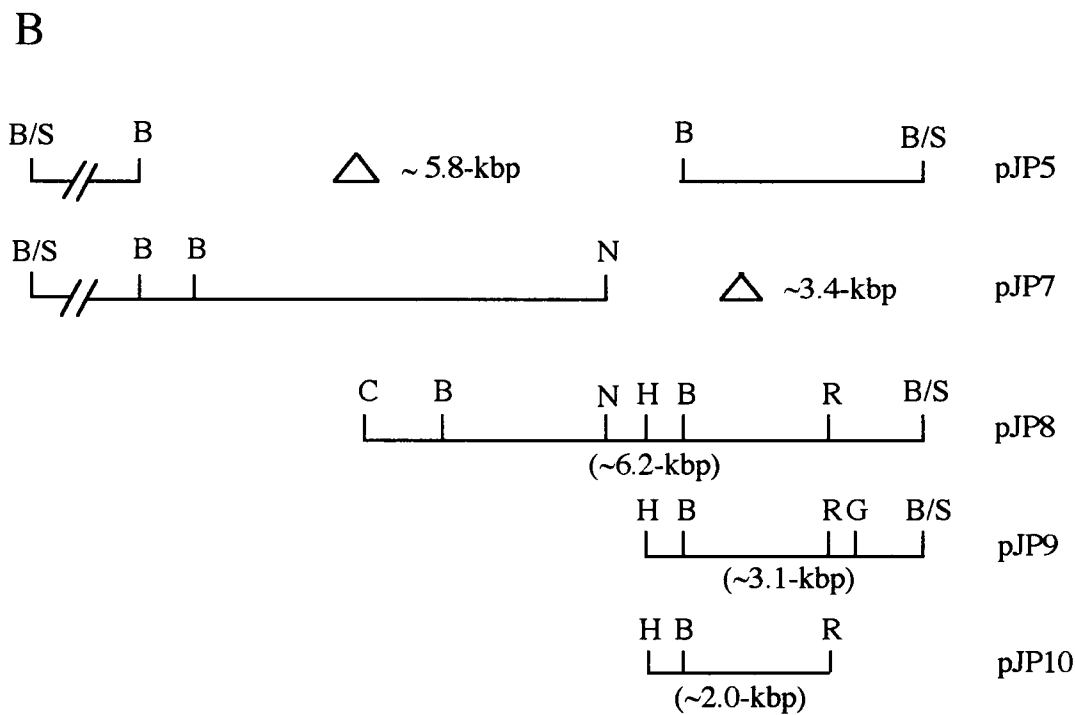
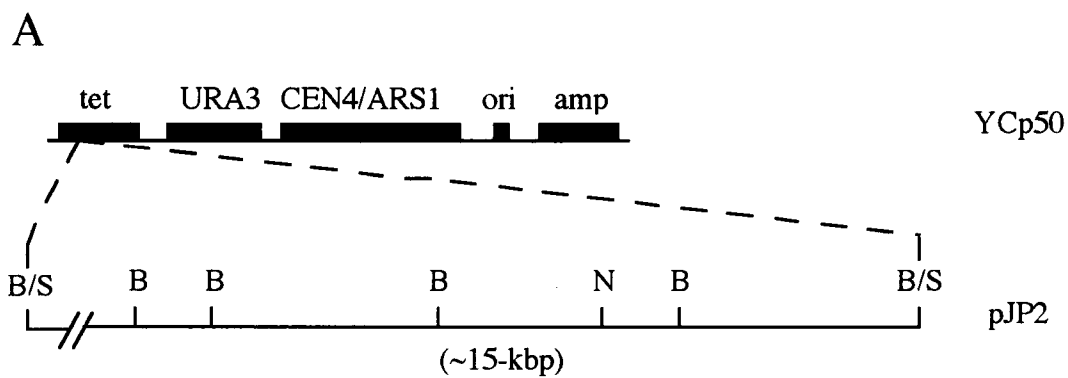


Figure 5.

Restriction endonuclease digestions of pJP9(fs-1), (fs-2), (fs-3), pJP23(I), and pJP23(II). Lane 1 is untreated plasmid pJP9 which migrates as supercoiled and relaxed circular DNA molecules. Lane 2 is pJP9 treated with *Bam* HI, which migrates as a single DNA molecule with a molecular size of approximately 11-kbp. Lane 3 is pJP9(fs-1) treated with *Bam* HI, which shows no cleavage of the plasmid. Lane 4 is pJP9 treated with *Eco* RI, which shows two DNA fragments of 9.0-kbp and 2.0-kbp. Lane 5 is pJP9(fs-2) treated with *Eco* RI, which shows a single DNA molecule with a molecular size of approximately 11-kbp. Lane 6 is pJP9 treated with *Bgl* II, which shows two DNA fragments of approximately 5.9-kbp and 5.1-kbp. Lane 7 is pJP9(fs-3) treated with *Bgl* II, which shows a single DNA molecule of approximately 11-kbp. Lane 8 is pJP23(I) treated with *Eco* RI, which shows a 1.3-kbp DNA fragment as predicted. Lane 9 is pJP23(II) treated with *Eco* RI which shows a 1.4-kbp DNA fragment as predicted. Lane 10 is λ phage DNA treated with *Hind* III. The λ phage *Hind* III DNA fragments are 23.1-kbp, 9.4-kbp, 6.6-kbp, 4.4-kbp, 2.3-kbp, 2.0-kbp, 0.56-kbp, and 0.13-kbp (not visible) in length. Lane 3 demonstrates the loss of the *Bam* HI site in plasmid pJP9(fs-2). Lane 5 demonstrates the loss of an *Eco* RI site in pJP9(fs-2). Lane 7 demonstrates the loss of a *Bgl* II site in pJP9. The loss of the correct *Eco* RI and *Bgl* II site in pJP9(fs-2) and (fs-3), respectively, was confirmed by additional endonuclease digests (Data Not Shown). Lanes 8 and 9 demonstrate the *Eco* RI generated DNA fragment patterns expected from the two orientations of the *LEU2* gene in plasmid pJP23.

1 2 3 4 5 6 7 8 9 10

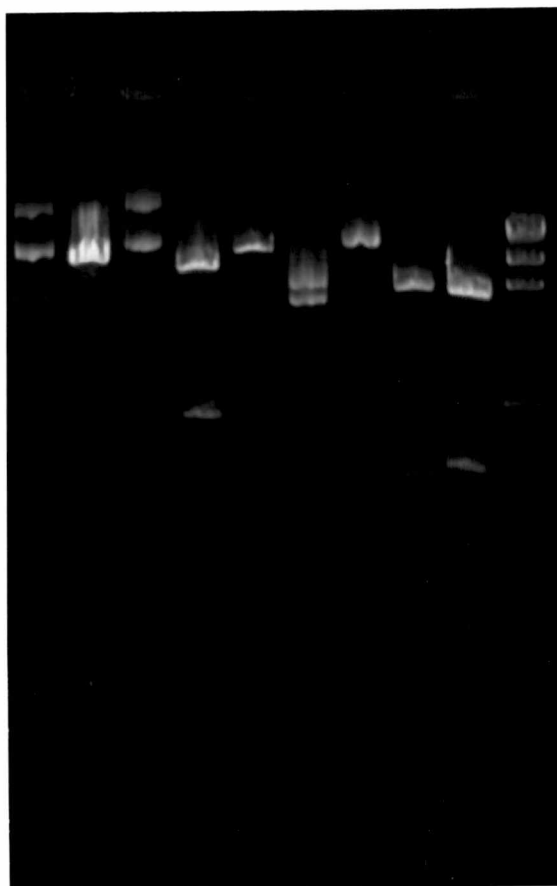
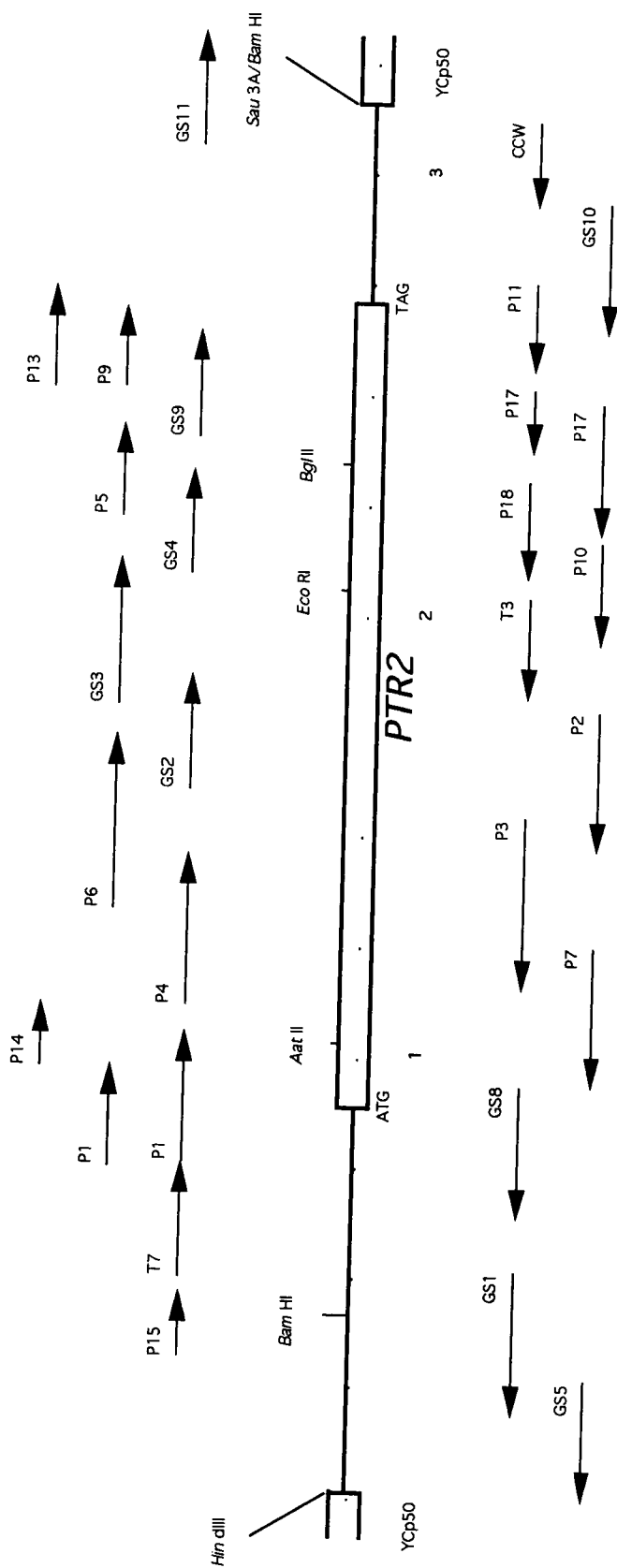


Figure 6.

PTR2 sequencing strategy. Shown is the 3.1-kbp *Hind* III - *Sau* 3A DNA fragment insert of plasmid pJP9. Each arrow represents a sequenced region of the *ptr2-2* complementing region of plasmids pJP9 or pJP15 and is designated by the oligonucleotide primer used in the DNA sequencing reactions. Both DNA strands of the *PTR2* open reading frame were sequenced. The *Hind* III and *Sau* 3A/*Bam* HI insert-vector junction sites are shown. The closed box represents the protein coding region of the *PTR2* gene and the open boxes represent plasmid vector, YCp50, regions. Relevant restriction endonuclease sites are indicated. Primers designated GS or P are complementary to sites within the cloned 3.1-kbp DNA fragment insert. Primers designated T3 and T7 are complementary to sites that flank the cloned DNA insert of pJP15. The primer designated CCW is complementary to a site within the *tet^R* gene of pJP9.



(3143-bp)

HindIII

AAGCTTGGGGTAACGCAATCAGTATTGTTTCTGAGATTTTTTTTCCCGGAAAAGCTAAGAGTTTTCTGGCGAGA
-896

TAAGATAACGGCCCCGAAGGCAGCGGCGAACTGACTTACCTCCGTCGGCTCCGGCGTACGGCACCAAAAAAT
-825

CCCGGCAATAACTTGACACCAATGGGCTAACGGCGTCCGCAGAACCGTGACGCAATCTGCGGTTTGTGTCA
-754

AGACAGAGAAGTGGGCTGCTTCTCCCTTTCAACACTCCCCTCCCATGGCTGAATACTCAGACGGTTTTCT
-683

GTGGTGGAAATGGCATCAAAAGTGCCACTGGCTGGCGAATCCAGACTATTTTGTGTCAAAGAAACATGCGT
-612

BamHI

ACCTGCACAATAAAAAGAAAAGGCCCGCCCTACTGACATCCTGTTTCTGAGATCCTCGAAGAACAGGAAAAAG
-541

GACAACCGTCCTTTTCACTTCACGTTCGCAGGGCTTTCCTCGATATAATGGCGATTACACAAGCGGTGGCG
-470

TCTTGAAAAAAGTTGGGTTTCTTATCTGTACGGCGATCACTCCCCCTCATATGATAGCTGTGTAGAGTGACT
-399

TGCTCTTTGATTGGCCTTACTTCTTGCATCTATCATCCTTTCTTTTACTCTCTCCAATCACCACAACATCAT
-328

ATCATTTCTACATTTTCAATTTTATTGTTCTGTACAATTTTTAGGTTTGTAATAATATATAAGTGATGTACATAT
-257

CTTCTATATCCTTTCCGTTTGTGTCTATTTTAGGGTTGGCTTATTACGCATTATTCTACTTTATTTTAC
-186

TTGCTTACTTGAAAAAGAAGGTATACCACAGTGCTGCTCCACTGCAAACAATATATAAATATATCAATTATT
-115

TTTTTTTTTCTTCTTTTGAATTAGATCACTAATAAACTCTTATAATG
-44 **M**

Figure 7.

DNA sequence of the promoter region of the *PTR2* gene. Endonuclease restriction sites *Hind* III and *Bam* HI are shown in bold type face at positions -896 and -494, respectively. The initiation codon, ATG, of the *PTR2* gene is also designated in bold. A large inverted repeat, CGaaGAAcaggaAAAAGGACaaccGTCCTTTTcacTTCaCG, present in the promoter region adjacent to the *Bam* HI site is underlined. Putative "TATAA" elements are located at positions -208 and -65, respectively, and are double underlined.

Figure 8.

Nucleotide sequence and predicted amino acid sequence of the *PTR2* gene. The 601 amino acid open reading frame is shown with the hydrophobic regions indicated by underlining. Each hydrophobic region is designated by Roman numerals I, II, III, etc. The initiation codon, ATG, and the termination codon, TAG, are shown in bold. Also shown in bold are the restriction endonuclease sites *Aat* II (121-bp), *Msc* I (776-bp), *Eco* RI (1148-bp), and *Bgl* II (1439-bp).

1

ATG CTC AAC CAT CCC AGC CAA GGC TCA GAT GAT GCT CAG GAC GAA AAG CAA GGC GAC TTC CCG
M L N H P S Q G S D D A Q D E K Q G D F P

64

AatII

GTC ATC GAA GAG GAG AAG ACC CAG GCT GTA ACG CTG AAG GAT TCG TAT GTT ACG GAC GAC GTC
V I E E E K T Q A V T L K D S Y V T D D V

127

GCC AAC TCC ACG GAA CGC TAC AAC TTG TCC CCT TCT CCG GAG GAC GAA GAC TTC GAA GGC CCC
A N S T E R Y N L S P S P E D E D F E G P

190

ACT GAA GAA GAA ATG CAG ACT TTA AGG CAC GTT GGT GGT AAA ATT CCT ATG AGG TGT TGG TTA
T E E E M Q T L R H V G G K I P M R C W L
I

253

ATT GCT ATT GTA GAG CTT TCC GAG AGA TTC TCC TAC TAC GGG CTT TCC GCA CCA TTC CAA AAC
I A I V E L S E R F S Y Y G L S A P F Q N

316

TAC ATG GAA TAT GGA CCT AAT GAC TCC CCA AAG GGT GTT CTG AGC TTG AAC AGT CAG GGT GCC
Y M E Y G P N D S P K G V L S L N S Q G A
II

379

ACT GGG TTG TCG TAT TTT TTC CAG TTT TGG TGT TAC GTT ACA CCA GTT TTC GGT GGT TAC GTT
T G L S Y F F Q F W C Y V T P V F G G Y V

442

GCG GAC ACC TTC TGG GGT AAA TAT AAT ACA ATT TGT TGC GGT ACC GCT ATT TAC ATT GCC GGT
A D T F W G K Y N T I C C G T A I Y I A G
III

505

ATT TTC ATT CTA TTT ATC ACT TCG ATT CCC TCC GTT GGT AAC AGA GAC AGT GCT ATT GGT GGG
I F I L F I T S I P S V G N R D S A I G G
IV

568

TTC ATT GCT GCC ATT ATT CTG ATC GGT ATT GCC ACT GGT ATG ATT AAA GCT AAC CTT TCC GTG
F I A A I I L I G I A T G M I K A N L S V

631

TTG ATT GCC GAC CAG CTT CCT AAG CGG AAA CCC TCC ATC AAA GTT TTA AAA TCG GGC GAA AGA
L I A D Q L P K R K P S I K V L K S G E R

694

GTC ATT GTC GAT TCA AAT ATT ACT TTA CAA AAC GTT TTT ATG TTC TTC TAT TTC ATG ATC AAT
V I V D S N I T L Q N V F M F F Y F M I N
V

757

MscI

GTC GGT TCT CTA TCA TTA ATG GCC ACT ACT GAA TTG GAA TAT CAT AAG GGG TTC TGG GCG GCC
V G S L S L M A T T E L E Y H K G F W A A
VI

820

TAT CTA TTG CCC TTC TGC TTC TTT TGG ATC GCT GTT GTC ACT TTG ATT TTT GGT AAA AAG CAA
Y L L P F C F F W I A V V T L I F G K K Q

883

TAC ATT CAA AGA CCA ATC GGA GAT AAA GTC ATC GCT AAA AGT TTT AAA GTT TGT TGG ATT TTA
Y I Q R P I G D K V I A K S F K V C W I L

946

ACT AAG AAT AAG TTC GAC TTC AAC GCT GCT AAA CCT TCT GTT CAT CCA GAA AAG AAC TAT CCA
T K N K F D F N A A K P S V H P E K N Y P

1009

TGG AAT GAC AAA TTT GTT GAT GAA ATT AAG AGA GCT TTG GCG GCT TGT AAA GTC TTT ATA TTC
W N D K F V D E I K R A L A A C K V F I F
VII

1072

TAC CCA ATT TAT TGG ACC CAA TAC GGT ACC ATG ATT TCC AGT TTC ATC ACT CAG GCC AGT ATG
Y P I Y W T Q Y G T M I S S F I T Q A S M

1135

EcoRI

ATG GAA TTA CAT GGA ATT CCC AAC GAT TTC TTA CAA GCA TTC GAT TCC ATT GCG TTG ATC ATT
M E L H G I P N D F L Q A F D S I A L I I
VIII

1198

TTC ATC CCA ATT TTT GAA AAA TTC GTA TAT CCT TTC ATT AGA AGA TAC ACT CCA CTA AAA CCA
F I P I F E K F V Y P F I R R Y T P L K P

1261

ATT ACA AAA ATT TTC TTC GGT TTC ATG TTT GGA TCT TTT GCC ATG ACA TGG GCT GCT GTT CTA
I T K I F F G F M F G S F A M T W A A V L
IX

1324

CAA AGT TTC GTT TAC AAG GCT GGT CCA TGG TAT AAT GAA CCT CTG GGT CAC AAC ACC CCA AAT
Q S F V Y K A G P W Y N E P L G H N T P N

1387

BglII

CAT GTC CAC GTT TGC TGG CAA ATA CCC GCA TAT GTC TTG ATT TCT TTT TCA GAG ATC TTT GCC
H V H V C W Q I P A Y V L I S F S E I F A
X

1450

TCT ATC ACT GGG TTG GAA TAC GCT TAT TCC AAA GCC CCA GCT TCC ATG AAA TCG TTT ATT ATG
S I T G L E Y A Y S K A P A S M K S F I M
XI

1513

TCC ATT TTC TTA TTG ACT AAC GCC TTT GGT TCT GCA ATC GGT TGT GCA TTG TCC CCA GTG ACC
S I F L L T N A F G S A I G C A L S P V T

1576

GTT GAT CCT AAA TTT ACA TGG TTA TTC ACT GGT TTG GCT GTT GCC TGC TTT ATT TCT GGT TGT
V D P K F T W L F T G L A V A C F I S G C
XII

1639

TTG TTC TGG TTG TGC TTC AGG AAG TAT AAT GAT ACA GAG GAA GAA ATG AAC GCT ATG GAC TAC
L F W L C F R K Y N D T E E E M N A M D Y

1702

GAA GAA GAA GAC GAA TTT GAT CTC AAT CCA ATT TCC GCA CCT AAA GCT AAC GAT ATT GAA ATA
E E E K E F K L N P I S A P K A N D I E I

1765

TTA GAA CCA ATG GAA AGT CTA AGA TCC ACC ACC AAA TAT TAG
L E P M E S L R S T T K Y END

(*PTR2* Coding Region)

CTA AGA TCC ACC ACC AAA TAT TAG TGC GTTTAACTTACTGTCTTTTTTTTTTTTTTTTTT
 L R S T T K Y END

TTTTAACCATATTACGTTGACGGGAGTTTTCTGTGCTGCTGTTATTTGTTTCACAGAACTCTCTCCTCTTC

CATTGCTATCGAACAGATAAAAATGGAATTTCTTTATGTATAATTACTATTGTATAATATTCTGAAATCTAA
 A A

ATTTAAGCTCATATATCTAAACACTACAACATGGAAGCAGCTTGTCTCTTCAGGCTCACTGTATCCGTTT
 A

ACTCTTATTTTCGCGTATTTGCATACCATTTATCTCAAATGACTTGTTATTATATCTTGATATATATTGTATT

ATAGAACAATTTGTGAAGAGAGGTCCATATTAAGATTTCTCCCATTTACAAATAGAATAGAAAAGGAAAT
 B

ATATATGGTAGTAAAAAAATCGTGATGTATACAAACTTTGGATTTGTGGAGATC
 B B *Sau 3A*

Figure 9.

Transcription termination region of the *PTR2* gene. The 3'-flanking region of the *PTR2* gene that overlaps with a known DNA sequence coding for the 3' region of the *UBI2* gene. The protein coding regions of the two genes do not overlap. The stop codon for the *UBI2* gene is located 13-bp downstream from the *Sau 3A* site and is not shown. Putative transcription termination sites are shown underlined. Two such tripartite elements are present and are designated A and B. Also shown is the *Sau 3A* site that forms the junction between the cloned *PTR2*⁺ DNA insert and the plasmid vector, YCp50.

Figure 10.

Maps of the gene disruption plasmids pJP23(I) and (II). Shown are the restriction maps of plasmids pJP9, pJP15, pJP23(I), and pJP23(II). Plasmid pJP9 contains a functional copy of the *PTR2* gene carried on CEN plasmid-vector YCp50. Plasmid pJP15 contains a 1.6-kbp *Bam* HI - *Eco* RI DNA fragment of the *PTR2* gene, derived from pJP9, carried on plasmid-vector pRS3062. A 2.0-kbp DNA fragment containing the *LEU2* gene was inserted into plasmid pJP15 which had a 653-bp *Aat* II - *Msc* I DNA fragment deleted (Materials and Methods). The *LEU2* gene is shown in both orientations designated pJP23(I) and pJP23(II). The 2.6-kbp *Nde* I - *Dra* I DNA fragment of pJP23(I) and pJP23(II), containing the inserted *LEU2* gene in both orientations and flanked at the termini by *PTR2* DNA sequence, was excised and used as substrate in transforming the *Ptr2*⁺ strain PB1X-2A. The arrow below pJP9 represents the region coding for the *PTR2* gene. The arrows below pJP23(I) and (II) represent the orientation of the regions coding for the *LEU2* gene. Restriction endonuclease sites: H, *Hind* III; B, *Bam* HI; RI, *Eco* RI; A, *Aat* II; D, *Dra* I; M, *Msc* I; and G, *Bgl* II.

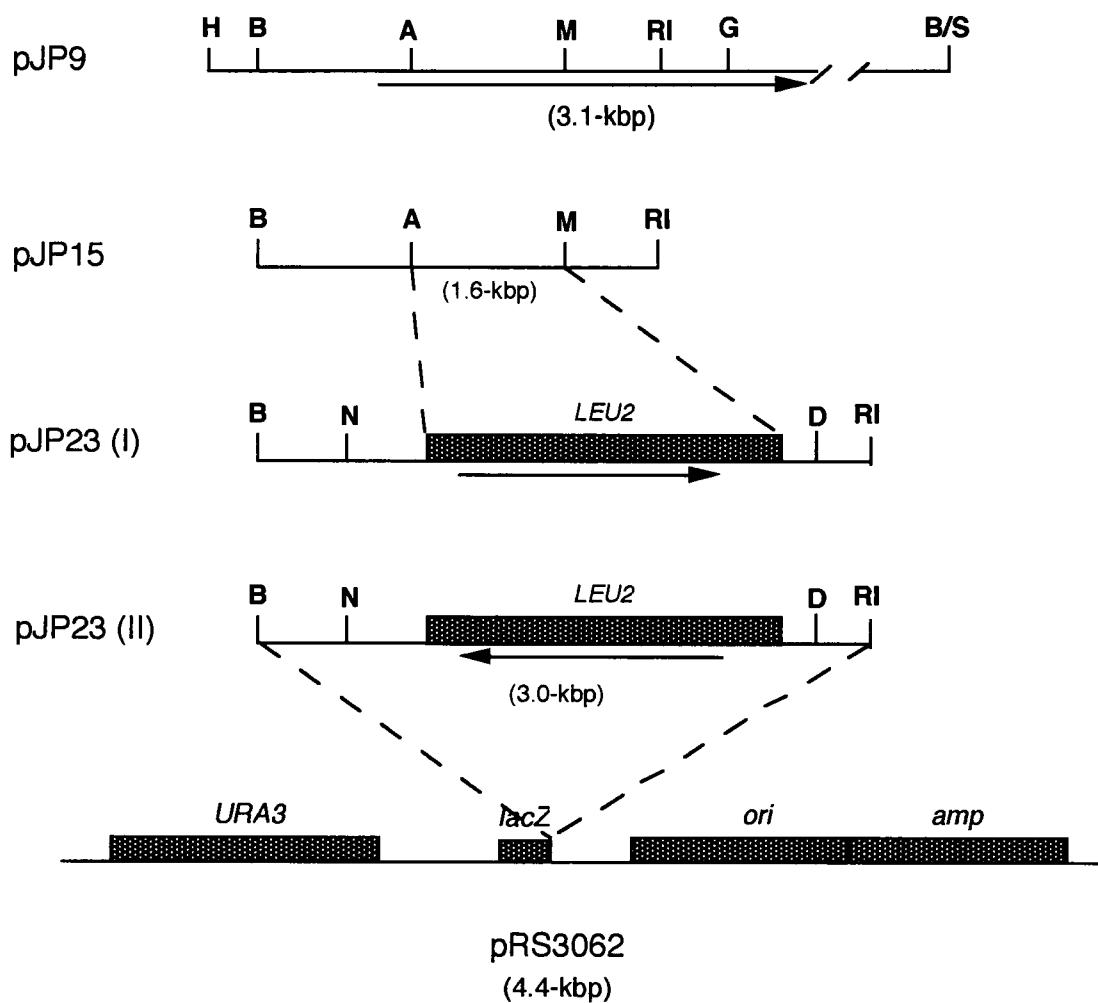
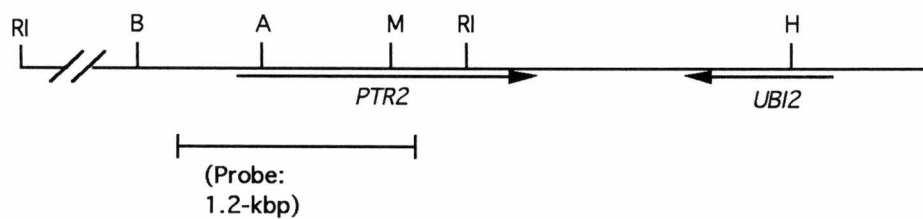


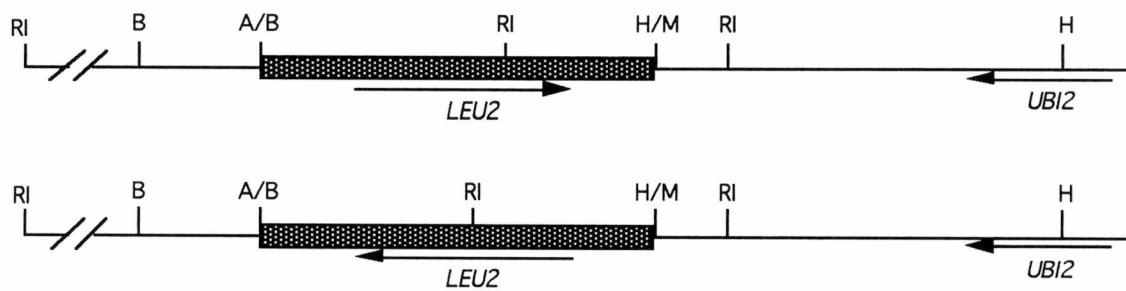
Figure 11.

Structure of the *PTR2* locus. The structure of the *PTR2* locus was deduced from plasmid maps, DNA sequence data, and Southern hybridization analysis. Panel A shows the region of the right arm of Chromosome XI that contains the *PTR2* and *UBI2* genes. The arrows indicate the direction of transcription of *PTR2*, *LEU2*, and *UBI2*. Also shown is the region of *PTR2* that corresponds to the radiolabeled probe used in Southern hybridization experiments. Panel B shows the predicted structure of the disrupted *PTR2* locus with the *LEU2* gene inserted in both orientations corresponding to plasmids pJP23(I) and pJP23(II). The region of the *PTR2* gene extending from the *Aat II* restriction site to the *Msc I* restriction site was deleted and replaced by 2.0-kbp DNA fragment encoding the *LEU2* gene of *S. cerevisiae* (Materials and Methods). The structure of the disrupted locus was confirmed by Southern hybridization analysis. Panel C shows the results of a Southern blot in which whole cell DNA from wildtype strains PB1X-2A, PB1X-2A(Δ I), and PB1X-2A(Δ II) were digested with *Eco* RI, fractionated on a 0.9% agarose gel, transferred to a nylon membrane, then hybridized with a radiolabeled *PTR2* specific PCR generated probe (Materials and Methods). Lane A demonstrates a 4.4-kbp *Eco* RI generated DNA fragment from the wildtype *S. cerevisiae* strain, PB1X-2A. Lane B demonstrates a similar 4.4-kbp *Eco* RI generated DNA fragment and, in addition, demonstrates a 1.2-kbp fragment expected from the disrupted *PTR2* locus of strain PB1X-2A(Δ I). Lane C also demonstrates a 4.4-kbp *Eco* RI generated fragment and a 1.4-kbp fragment expected from the disrupted strain PB1X-2A(Δ II). The two disruption strains differ only in the orientation of the *LEU2* insert. 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.

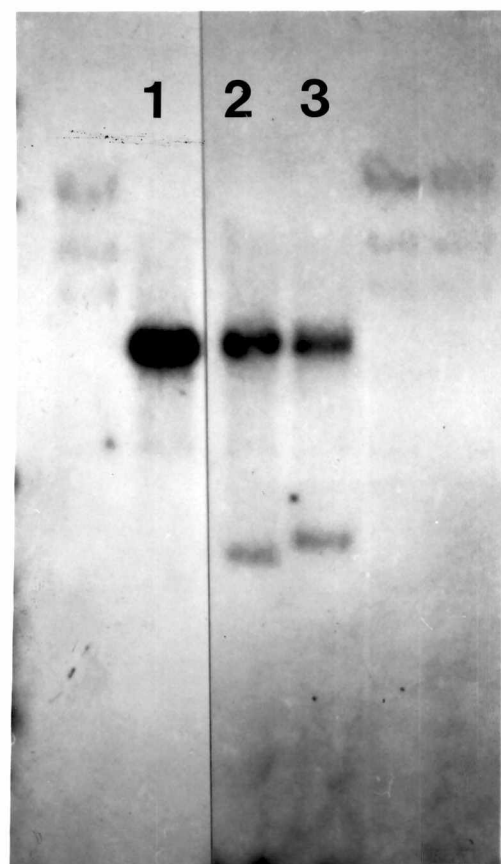
A



B



C



◀ 23-kbp
 ◀ 9.4-kbp
 ◀ 6.6-kbp
 ◀ 4.4-kbp
 ◀ 2.3-kbp
 ◀ 2.0-kbp
 ◀ 0.6-kbp

Figure 12.

Comparison of the *PTR2* and *CHL1* encoded proteins. A search of the GenBank database using the BLAST algorithm revealed significant similarities between the proteins encoded by the *PTR2* gene of *S. cerevisiae* and the *CHL1* gene of *A. thaliana*. The proteins exhibit 26% identity and 52% similarity over the entire sequence with regions of higher identity, such as 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 eight standard deviations over a random fit using a stringency of 20 randomizations of the BESTFIT algorithm. Amino acids which show identity between the two proteins are underlined.

PTR2 MLNHPSQGSDDAQDEKQGDFPVIEEEKTQAVTLKDSYVTDDV 42
 CHL1 LPETKSDDILLDAW..... 16

 ANSTERYNLSPSPEDDEFEGPTEEEEMQTLRHVGGKIPMRCWL 84
DFQGRPADRSLTGGWASAA.....M 36

 IAIVELSERFSYYGLSAPFQNYMEYGPNDSPKGVLSLNSQGA 126
 ILCEIAVERLTTLGIGVNLVTYL.....TGTMHLGNATA 70

 TGLSYFFQFWCYVTPVFGGYVADTFWGKYNTICCGTAIYIAG 168
 ANVTNFLGTSFMLCLLGGFIADTFLGRYLTIIFAIIQATG 112

 IFILFITSI.....PSVGNRDSAI..GGFIAAII 195
 SVILTLSTIIPGLRPPRCNPTTSSHCEQASGIQLTVLYLALY 154

 LIGIATGMIKANLSVLIADQLPKRKPSIKVLKSGERVIVDSN 237
 LTAGGTGGVKASVSFGSDQFDETEPKER.....SK 185

 ITLQNVFMFFYFMINVGSLSLMATTELEYHKG.....FW 271
 MTY..FENREFECINVG..SLLAVTVLVYVQDDVGRKWGYGI 223

 AAYLLPFCFFWIAVVTLIFGKKQYIQRPIGDKVIAKSFKVCW 313
 CAFAIVLALSVFLAGTNRYTFKKLIGSPMTQ..VAAVIVAAW 263

 ILTKNKF.....DFNAAKPSVHPEKNYPWNDKF.... 341
 RNRKLELPADPSYLYDMDIIAAEGSMKGKQKLPHTEQFRSL 306

VDEIKRALAACKVFIF 357
 DKAAIRDQEAGVTSNVFNKWLSTLTDVEEVKQIVRMLPIWAT 349

 YPIYWTQYGTNISSFITQASMME...LHGIPNKFLQAFDSIA 496
 CILFWTVHAQLTTLSVAQSETLDRSIGSFEPASMAVEYVGG 392

 LIIFIPIFEKFVYPFIRRY...TPLKPITKIFFGMFGSFAM 435
 LLLTTAVYDRVAIRLCKKLFNYPHGLRPLQRIGLGLFFGSMAM 435

 TWA AVLQSFVYKAGPWYNEPLGHNTPNHVHVCWQUPAYVLISF 478
 AVAALVELKRLRTAHAGPTV...KTLPLGFYLLIPQYKUVGI 475

 SEIFASITGLEAYSKAPASMKSFIMSIFLLTNAFGSAIGCAL 521
 GEALIYTGQLDFFLRECPKGMKGMSTGLLLSTLALGFFFSSVL 518

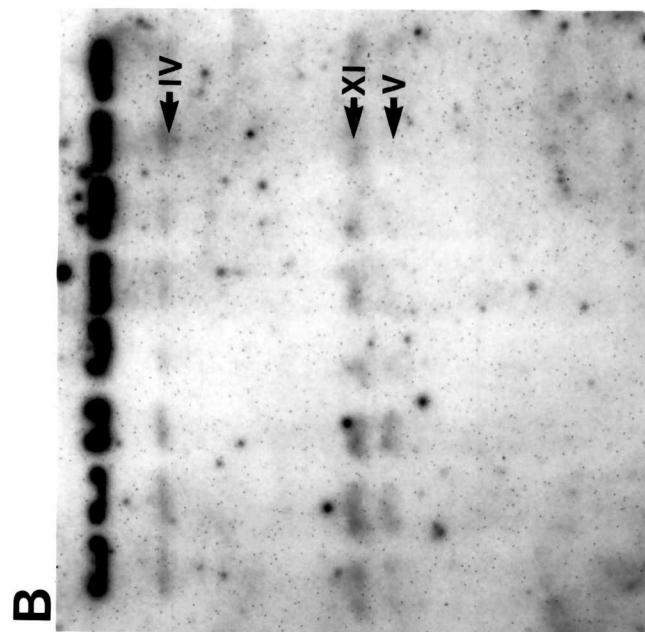
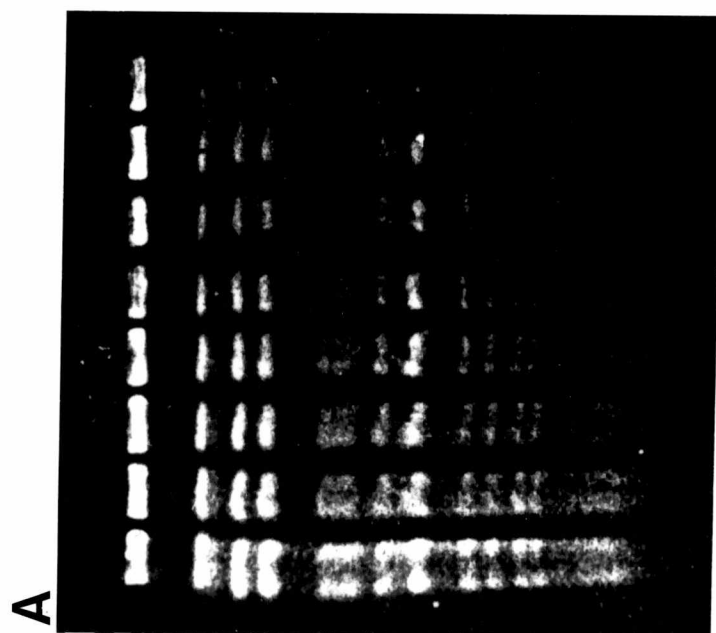
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 ..VTIVEKFTGKAHPWIADDL NKGRLYNFYWLVAVLVALNFLI 559

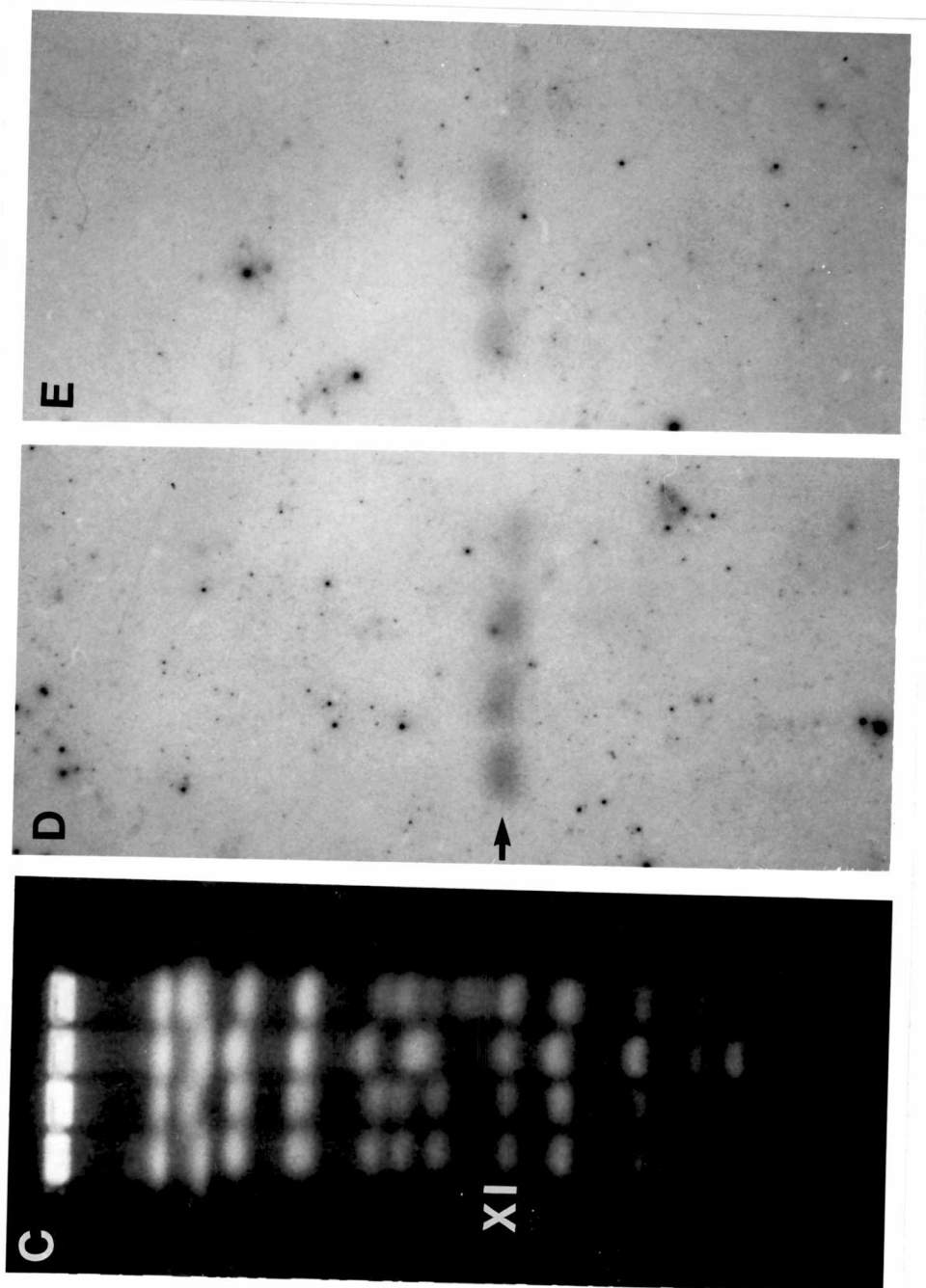
 WLCFRKYNDTEEMNAMDYEEDEFDLNPISAPKANDIEILEP 591
 FLVESKWVYKEKRLA...EVGIELDDEP.SIPMGH..... 591

 MESLRSTTKY 601

Figure 13.

Chromosomal assignment of the *PTR2* gene (Materials and Methods). Shown in panel A is a photograph of electrophoretically fractionated chromosomes from *S. cerevisiae* S288C. The eight lanes, progressing from left to right, contain decreasing amounts of chromosomal DNA. Shown in panel B is an autoradiogram of a blot prepared from the chromosomes in panel A and probed with radiolabeled pJP9. DNA sequences on three chromosomes hybridize with the radiolabeled pJP9 probe. Chromosome IV hybridizes with the *CENIV* and *ARS1* sequences of pJP9. Chromosome V hybridizes with *URA3* sequences of pJP9. Chromosome XI hybridizes with the *PTR2*⁺ insert sequence of pJP9. The prominent signals near the top of panels A and B are due to the failure of some chromosomes to migrate from the gel wells during electrophoresis. Chromosomes IV, V, and XI are indicated. Shown in panel C is a photograph of electrophoretically fractionated chromosomes from *S. cerevisiae* X2180-1B, X2180-1A, DBY747, and CG375/380. Shown in panels D and E are autoradiograms of blots prepared from the chromosomes in panel C and probed with a radiolabeled 1.6-kbp, *PTR2*-specific DNA probe and a radiolabeled 4.0-kbp, Chromosome XI-specific DNA probe, respectively. Both probes hybridize with the same chromosome from the four strains tested.





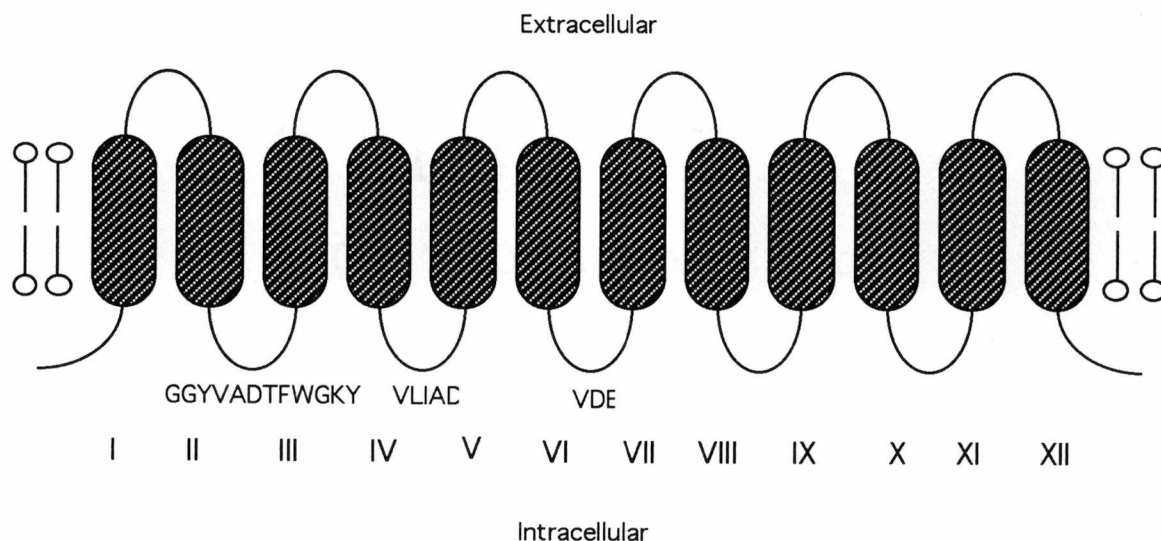


Figure 14.

Schematic representation of the Ptr2 protein. The shaded barrels represent the twelve hydrophobic domains, shown here as membrane spanning regions and designated with Roman numerals. The connecting lines represent regions of relative hydrophilicity. Shown are the regions resembling the Walker A and B motifs (Discussion). Also shown is the region containing the VDE element which may also participate in binding ATP or GTP in concert with the Walker A element (Discussion). Extracellular and Intracellular domains are indicated.

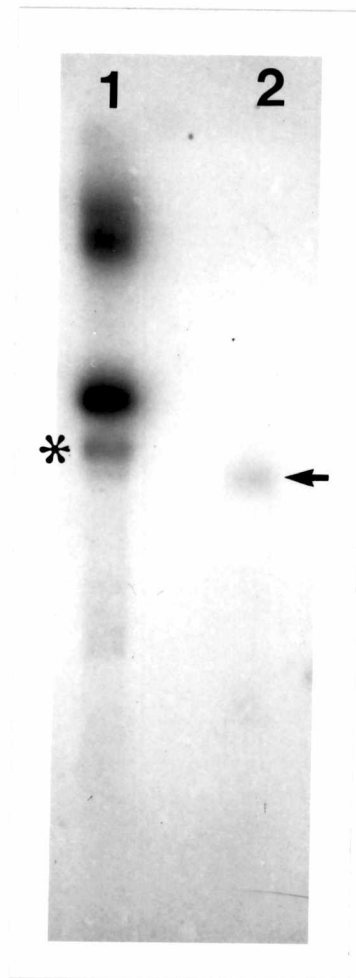


Figure 15.

Northern blot demonstrating the *PTR2* transcript. Lane 1, RNA size standards of 9.49, 7.46, 4.40, 2.37 (asterisk), and 1.35-kb in length, respectively. Lane 2, 10 μ g of total RNA from *S. cerevisiae* S288C grown in YEPD (Materials and Methods). Lane 2 demonstrates a transcript of approximately 1.8-kb in length (arrow).

VITA

Jack R. Perry was born in Nashville, Tennessee on April 8, 1959. He attended public schools and graduated from Cohn Public High School in May, 1977. He entered Tennessee State University and in December, 1982 received the degree of Bachelor of Science in Biology. For the following two years he studied mathematics at Tennessee State University. In June of 1984 he entered The University of Tennessee, Oak Ridge Graduate School of Biomedical Sciences where he studied the genetic basis for error-prone DNA repair in *Saccharomyces cerevisiae*, under the mentorship of Dr. Frank W. Larimer, at the Oak Ridge National Laboratory. In 1986, he began studies in the Laboratory of Dr. Jeffrey M. Becker to elucidate the genetic basis for the transport of small peptides across the plasma membrane of *S. cerevisiae*. In August of 1990 he entered East Tennessee State University, College of Medicine and in May, 1994 received the degree of Doctor of Medicine while continuing his doctoral research at The University of Tennessee. In May of 1995 he completed his requirements for the degree of Doctor of Philosophy in the Life Sciences, Program in Cellular, Molecular, and Developmental Biology.

He is currently employed as a Clinical Instructor and House Staff Officer in the Department of Pathology and Laboratory Medicine at the University of Cincinnati, College of Medicine, Cincinnati, Ohio.