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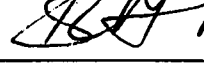
Signature 

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Search for the human homolog of *S. cerevisiae PTR2* gene

A Thesis
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ABSTRACT

Peptide transport is an evolutionarily conserved phenomenon with functional significance in cell nutrition. An attempt to identify a human homolog of the *S. cerevisiae* peptide transport gene (*PTR2*) was made in order to study the molecular features of the putative human gene. *S. cerevisiae ptr2* mutant PB1X-9B was transformed with a HeLa cell cDNA library and ptr2$^+$ transformants were selected directly on dipeptide medium. A single clone with a 10kb insert was obtained. Subsequently, the complementing region was subcloned as a 6kb fragment of the insert. Southern and DNA sequence analyses showed that the isolated clone was identical to the *S. cerevisiae PTR2* and not derived from HeLa cell DNA. The explanation for this result is not apparent.

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I. Introduction

The evolutionary conservation of peptide transport mechanisms across species barriers underscores its significance in the acquisition of peptides in cell nutrition. Although several types of cellular transport processes are known to exist, the focus of this study was to elucidate the molecular characteristics of mammalian dipeptide specific transport proteins.

This study was undertaken to identify a human homolog of the *Saccharomyces cerevisiae* peptide transport gene(*PTR2*) using a cervical carcinoma (HeLa) cell cDNA library by functionally complementing the *S. cerevisiae ptr2* mutation. The cloning of the human peptide transport gene will pave the way to study the molecular features of its encoded protein in comparison with the peptide transporters from other organisms.

Theoretically, the maximum number of the naturally occuring dipeptides and tripeptides that could be transported across the plasma membrane is 400 and 8000, respectively, with many potential attachment points for toxic moieties in these peptides. The determination of the substrate specificity of the human peptide transporter would be a step towards the design of peptide drugs toxic to fungi that are not taken up by human cells. An obvious extension of the study is to determine whether the human peptide transporter can be exploited as a vehicle in the uptake of biologically and pharmacologically useful compounds. A thorough understanding of the peptide transport systems in human and fungal cells may aid in the design of effective anti-fungal drugs.

II. Review of Literature

Peptides containing usually no more than a few amino acids are hydrolysis products of proteins. Peptides are found abundantly in nature as a result of micro biological activity. The transport of peptides into bacteria, fungi and mammalian cells is a physiological process distinct from amino acid uptake (Higgins, 1992: Becker and Naider 1980, and Matthews, 1991). Peptides can be used to replenish nitrogen content of the cell (Suomalainen and Oura, 1971). Additionally, amino acids derived from intracellular peptide hydrolysis can be used as precursors of other peptides or proteins for cellular activities (Goodell and Higgins, 1987).

Modes of Peptide Transport

Cell membranes form a protective barrier for the celullar contents and also regulate the entry or the exit of various biomolecules into and out of the cell. Neuropeptides are generally oligopeptides. They are known to participate in intercellular communication in animals (Scharrer, 1987). The *S. cerevisiae* mating pheromones, a and α-factors are also oligopeptides. These signaling oligopeptides are usually taken in by the target cells through receptor-mediated endocytosis (Segaloff and Ascoli, 1988; Reizman *et al.*, 1986). Such vesicular transport of peptides is distinct from carrier mediated transport of peptides.

In early studies it had been postulated that peptide utilisation was mediated by either extracellular peptidases or cell membrane bound peptidases (Matile *et al.*, 1971; Stotzler *et al.*, 1977). Completely hydrolysed peptides were transported into the cell as amino acids by amino acid transporters. However, many studies have been conducted that establish peptide utilisation in the absence of extracellular or membrane bound peptidases. Payne (1974) and Sussman and Gilvarg (1971) reported the intracellular localisation of peptidase activities in bacteria. Becker and co-workers (1973) found peptidase activity against trimethionine in the extracts of yeast whole cells and osmotically shocked cells but not in the extracellular milieu of these cells. In the intestinal brush border membranes of man, rat and rabbit, Morita *et al.* (1983) found no peptidase activity against proline-containing dipeptides. This observation was made notwithstanding the fact that the widely distributed dipeptidyl peptidase IV is known to cleave proline-containing oligopeptides. Steinhardt and Adibi (1986) detected no hydrolysis of glycyl-dipeptides in human jejunum. Tiruppathi *et al.* (1990) reported the uptake of Phe-Pro-Ala in rat renal brush border membrane lacking in dipeptidyl peptidase IV.

These and other studies buttress the fact that peptide transporters with specific substrate specificities exist and complement transport systems available for individual amino acids. It is reasonable, therefore, to think in terms of membrane bound peptide transport proteins analogous to amino acid or glucose transporters.

Peptide Transport is Independent of Amino Acid Transport

Several lines of evidence are available to demonstrate that peptide transport is independent of amino acid transport in eucaryotic cells. Two experiments indicated that peptides are transported intact in yeast. It was shown that certain peptides capable of supporting the growth of yeast were not hydrolysed and also that amino acids do not compete with the uptake of peptides. Similarly peptides were found not to compete with the transport of amino acids (Marder *et al.*, 1977). The uptake of radiolabeled dileucine was shown to be uninhibited in the presence of leucine in yeast (Island *et al.*, 1987; Perry *et al.*, 1994).

The dipeptides Phe-Phe and Lys-Lys were found to be transported across the intestinal brush border membrane of people with genetic disorders of amino acid transport such as Hartnup and Cystinuria syndrome and to supply the respective amino acids (Asatoor *et al.*, 1972 and Hellier *et al.*, 1972). Rubino and colleagues (1971) showed that while the amino acids Phe, Leu and Met were able to compete out Gly, they were not able to inhibit the influx of Gly-Pro dipeptide across the brush border membrane of rabbit ileum *in vitro* Similarly, the peptides Phe-Pro and Gly-Gly were shown not to inhibit the uptake of Gly in the same study. Even though excess amounts of Gly saturates the ability of human intestine to absorb Gly, the uptake of Gly-Gly was shown to be independent of the amino acid transport (Cook, 1973). Similar results have been obtained in rat intestine with Met and Met-Met (Crampton *et al.*, 1973). Further, the energetics of amino acid and peptide transport are different. Conclusive evidence for the energisation of the intestinal peptide

transport was provided by two separate studies. While the transport of amino acids is now known to be Na^+-dependent, Dantzig and Bergin (1990), and Calonge *et al.* (1990) demonstrated the coupling of peptide transport to the proton motive force.

Peptide Transport in Procaryotes

The transport of small peptides has been studied in much detail in bacteria, especially in *Escherichia coli* and *Salmonella typhimurium* (Higgins, 1992). In these procaryotic organisms, the oligopeptide permease(Opp) system is comprised of oppA, oppB, oppC, oppD and oppF genes (Andrews and Short, 1985, Hiles *et al.*, 1987 and Hogarth and Higgins, 1983). This system transports peptides having 5 to 6 amino acids without regard to amino acid side chain (Payne, 1968 and Payne and Gilvarg, 1968). A tripeptide permease system with an affinity for hydrophobic amino acid residues was reported by Gibson *et al.* (1984). A dipeptide permease system also exists for *S. typhimurium.* (Abouhamad *et al.*, 1991)

Peptide Transport in Eucaryotic Microorganisms

The presence of peptide transport systems in eucaryotic microorganisms namely, *S. cerevisiae* and *C. albicans* has been very well established. The transport of a variety of peptides in *C. albicans* has been shown to be energy dependent (Logan *et al.*, 1979, and McCarthy *et al.*, 1985) with little side chain specificity (Lichliter *et al.*, 1976). The

transport characteristics of small peptides in *S. cerevisiae* is similar to that of *C. albicans* with regard to energy requirement and side chain specificity (Becker and Naider, 1980). According to Becker and Naider (1977) trimethionine uptake in *S. cerevisiae* was inhibited in the presence of energy uncouplers such as sodium azide, dinitrophenol and potassium cyanide. Furthermore, dipeptides containing toxic amino acids such as ethionine or DL-m-fluorophenylalanine inhibited the growth of wild type *S. cerevisiae* (Island *et al.*, 1987).

The absence of amino acid side chain specificity implies a broad substrate specificity in the transport of peptides across the plasma membrane. It is well known that non-permeating toxic moieties can be conjugated to the functional groups of amino acids. Such compounds may easily enter into the cell through the peptide transport system (Gilvarg, 1981, and Ringrose, 1985). Such a strategy has been proposed for the development of anti-fungal compounds (Becker *et. al.*, 1977; Ti *et al.*, 1980 and Becker and Naider, 1994). In fact, peptidyl nucleoside antibiotics like nikkomycins and polyoxins are transported through the peptide transport system in *C. albicans* (Becker *et al.*, 1983., McCarthy *et al.*, 1985., Mehta. *et al.*, 1984 and Yadan *et al.*, 1984).

Peptide Transport in Higher Eucaryotic Cells

The uptake of peptides has been observed in many cells of mammalian origin. Radiolabeled peptides like Gly-Phe, Gly-Leu (Zlokovic *et al.*, 1983), Glutathione and Leu-enkephalin (Cornford *et al.*, 1978) are transported into rat brain at

the blood brain barrier. The presence of specific peptide transporters in the endothelial cell membrane has not been shown conclusively. However, using perfusion technique, the analysis of the uptake of (^{3}H)Leucine-enkephalin by the guinea pig brain revealed saturation kinetics (Zlokovic *et al.*, 1989). This suggested the existence of specific carrier mediated transport for peptides into brain.

Hydrolysis-resistant peptides of non-physiological origin have been shown to be actively transported in hamster jejunum (Addison *et al.*, 1975). While the tripeptide Gly-Sar-Sar was transported actively, its uptake was slow when compared to that of the dipeptide Gly-Sar. Adibi and Morse (1977) demonstrated that the human jejunum could not transport tetra-Gly or higher peptides. Smithson and Gray (1977) concluded that the rat intestine was unable to transport Gly-Leu-Gly-Gly unless it underwent hydrolysis. In the hamster intestine, the transport of Carnosine (beta-alanyl-L-histidine), a hydrolysis resistant peptide found in meat, showed saturation kinetics (Matthews *et al.*, 1974).

Substrate Specificity in Peptide Transport

The preference for L-amino acids has been shown in many studies investigating peptide transport. According to Shallow *et al.* (1991) *C. albicans* did not transport peptides containing D-residues. Marder *et al* (1977) and Nisbet and Payne (1979b) found that *S. cerevisiae* did not utilise L-Ala-L-Ala-D-Ala. A similar result was obtained with L-Met-L-Met-D-Met (Becker and Naider, 1977). However, peptides with the

D-amino acids as the N-terminal residue were found to be good substrates (Logan *et al.*, 1979; Milewski *et al.*, 1991a).

Transmembrane transport of Tyr-D-Ala-Gly was shown to be very slow in rat intestine(Heading *et al.*, 1979). This result was in conformity with the observation that peptides containing D-amino acid residues resist hydrolysis and are transported poorly by the small intestine (Burston *et al.*, 1972; Asatoor *et al.*, 1973). Boyd and Ward (1982) reported that an N-terminal D-amino acid residue may have more effect on transport than a C-terminal D-amino acid residue in a peptide.

A broad substrate specificity is seen in the transport of peptides containing L-amino acid residues in both *C albicans* and *S cerevisiae*. However, the transport system shows a preference for hydrophobic amino acids (Becker and Naider, 1994). Wide variation in the utilisation of specific peptides has been reported using different microbial strains and different types of experiments to assay transport. While Nisbet and Payne, 1979a reported that the dipeptide lysyl-lysine is transported by *S. cerevisiae*, Marder *et al.* 1977 and Becker *et al.* 1973 have shown that the dipeptide does not serve as a good substrate in satisfying the auxotrophic requirements of *S. cerevisiae*. Naider and co-workers (1974) showed that tripeptides containing glycine and methionine supported either moderate or excellent growth of a *S. cerevisiae* methionine auxotroph.

In *C. albicans* the dipeptide Ala-Ala was reported to be transported at a rate four times greater than Ala-Gly while the rate of transport remained the same for tripeptides Ala-

Ala-Ala and Leu-Gly-Gly (Milewski *et al.*, 1991b). In another study, Milewski *et al.* (1991a) reported higher transport rates for dipeptides such as Leu-N^3-(4-methoxyfumaroyl)-L-2,3 diaminopropanoic acid (Leu-FMDP) that contain a hydrophobic amino acid residue like leucine. The poor efficacy of Lys-FMDP as an anti-*candidal* agent was attributed to its slow intra-cellular cleavage. Becker and Naider (1994) suggest that an effective peptide drug should not only be transported quickly but also be cleaved rapidly. While *S. cerevisiae* generally transports peptides not larger than tri-peptides (Marder *et al.*, 1977) *C. albicans* has been found to transport pentapeptides containing toxic amino acids by growth inhibition studies (Basrai *et al.*, 1992).

The extremely slow transport of Gly-Sar-Sar-Sar relative to Gly-Sar or Gly-Sar-Sar by rat jejunum was interpreted as the uptake being limited to di- and tri-peptides (Addison *et al.*, 1975). Adibi and Morse (1977) had earlier found that the human jejunum was unable to transport tetra-Gly or higher peptides. The existence of multiple peptide transport systems became evident when Gly-Gly and Gly-Gly-Gly did not inhibit the uptake of Gly-Leu in guinea pig intestine (Himukai and Hoshi, 1980 ; Himukai *et al.*, 1982). According to Matthews (1991) the transport of basic and acidic dipeptides, namely Lys-Lys and Glu-Glu, was by the same system that transported Gly-Sar and Gly-Gly in hamster jejunum. Recently, Brandsch *et al.* 1994 found that the Gly-Sar uptake in Caco-2 human colon carcinoma cell line was inhibited by di- and tri-peptides and by cephalexin, a peptide-like antibiotic of the beta-lactam family.

Regulation of Peptide Transport

Peptide transport in *S. cerevisiae* and *C. albicans* is profoundly influenced by the source of nitrogen. Several workers have reported decreased peptide uptake by *S. cerevisiae* in the presence of ammonium ions in the growth medium. On the other hand, organic sources of nitrogen such as allantoin, isoleucine or proline were shown to facilitate peptide uptake (Becker and Naider, 1977; Moneton *et al.*, 1986; Nisbet and Payne, 1979b). According to Becker *et al.* (1983), and Mehta *et al.* (1984) the source of nitrogen influenced the toxicity of a peptidyl-nucleoside that shares the peptide transport system in *C. albicans*. Increase in the utilisation of di- and oligopeptides was also shown in the presence of peptide mixtures added to culture medium (Payne *et al.*, 1991).

Island *et al.* (1987) have shown that the dipeptide transport in *S. cerevisiae* is regulated by micromolar amounts of amino acids. A dramatic increase in the utilisation of Leu-Leu was noted in the presence of 150μM tryptophan. Leucine, tryptophan and lysine resulted in the greatest growth inhibition of *S. cerevisiae* by toxic peptides in the presence of allantoin. Basrai *et al.* (1992) reported a similar increase in sensitivity to toxic peptides for *C. albicans* in the presence of lysine, arginine, histidine, glutamine, aspartate and serine. Amino acids appear to regulate the transcription of the *PTR2* gene in *S. cerevisiae* leading to an increase in *PTR2* mRNA in the presence of tryptophan, leucine, or lysine (Steiner, Unpublished results).

Recently, it has been shown that the *PTR2* transcript is not made in *S. cerevisiae ptr1* mutant indicating that the peptide transport is under transcriptional control (Alagramam, unpublished results). Since the *PTR1* has potentially DNA binding domains such as a Leucine-zipper, a Zinc-finger-like motif, and an acidic tail, it has been proposed that *PTR1* could be the transcriptional activator of *PTR2*. The precise mechanism of regulation is, however, yet to be understood.

Brandsch *et al.* (1994) were the first to provide evidence for a protein kinase C(PKC)-mediated regulation of the peptide/H^+ co-transport system in the Caco-2 cell line of the human intestine. Significant inhibition of the transport system was found when the cells were treated with PKC activators like phorbol 12-myristate 13-acetate(PMA), phorbol 12,13-dibutyrate(PDBu) and not with 4α-phorbol and 4α-phorbol 12,13-didecanoate(PDD) that do not activate PKC. The inhibition was reported to be specific since the transport of leucine was not affected under identical conditions. It was proposed that the function of the transporter was regulated as a result of change in its phosphorylation state directly by PKC or indirectly through additional protein kinases/phosphatases.

Genetic Components of Peptide Transport in Eucaryotes

Island *et al.* (1991) isolated and characterised *S. cerevisiae* peptide transport mutants. Genetic analysis facilitated their classification into three distinct complementation groups namely, *PTR1*, *PTR2* and *PTR3*. The first report of isolating a

structural gene required for peptide transport in a eucaryotic cell came from the work of Perry *et al.* (1994).The *S. cerevisiae PTR2* gene was found to have an open reading frame of 1,803bp (601 amino acids) coding for a 68.1kDa hydrophobic peptide typical of a membrane protein. Basrai *et al.* (1994) identified the *PTR2* homolog from *C. albicans*. In the recent past, *S. cerevisiae PTR1* gene was also cloned by rescuing the *ptr1* mutation in *S. cerevisiae* PB4X-5B (Alagramam *et al.*, 1994). The gene was unexpectedly found to be identical to *UBR1* gene. The gene product of *UBR1* is a recognition component of N-end rule pathway of the ubiquitin system that targets proteins for degradation (Varshavsky, 1992). The *S. cerevisiae ptr2* mutant PB1X-9B has been successfully exploited in cloning the peptide transport gene from *Arabidopsis thaliana* (Steiner *et al.*, 1994) The *AtPTR2* codes for a protein of 610 amino acids. The cloning of a second peptide transport gene from *Arabidopsis thaliana* which is not sensitive to toxic dipeptides, has also been accomplished (Wei-Song and Steiner, Unpublished results).

Recently, Fei *et al.* (1994) reported the cloning of rabbit intestinal peptide transport gene(*PepT1*) by functional expression of the transporter mRNA in *Xenopus laevis* oocytes. The protein, consisting of 708 amino acids, was found to have about 28% similarity at the amino acid level to the gene product of *S.cerevisiae PTR2*. In a Northern analysis, the presence of large amounts of oligopeptide transporter mRNA was indicated in rabbit kidney, liver and intestine and small amounts in brain. Using *PepT1* cDNA, hybridisation cloning of the human intestinal peptide transport gene showing 86% identity at the nucleotide level to the rabbit *PepT1* gene has been carried out (Leibach *et al.*, personal communication). Dantzig *et al.* (1994) reported the cloning of a human

intestinal peptide transport gene using transfected Chinese hamster ovary cells. What is unusual about this membrane transport protein which belongs to cadherin family, is that it has a single transmembrane domain. In contrast, the rabbit *PepT1* and the *S. cerevisiae PTR2* have 12 transmembrane domains. At the current time, the gene cloned by Dantzig *et al.*(1994) is thought to be misidentified as encoding a peptide transporter.

Functional Significance of Mammalian Peptide Transport

In mammals, peptide translocation has been seen in several tissues. It is very obvious that peptides have a primary role in protein nutrition (Matthews and Adibi, 1976). Small peptides that are liberated as protein digestion products in the intestine are transported across the intestinal cell membrane. The renal peptide transport system is understood to function in retrieving peptides excreted into urine (Inui *et al.*, 1984).

Another significant role for the peptide transport systems in intestine and kidney is in the uptake of many orally active antibiotics (Iseki *et al.*, 1989 and Muranushi *et al.*, 1989). The structural characteristics of these pharmacologically active compounds are similar to those of natural substrates of peptide transport system. The intestinal peptide transport system can function as a vehicle in the absorption of these peptide antibiotics. In rat and rabbit intestinal vesicles, the inhibition of the uptake of cephradine, an amino-ß-lactam antibiotic, by di- and tri-peptides clearly showed that they share the same peptide carrier pathway (Yuasa *et al.*,1993).

In a similar study cephalexin was shown to be inhibited by dipeptides and not by amino acids in rat renal brush border membrane vesicles (Inui *et al.*, 1984). It is also believed that a placental peptide transport system participates in the transport of peptide antibiotics from the mother to the fetus (Ganapathy *et al.*, 1985).

Peptide-based enteral or parenteral solutions can be administered to patients suffering from impairment of gastrointestinal function (Adibi, 1987., Grimble and Silk, 1989 ; Furst *et al.*, 1990). Such a possibility offers clinical relevance to the mammalian peptide transport system, especially when some amino acids are known to be unstable in solution. For instance, glutamine, owing to its instability in solution, was not initially included in parenteral nutrition (Adibi *et al.*, 1993). However, the dipeptide transport system provides a suitable route to include the amino acid in patient care. The recovery of intravenously injected glycyl-glutamine from the human kidney filtrate was attributed to carrier-mediated transport systems (Adibi *et al.*, 1993). Daniel *et al.* (1991) have shown the inhibition of glycyl-glutamine uptake by other peptides indicating that peptides compete for the same transport system.

S. cerevisiae as a Model Eucaryotic Cell

S. cerevisiae shares with eucaryotic cells many fundamental properties of cell biology enabling it to serve as a model eucaryotic cell (Botstein and Fink, 1988). Baker's yeast, as it is popularly called, has been studied extensively and its genetic characteristics have been well documented. The

facility with which the genotype of the organism can be manipulated using powerful genetic techniques makes it attractive in studying the relation between gene structure and protein function (Herskowitz, 1985). The technique of functional complementation has been successfully used in heterologous expression of several genes of mammalian origin in the background of appropriate *S. cerevisiae* mutations (Lew *et al.*, 1991; Schild *et al.*, 1990; Colicelli *et al.*, 1991; Dougherty *et al.*, 1992 and Becker *et al.*, 1991). The HeLa cell cDNA library has been used to clone the human counterpart of Hap2 transcription factor in yeast (Becker *et al.*, 1991). Several peptide transport genes have been cloned using *S. cerevisiae* peptide transport mutant strains in our laboratory.

The identification of a human peptide transport gene by functional complementation of the *S. cerevisiae ptr2* mutation would further the comparative studies of peptide transport characteristics between many species. The results of such an effort would be expected to provide a rational basis in the design and development of anti-fungal compounds.

III. Materials and Methods

HeLa Cell cDNA Library

The cDNA library was obtained from the laboratory of Dr.L.Guarente, Massachussets Institute of Technology, Cambridge. The cDNA had been inserted into BstX I site of the pDB20 vector after ligation to phophorylated, partially double-stranded BstX I adapters. The cDNA's are under the control of the ADH promoter (ADHp) and terminator (ADHt) sequences. The pDB20 is a 7.2kb yeast- *E. coli* shuttle vector containing the *URA3* yeast selectable marker, Amp^r as the *E. coli* selectable marker, Ori as the *E. coli* origin of replication and 2μm sequence for high copy propagation in yeast (Becker *et al.*, 1991). The detailed map of the vector is given in Figure 1.

Strains and Media

Saccharomyces cerevisiae strains PB1X-9B (*MATa ura3-52 leu2-3 lys1-1 his4-38 ptr2-2*), PB1X-2AΔ(*Mata ura3-52 leu2-3 lys1-1 his4-32 ptr2::LEU2*), PB1X-2A (*Mata ura3-52 leu2-3, 112 lys1-1 his4-38*) (Perry *et al.*, 1994) and PB4X-5B(*Mata ura3-52 leu2-3, 112 lys1-1 his4-38, ptr1-2*) (Alagramam *et al.*, 1994) were constructed earlier in our laboratory using standard procedures (Sherman, *et al.*, 1986). The strain S288C (*Matα SUC2 mal mel gal2 CUP1*) was obtained originally from the Yeast Genetic Stock Center (Berkeley, CA). All the yeast

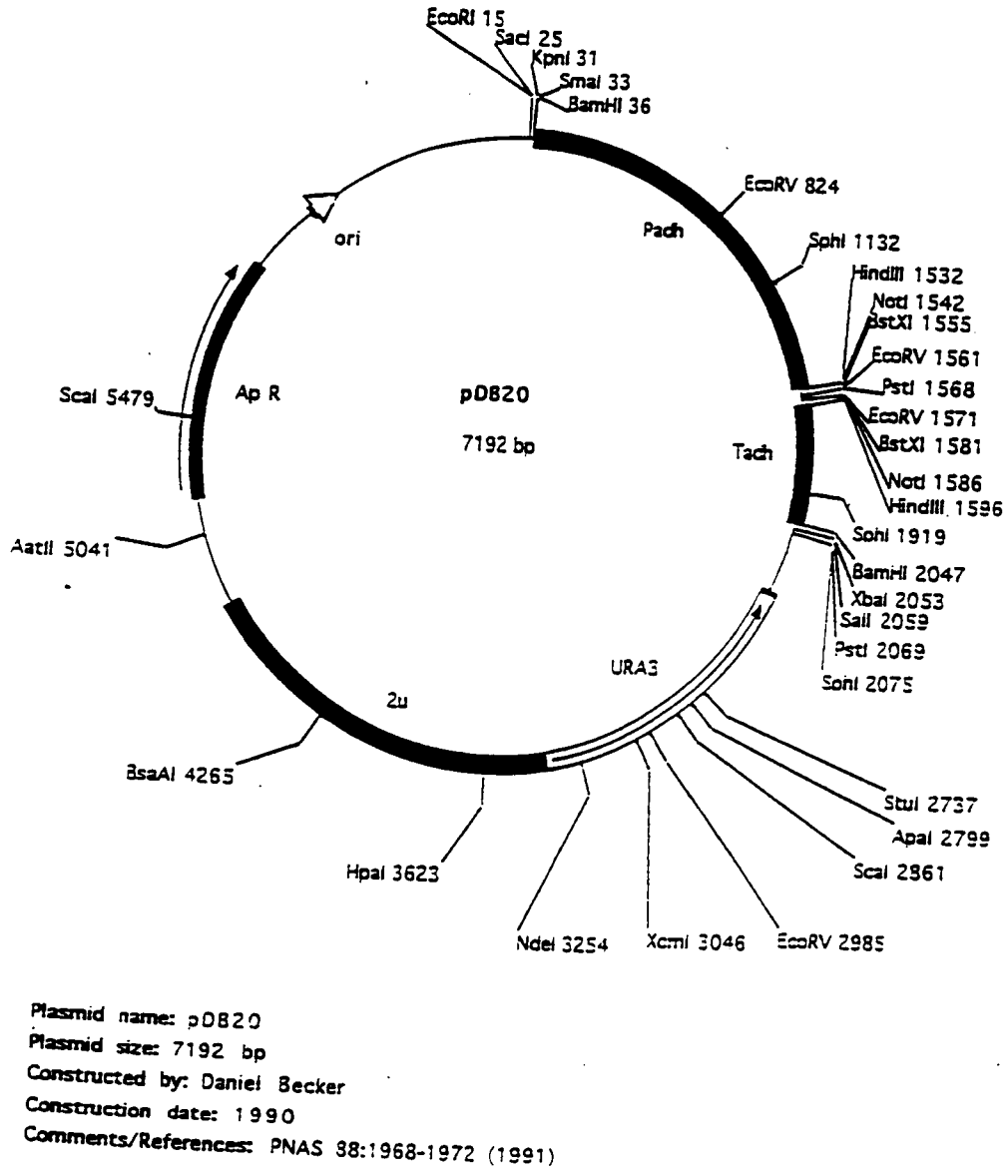


Figure 1. Plasmid map of the pDB20 used to construct the HeLa cell cDNA library.

strains were propagated on YEPD medium containing yeast extract(1.0%), Peptone (2.0%) and dextrose(2.0%) on a weight per volume basis. *E. coli* strain DH5α was maintained on Luria-Bertani(LB) medium and the plasmid-bearing derivatives were selected and maintained on LB medium containing 50 µg of ampicillin per mL.

The strains PB1X-9B and PB1X-2AΔ after transformation with plasmids encoding peptide transport phenotype were maintained on dipeptide medium(See below). Alternatively, they were also maintained on synthetic defined medium(SDM) composed of 0.67% yeast nitrogen base containing ammonium sulfate and without amino acids (Difco Laboratories, Detroit, MI), 2.0% dextrose. Amino acids lysine, leucine and histidine were added at 30µg/mL. Uracil was added, where needed, at a concentration of 10µg/mL to the SDM to obtain SDM plus uracil medium.

The dipeptide medium was composed of 0.17% yeast nitrogen base without amino acids or ammonium sulfate (Difco Laboratories, Detroit, MI), 0.1 % allantoin as nitrogen source instead of ammonium sulfate, 2.0% dextrose, and unless otherwise stated, the dipeptide Lys-Leu at 60µg/mL. In the dipeptide medium, Lys-Leu satisfies the auxotrophic amino acid requirements of lysine and leucine for the growth of the transformed yeasts. Histidine was also added at 30µg/mL to the medium.

Transformation

The *ptr2* mutant PB1X-9B was transformed with pDB20 based HeLa cell cDNA library as described (Geitz *et al.*, 1992). The

yeast cells were grown to a density of 1 x 10^7 cells/mL, diluted to 1 x 10^6 cells/mL in YEPD medium and regrown to 1 x 10^7 cells/mL. The cells were washed with sterile water and 1x lithium acetate/Tris-EDTA buffer (10x filter sterile Tris-EDTA stock is 0.1M Tris-HCl plus 0.01M EDTA at pH 7.5 and 10x filter sterile lithium acetate stock is 1 M lithium acetate adjusted to pH 7.5 with dilute acetic acid). 50µL of the cells at a density of 1 x 10^9 cells/mL was transformed with 1.0µg of the HeLa cell cDNA library and incubated in 40% polyethylene glycol 4000 solution containing 1x lithium acetate/Tris-EDTA buffer at 30^0C for 30 min. The cells were heat shocked at 42^0C for 15 min. following which the polyethylene glycol solution was removed and the cells were incubated in YEPD medium for 1.0 hr at 30^0C. The YEPD medium was removed and the yeast cells washed with 1x Tris-EDTA buffer and finally resuspended in 1.0mL of 1x Tris-EDTA buffer. The Ptr^+ transformants that were directly selected on the Lys-Leu dipeptide medium made with 1.5% agar generally took five days at 30^0C to develop into visible size colonies. The detailed scheme for selecting human peptide transport gene is illustrated in Figure 2.

Competent *E. coli* DH5α cells obtained by treating them with $CaCl_2$ solution (Hanahan, 1983) were transformed with the plasmids of interest and the transformants were selected on LB-ampicillin medium.

Plasmid Curing

The transformant yeast strains were grown overnight at 30^0C in 5.0 ml of the SDM plus uracil medium. The cells were spun

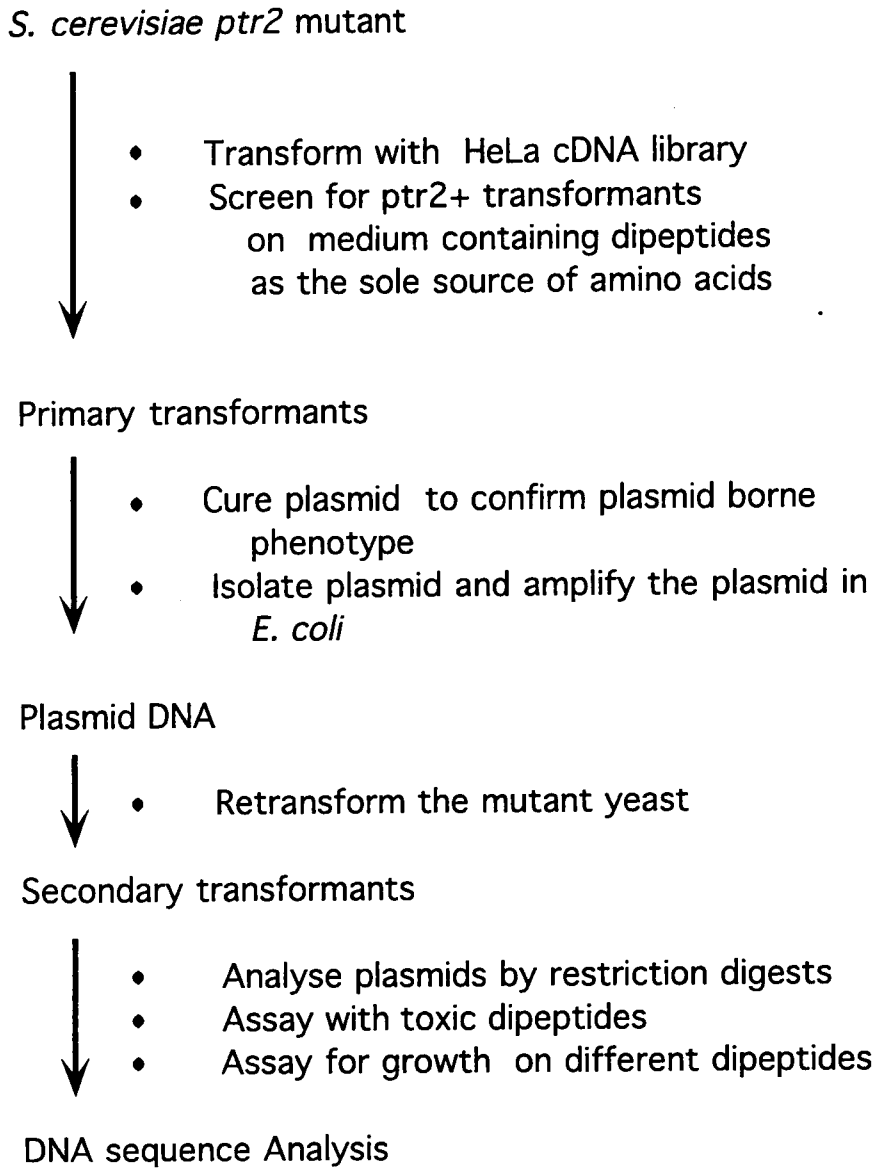


Figure 2. Scheme for cloning human peptide transport gene by functional complementation.

down and appropriate dilutions of each transformant made to obtain approximately 50 colonies when plated on YEPD medium. In the absence of the selective pressure, the retention of the plasmid in the transformant yeasts while growing on rich medium would become superfluous. These colonies were replica plated onto three media: the SDM, Lys-Leu dipeptide, and YEPD. The plates were incubated at 30^0C for five days. The concomitant loss of the ability to grow on the SDM and the dipeptide medium was monitored.

Amplification and Isolation of Plasmid DNA

The plasmid DNA was shuttled from the transformant yeasts into *E. coli* according to an established procedure (Hoffman and Winston, 1987). A crude preparation of yeast plasmid DNA was obtained by lysing the yeasts using glass beads, phenol-chloroform and a denaturing solution containing 1.0% SDS, 100mM Tris, 2.0% Triton-X-100, 100mM NaCl and 1mM EDTA. Competent cells of *E. coli* DH5α strain were transformed with plasmid isolated from yeast and the transformants selected on LB-amp medium. Amplification of plasmid DNA was done by growing overnight cultures of *E. coli* transformants. The plasmid DNA was isolated from *E. coli* by the alkaline lysis method (Sambrook *et al.*, 1989).

Restriction Enzymes and Biochemicals

Restriction endonucleases and T4 DNA ligase were purchased either from the New England Biolabs (Beverly, MA) or Promega

(Madison, WI) and used according to the suggestions of the supplier. The dipeptides employed in the study were purchased from the Sigma Chemical Co. (St.Louis, MO) or Bachem (Philadelphia, PA). Plasmid purification columns were purchased from Qiagen Inc. (Chatsworth, CA). ^{35}S-dATP used in DNA sequencing was purchased from Dupont, NEN Research product (Boston, MA). XAR autoradiography film was purchased from Eastman Chemical Company (Rochester, NY).

Agarose Gel Electrophoresis

Restriction digested plasmid samples were run on 0.8% agarose gels and electrophoresed for 2 hours at 100v. The gels were stained in ethidium bromide solution (0.5 µg/mL) for 20 to 30 minutes. After destaining in de-ioninsed water, the gels were visualised and photographed by placing them on an ultraviolet transilluminator.

Toxic dipeptide assay

Ptr$^+$ yeast transformants, and the wild type strain S288C were grown in SDM medium, and the *ptr2* mutant strain PB1X-9B in SDM plus uracil medium overnight at 30^{0}C. The cells were harvested at a density of 1.0 x 10^7 cells/mL and washed in sterile water before re-suspending them in sterile water at 5.0 x 10^6 cells/mL. 4.0 mL fractions of the cells were mixed with 12.0 mL of 1.1% Noble agar kept in a molten state at 50^{0}C and poured as an overlay onto synthetic defined medium containing only the auxotrophic amino acids and allantoin.

Sterile disks containing 0.38 µmoles of toxic dipeptides or toxic amino acid analogs were placed on the surface of the growth medium. The sensitivity of the transformants to the compounds are proportional to the diameter of zones of inhibition of growth measured after 40 hrs of incubation at 30°C. The range of the zones of inhibition did not exceed ± 10 per cent of the diameter.

DNA Sequencing

Nucleic acid sequencing based on Sanger's dideoxy sequencing method (Sanger *et al.*, 1977) was employed using the Sequenase 2.0 kit purchased from USB Inc.(Cleveland, OH). The primer to the ADHp (5'GTC ATT GTT CTC GTT CCC3') was purchased from Genosys Biotechnologies Inc.(The Woodlands, TX). Appropriate primers were designed and purchased from Integrated DNA Technologies Inc.(Coralville, IA) to obtain nucleotide sequence of pMS2 by the primer walking method from the ADHp terminus. These primers include IP1 (5'GTA GAT ACG TTG TTG3'), P2 (5'CGA GCA AAT GCC TGC3'), P3 (5'CTG GCG TTA CCC AAC3') and P4 (5'GTT TAG TAT ACA TGC3') Primer to the ADHt with the sequence 5'AAG CCG ACA ACC TTG 3' was also purchased from the same source. The primers *PTR2*-P1 (5'CAC CAC AAC TCA TAT3') to the plus strand of the promoter region and *PTR2*-P2 (5'TCC GAT TGG TCT TTG AAT G3') to the minus strand of the open reading frame of *PTR2* gene were used to obtain *PTR2* nucleotide sequence in the plasmids pJP9 and pMS2 (Perry *et al.*, 1994). DNA sequence analyses were done using BLAST algorithm (Altshul *et al.*, 1990) and the GCG sequence analysis software (Devereux *et al.*, 1984).

IV. Results

Transformation of *S. cerevisiae ptr2* Mutant with the HeLa Cell cDNA Library

S. cerevisiae PB1X-9B (*ptr2*$^-$) was transformed with pDB20 containing the HeLa cell cDNA library and the transformation efficiency was calculated to be 0.8 x 10^6 Ura$^+$ transformants/μg of plasmid DNA. Four transformants grew on Lys-Leu dipeptide medium. Each of the transformants recovered from the dipeptide medium was plated on synthetic defined medium (SDM) lacking at least one auxotrophic supplement and also on the dipeptide medium and SDM plus uracil medium. The transformants were able to grow on the SDM, the SDM plus uracil and the dipeptide media but not on media lacking in any one of the auxotrophic amino acids (Table 1). The results indicated that the transformants regained peptide transport ability and were not the result of loss of auxotrophy caused by reversion.

Plasmid Curing of Transformants

The loss of episomal DNA was achieved by growing the transformants in SDM plus uracil medium. In the absence of selective pressure, the plasmids were cured out of the transformants. Transformants unable to grow on the SDM were tested for their ability to grow on the dipeptide medium. In each case, the loss of uracil prototrophy was found to be accompanied with the loss of the phenotypic complementation

Table 1. Growth assay for the four transformants from HeLa cell cDNA library on media lacking auxotrophic supplements

Strain	SDM Lacking[a]			SDM	SDM +Ura	Dipeptide Medium
	His	Lys	Leu			
Controls						
S288C(*PTR2*)	+[b]	+	+	+	+	+
PB1X-9B(*ptr2*)	-[c]	-	-	-	+	-
PB1X-2AΔ(*ptr2Δ*)	-	-	+	-	+	-
PB1X-9B(pJP9)[d]	-	-	-	+	+	+
Transformants						
Transformant 1	-	-	-	+	+	+
Transformant 2	-	-	-	+	+	+
Transformant 3	-	-	-	+	+	+
Transformant 4	-	-	-	+	+	+

a = Synthetic defined medium contains all other auxotrophic amino acids. SDM was prepared as described in materials and methods.

b = Growth is indicated by "+"

c = No Growth is indicated by "-"

d = pJP9 is a YCp50-based vector with a genomic copy of *S. cerevisiae PTR2*

of *S. cerevisiae ptr2* mutation. 2 out of 51 colonies of transformant 1 and 3 out of 40 colonies of transformant 2 lost the plasmid borne uracil marker and dipeptide uptake phenotype. On the other hand, a greater number of colonies of transformants designated 3 and 4, 15 out of 55 and 17 out of 64 respectively, lost both phenotypes.

Recovery of Plasmids Encoding Peptide Transport Phenotype

The plasmid DNA was recovered in *E. coli* from three of the four yeast transformants. No *E. coli* transformant was obtained in the case of transformant 1 (Table 2).

Re-transformation of *S. cerevisiae ptr* Mutants with the Recovered Plasmids

The plasmids isolated from the *S. cerevisiae* transformants were re-introduced into the two *ptr2* mutants to show that the rescue of *ptr2* mutation was plasmid borne. The plasmids were also used to transform the *ptr1* mutant PB4X-5B. Only one plasmid, designated as pMS1, out of the five plasmids that were isolated, was able to restore the dipeptide transport function in the *ptr2* deletion strain PB1X-2AΔ and the *ptr2* mutant PB1X-9B as is evident from their growth on both Lys-Leu and His-Lys dipeptide medium (Table 3). However, all the isolated plasmids were able to restore the uracil prototrophy as evidenced by the growth of the transformants on the SDM. In order to verify that the plasmid borne complementation was

Table 2. Shuttling plasmids from yeast transformants into *E.coli*

PB1X-9B Transformants	*E. coli* Transformants
Transformant 1	0
Transformant 2	2
Transformant 3	1
Transformant 4	2

Table 3. Complementation in *S. cerevisiae ptr* mutants by pMS1

Mutants	Plasmid	SDM[a]	Dipeptide Media	
			Lys-Leu	His-Lys
PB1X-9B[b]	pMS1	+	+	+
PB1X-2AΔ[c]	pMS1	+	+	+
PB4X-5B[d]	pMS1	+	-	-

a = Synthetic defined medium was prepared as described in materials and methods

b = *ptr2* mutant

c = *ptr2* knock out mutant

d = *ptr1* mutant

specific to *ptr2* mutation, a cross complementation analysis was done. pMS1 was introduced into *ptr1* strain, PB4X-5B. The results showed that the plasmid pMS1 was not able to complement the *ptr1* mutation despite being able to confer uracil prototrophy.

Restriction Digestion and DNA Sequencing of pMS1

The *ptr2* complementing plasmid pMS1 was restriction digested with Hind III and Not I endonucleases to determine the size of the insert. The Hind III and Not I recognition sites are present in the multiple cloning site of pDB20, whose derivative is pMS1. The Not I digestion released a single insert of about 10kb in size that corresponded to the 6kb and 4kb insert fragments released by Hind III digest (Figure 3). Another small Hind III fragment of the insert of approximately 0.4kb is barely visible on the gel. The 7.1kb pDB20 vector backbone migrated as expected. DNA sequencing of pMS1 with primers to ADHp and ADHt showed the presence of Hind III and Not I recognition sites. BstX I site in the multiple cloning site of pDB20 into which the cDNA library had been cloned was not indicated in the sequencing data (data not shown).

Transformation of *ptr2* Mutants with the pMS2 Subclone

The insert size of approximately 10kb was unusually large for a cDNA library. An attempt, therefore, was made to define the

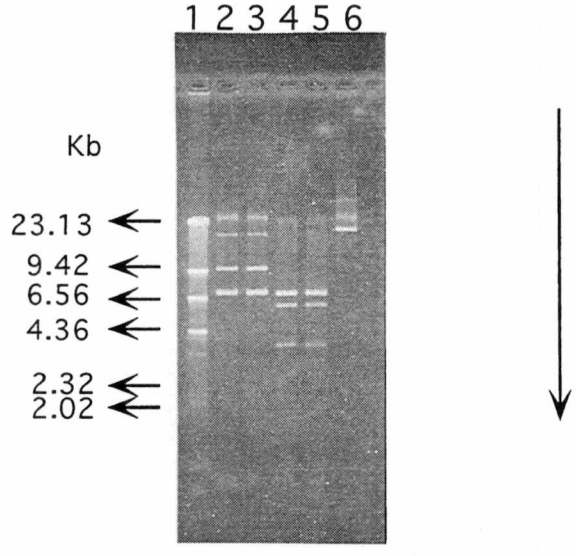


Figure 3. Restriction digestion of pMS1. Lane 1, lambda Hind III molecular size markers; Lanes 2 & 3, Not I digest of pMS1; Lanes 4 & 5, Hind III digests of pMS1; and Lane 6, uncut pMS1.

complementing region to 6.0kb or 4.0kb insert fragments of the Hind III digest of pMS1. pMS constructs with the respective fragments were made by restriction digestion of pMS1 with Hind III followed by ligation into pDB20. The constructs were recovered in *E. coli*. The construct pMS2 (Figure 4) with the 6kb Hind III fragment of the original insert was able to restore the peptide transport function in the *ptr2* mutants (Table 4). Expectedly, DNA sequencing of pMS2 using primers to ADHp and ADHt indicated the loss of the Not I sites which are internal to Hind III sites in the multiple cloning site and in the insert (data not shown).

Sensitivity of PB1X-9B Transformant of pMS2 to Toxic Dipeptides

The results of the toxic dipeptide assay are summarised in Table 5 in terms of zone of growth inhibition by toxic peptides and toxic amino acid analogs (Also see Figure 5). The PB1X-9B transformed with pMS2 had markedly greater sensitivity to Leu-f-Phe as compared to wild type S288C or PB1X-9B(pJP9) (Figure 5, Disk 3 in two bottom plates). Zones of inhibition in PB1X-9B(pMS2) caused by Ala-Eth and Leu-Eth (Figure 5, Disk 1 and 2 in left bottom plate) were not completely clear since several colonies had grown within them. *ptr2* mutant *S. cerevisiae* PB1X-9B transformed with pJP9 plasmid (Figure 5, bottom right plate) that encodes *PTR2* gene showed sensitivity to the toxic dipeptides Ala-Eth, Leu-Eth and Leu-f-Phe just as the wild type S288C strain (Figure 5, upper right plate). The susceptibility of either strain to Leu-f-Phe was less than their sensitivity to Ala-Eth or Leu-Eth. The transformants and the S288C strain were sensitive to

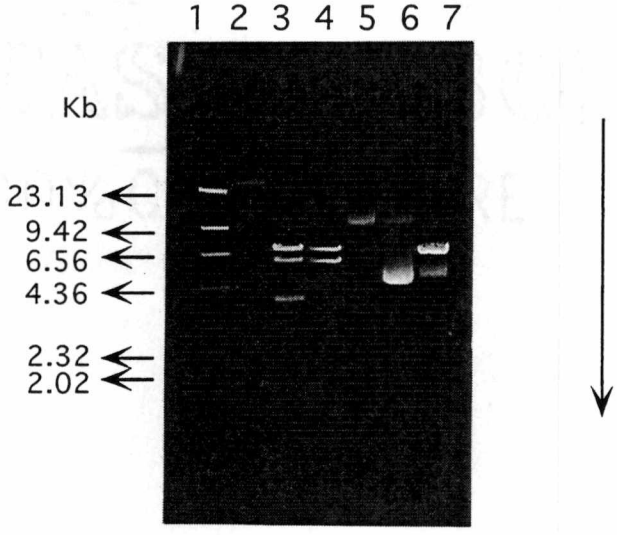


Figure 4. Hind III digests of pMS1, pMS2 and pDB20 plasmid samples. Lane 1, lambda Hind III markers; Lane 2, Uncut pMS1; Lane 3, Cut pMS1; Lane 4, Cut pMS2; Lane 5, uncut pMS2; Lane 6, uncut pDB20, and Lane 7, Cut pDB20.

Table 4. Transformation of *ptr2* mutants with pMS2 subclone

Plasmid	SDM	Dipeptide medium
pDB20	+	-
pMS1[a]	+	+
pMS2[b]	+	+

a = 10kb insert

b = 6kb Hind III fragment (Figure 4)

Table 5. Toxic Dipeptide Assay: Diameter of zones of inhibition(in cm)[a]

Strains	Toxic dipeptides			Toxic amino acid analogs	
	Ala-Eth	Leu-Eth	Leu-f-Phe	Eth	f-phe
PB1X-9B(*ptr2*)[b]	0.0	0.0	0.0	3.6	0.6
S288C(WT)[c]	3.3	3.3	3.0	4.1	1.4
PB1X-9B(pJP9)[d]	4.0	3.9	3.2	4.0	1.2
PB1X-9B(pMS2)[e]	4.3	4.0	3.7	4.2	1.0

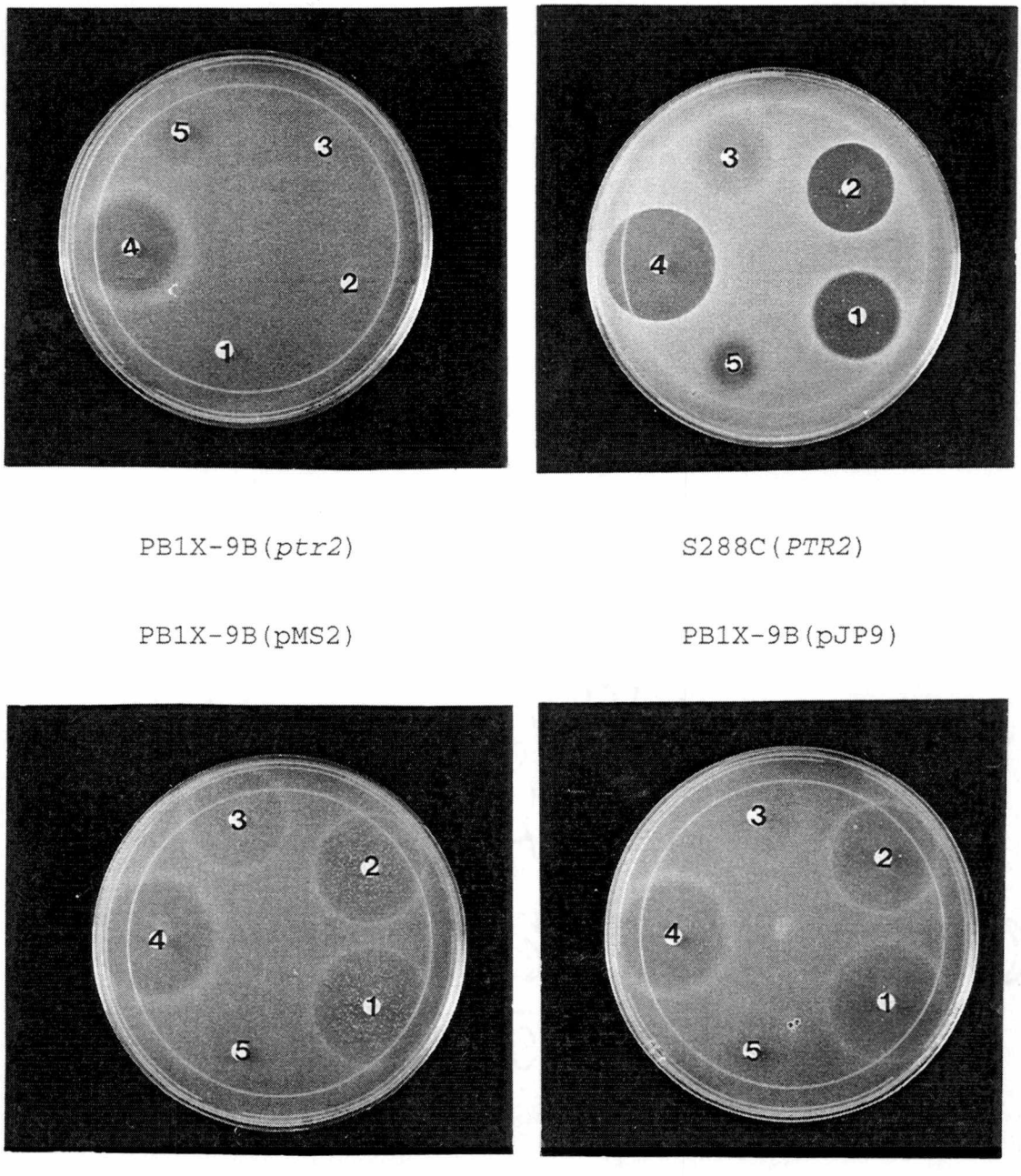
a = Diameter of zones of inhibition refer to those on the plates in Figure 5

b = *S. cerevisiae* peptide transport (*ptr2*) mutant

c = *S. cerevisiae* with wild type *PTR2* gene

d = pJP9 encodes *S. cerevisiae PTR2* gene

e = pMS2 ,a derivative of pDB20, shows peptide transport function.



PB1X-9B(*ptr2*)

S288C(*PTR2*)

PB1X-9B(pMS2)

PB1X-9B(pJP9)

Figure 5. Toxic dipeptide assay. The minimal media plates have a lawn of the respective *S. cerevisiae* strains with the toxic dipeptides, Alanyl-Ethionine(1), Leucyl-Ethionine(2) and Leucyl-f-Phenylalanine(3), and the toxic amino acid analogs, Ethionine(4) and f-Phenylalanine(5) spotted at 0.38 µmoles amounts.

the toxic amino acid analogs ethionine and f-phenylalanine. The *ptr2* mutant, PB1X-9B, was resistant to the toxic dipeptides while being sensitive to the toxic amino acid analogs.

DNA Sequence and Southern Analyses of pMS2

In order to obtain the sequence of the putative cDNA insert encoding the dipeptide transport phenotype, sequencing was initiated beginning with a primer to ADHp in pMS2. Analyses of over 800bp sequenced using BLAST algorithm indicated DNA sequences of ADHp followed by partial gene sequences of genes encoding alcohol dehydrogenase, Lac Z and *URA3* interspersed with DNA of unknown origin. Using primer to the ADHp, a sequence of 34 bases corresponding to the bases from 706 to 739 of ADHp region was obtained. 4 bases later, a Hind III site (6 bases) and 16 bases of unknown origin followed. Sequencing further by primer walking method, 428 bases corresponding to 1687-2115 bases of the ADH gene followed by 42 bases of indeterminate origin and then 160 bases of two segments corresponding to 17-98 and 100-177 bases of the lac Z gene interrupted by three bases were obtained. Next followed 51 bases of indeterminate origin and 79 bases corresponding to 904-825bp of the minus strand of *URA3* gene.

At this stage identification of the source of the insert DNA was critical. A Southern analysis using the 6kb insert fragment of pMS2 as a radioactive probe gave no hybridisation band with the Hind III digest of HeLa genomic DNA. However,

the probe hybridised to the *S. cerevisiae PTR2* gene fragment from pJP9 and to the 6 kb insert fragment released upon Hind III digestion of pMS1 and pMS2 (data not shown).

Restriction Digestion of Plasmid Samples with Restriction Enzymes that have Recognition Sites Internal to *PTR2* Gene

Restriction digestion of pMS1, pMS2 and pJP9 plasmid samples with enzymes Kpn I and Nde I gave identical fragments corresponding to those of the *S. cerevisiae PTR2* gene in all plasmids (Figure 6). Kpn I released aprroximately 600bp *PTR2* fragment while Nde I cut out two fragments of the *PTR2* seen at the bottom of the gel.

DNA Sequencing of pMS2 and pJP9 Plasmids with *PTR2* Primers

Two primers, one to the plus strand of the *PTR2* promoter, and another internal to the minus strand of the open reading frame of the *PTR2* gene were employed in sequencing the pMS2 and pJP9 plasmids. Identical nucleotide sequences were obtained from the use of the two primers for pMS2 and pJP9 plasmids (Figure 7). 172 bases corresponding to -252 to -80 bases in the promoter region and 118 bases corresponding to 824 to 706 bases of the minus strand of the *PTR2* ORF were obtained (Perry *et al.*, 1994). This result confirmed that the insert in pMS2 thought to contain the HeLa cell *PTR2* homolog actually contained the *S. cerevisiae PTR2* gene.

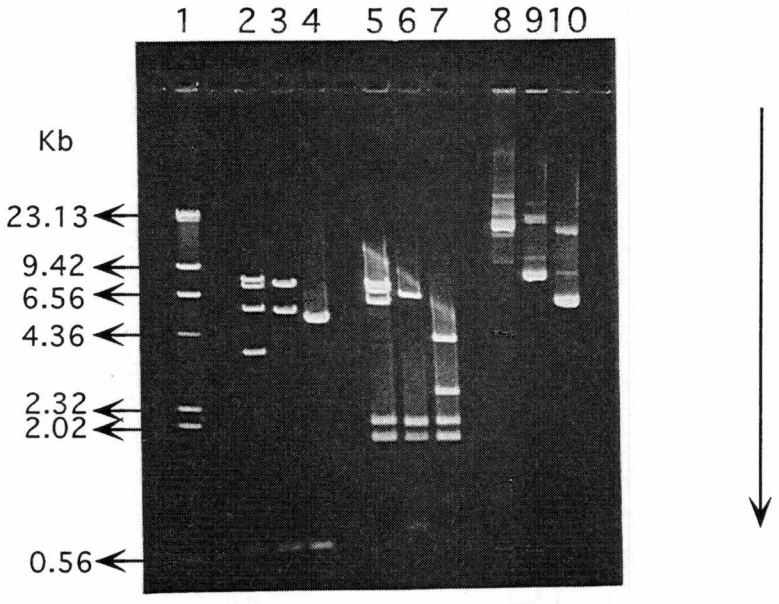


Figure 6. Restriction digestion of pMS1, pMS2 and pJP9 plasmid samples with Kpn I (lanes 2, 3 & 4) and Nde I (lanes 5, 6 & 7) endonucleases. Lane 1, lambda Hind III markers; Lanes 2, 5 & 8, pMS1; Lanes 3, 6 & 9, pMS2 and Lanes 4, 7 & 10, pJP9. Uncut samples are in lanes 8, 9 & 10.

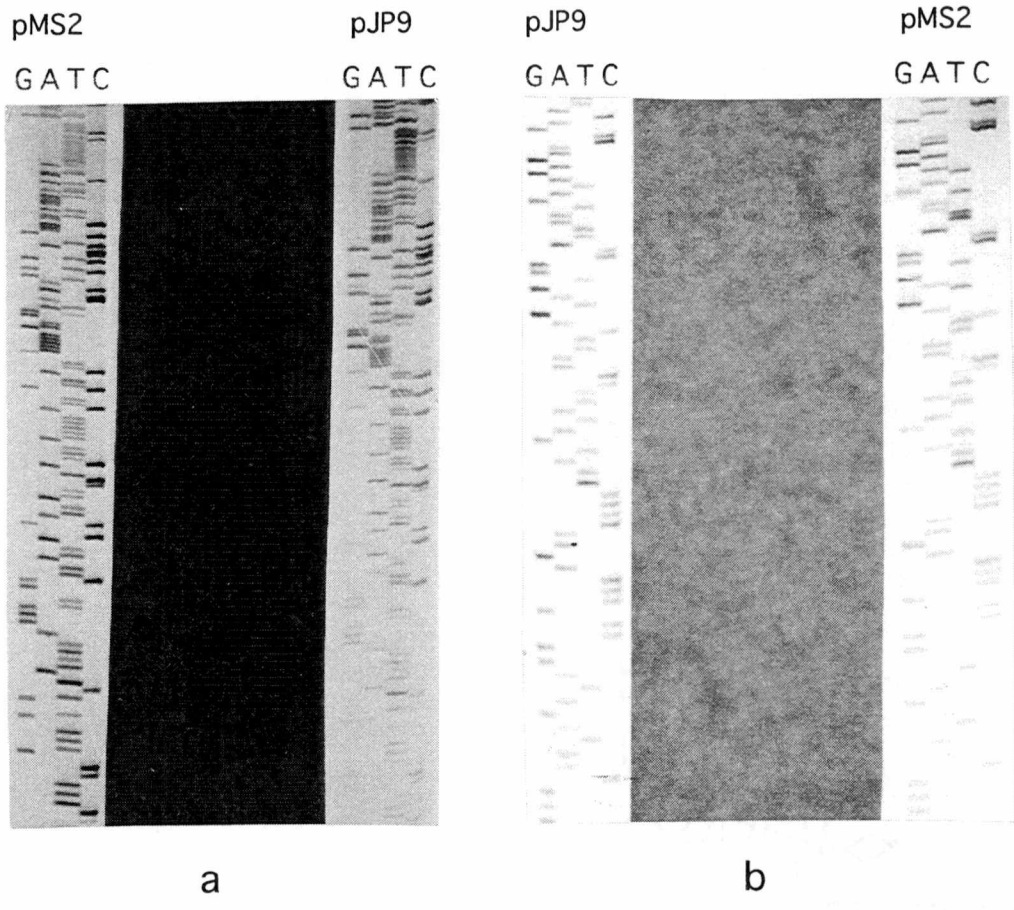


Figure 7. Identical nucleotide sequence of *PTR2* obtained from pJP9 and pMS2 plasmids. a) Promoter region b) ORF region.

V. Discussion

S. cerevisiae Transformants from HeLa cDNA Library and Identification of the Plasmid Borne Dipeptide Transport Phenotype

Several peptide transport genes have been successfully cloned from *S. cerevisiae* (Perry *et al.*, 1994; Alagramam *et al.*, 1994), *A. thaliana* (Steiner *et al.*, 1994) and *C. albicans* (Basrai *et al.*, 1994) using well characterised *S. cerevisiae* peptide transport mutants (Island *et al.*, 1991). The strategy of cloning mammalian genes by functional complementation of the *S. cerevisiae* mutations has been exploited by many workers (Lew *et al.*, 1991; Schild *et al.*, 1990; Becker *et al.*, 1991; Dougherty *et al.*, 1992 and Colicelli *et al.*, 1991). The *S. cerevisiae ptr2* mutant PB1X-9B was transformed with the HeLa cell cDNA library contained in pDB20. The calculated transformation efficiency was very high and close to a million transformants/μg of plasmid DNA. Such a high transformation efficiency allowed four different clones to be directly selected on the Lys-Leu dipeptide medium supplemented with histidine. This result is in conformity with transformation efficiencies of 5 x 10^5 transformants (Colicelli *et al.*, 1991; Dougherty *et al.*, 1992) and 2 x 10^7 transformants (Lew *et al.*, 1991) reported in the literature to identify human cDNA's that complement a yeast mutation.

The plasmid borne dependence of the dipeptide uptake phenotype was effectively determined by curing out the plasmids from the transformants. All the colonies that were

not able to grow on the SDM did not transport the dipeptide. The loss of uracil prototrophy accompanied by the loss of dipeptide uptake ability strongly indicated that both phenotypes were conferred by the plasmid.

Isolation of Plasmids and Re-transformation of the *ptr* Mutants

Re-establishing the dipeptide transport in the *S. cerevisiae* *ptr2* mutants with the isolated plasmids provides direct physical evidence for linking the phenotypic expression to plasmids. Out of the five plasmids that were recovered in *E. coli*, only one designated as pMS1 from transformant 3 was able to re-complement the peptide transport deficiency in the *ptr2* mutant PB1X-9B and the *ptr2* deletion strain PB1X-2AΔ. The transformants PB1X-9B(pMS1) and PB1X-2AΔ(pMS1) were able to utilise the dipeptides His-Lys and Lys-Leu as evidenced by their growth on the two dipeptide media (Table 3). However, in certain cases the plasmids could not re-establish dipeptide transport in the *ptr2* strains. The inability of plasmids to re-complement the mutation of the strain in which they were isolated is not uncommon (Lew *et al.*, 1991). According to Schild *et al.* (1990), interactions between the complete 2-μm sequences on the vector and the endogenous 2-μm sequences may lead to plasmid re-arrangements in yeast resulting in non-complementation of mutation. The complementation was specific to *ptr2* mutation as indicated by the results of transformation of *ptr1* mutant PB4X-5B. None of the plasmids were able to cross-complement the *ptr1* mutation in that strain which has the same auxotrophic requirements as that of the *ptr2* mutant, PB1X-9B.

Identification of the Plasmid Insert and Subcloning the Insert

Restriction analyses of pMS1 plasmid indicated a 10.0kb insert when digested with Hind III and Not I enzymes that have recognition sites in the multiple cloning site of the vector. The unusually large size of the cDNA insert prompted detailed analyses of the insert. Nucleotide sequence analyses of the ADHp and ADHt regions of the plasmid showed the expected DNA sequence and the presence of the Hind III and Not I restriction sites and the absence of the BstX I sites. The restriction sites indicated by the DNA sequence analyses was in conformity with the restriction analyses. These results suggested that a pDB20 plasmid derivative with a 10.0kb insert complementing the peptide transport deficiency in the *ptr2* strain had been isolated.

The 6.0 and 4.0kb fragments of the insert liberated by Hind III digestion provided an opportunity to determine which one of them harbored the complementing activity. pMS1 was restriction digested with Hind III and 6.0kb and 4.0kb insert fragments were re-ligated separately into pDB20 to obtain the subclones. Transformation of the *ptr2* mutant PB1X-9B with these constructs showed that dipeptide transport was contained in the 6.0kb fragment of the insert in pMS2.

Because the peptide transporters are wide spread, we expected to find evidence for peptide transporters in human cells However, the finding of peptide transport activity in HeLa cells was in contrast with the observation that no peptide uptake activity could be detected in HeLa cells (Leibach, Personal communication). When we first learned of Lebach's

observation, we believed that the physiological conditions under which dipeptide uptake was assayed in HeLa cells may not have permitted the expression of the peptide transport activity. Further, it is known that there are three "original" HeLa cell lines in the American Type Culture Collection repository, namely HeLa (CCl 2), HeLa 229 (CCL 2.1) and HeLa S3 (CCL 2.2) (Akiyama, 1987). Specific physiological conditions may determine the expression of peptide transport genes in each of the three cell lines. Thus, the absence or presence of peptide uptake in any one of them may depend upon their growth conditions.

DNA Sequence and Southern Analyses

Attempts to sequence the insert in pMS2 plasmid from the ADHp end were made by the primer walking method in the hope of finding the open reading frame(ORF) of the subcloned insert. Sequencing was suspended when no unique ORF was found as indicated by the comparison of the sequenced region to the DNA sequences in the database using the BLAST (Basic Local Alignment Search Tool) algorithm (Altshul *et al.*, 1990). After the Southern analyses and DNA sequencing of pMS2 with primers to *PTR2* promoter and *PTR2* ORF, it was clear that the peptide transport was encoded by the *S. cerevisiae PTR2* gene. However, the restriction digestion of the pMS1 clone and the distinctive pattern of peptide toxicity it conferred on transformants had earlier led us to believe that the insert was distinct.

The differing substrate specificities seen in regard to the toxic dipeptides for the same gene in the two different

plasmids, pMS2 and pJP9, may be an anomaly (See Figure 5 and Table 5). It is possible that the greater susceptibility of pMS2 transformed PB1X-9B to Leucyl-f-Pheylalanine could be due to the action of ADHp in concert with the *PTR2* promoter. In contrast, the growth of some colonies within the zone of inhibition caused by Ala-Eth and Leu-Eth, could be attributed to two reasons. It may be that the complexity of gene re-arrangements in pMS2 plasmid might have affected specific regions of the transporter responsible for the uptake of Ala-Eth and Leu-Eth. Alternatively, it could be argued that the pMS2 plasmid is unstable. The loss of an unstable plasmid from PB1X-9B could result in its inability to be sensitive to the toxic dipeptides as supported by the observation of resistant colonies growing within the zone of inhibition in toxic peptide halo assay.

How the *S. cerevisiae PTR2* gene could have integrated into the multiple cloning site of the pDB20 where the cDNA should have been present cannot be explained easily. Perhaps, integrative events involving contaminating pieces of DNA during or after the construction of the cDNA library might have caused this situation. Identification of false-positive clones could be avoided if a more detailed restriction analyses of not only the vector but also of the insert were done initially. Further, an attempt to partially sequence the insert using primers to the host gene whose mutation is being complemented, would indicate the heterologous nature of gene sequence under study.

In summary, a derivative of pDB20 plasmid was isolated that encoded the dipeptide transport phenotype. The clone designated pMS1 contained what was believed to be an unusually large cDNA insert of 10.0kb in size as determined

by Not I and Hind III restriction digestion. DNA sequence analyses also confirmed the Not I and Hind III recognition sites to be intact and the loss of the BstX I site. Subcloning showed that the dipeptide transport function was localised in the 6.0kb Hind III fragment of the insert. The subclone, now called pMS2 was found to exhibit some unique transport characteristics with the toxic dipeptides. However, it was found to contain the DNA sequences of *S. cerevisiae* *PTR2* gene as shown by the Southern analyses and DNA sequencing.

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