STRUCTURE-FUNCTION STUDIES OF HUMAN SLC36 AMINO ACID TRANSPORTERS

SAMYUKTHA MURALIDHARAN PILLAI

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PhD Supervisors:
Dr. David Meredith (Oxford Brookes University)
Dr. Richard Boyd (University of Oxford)

Structure-function studies of human SLC36 amino acid transporters

The Solute Linked Carrier family 36 (SLC36) of integral membrane transport proteins has four members which are also known as the human Proton coupled Amino acid Transporters (hPATs).This is due to hPATs 1 and 2 having been functionally characterized as proton coupled transporters that bring about a 1:1 symport of H^+ and an amino acid across the membrane (with optimum uptake at an extracellular pH of 5.5). Their main substrates were found to be the small neutral amino acids glycine, alanine and proline; however, in addition to these, PATs 1 and 2 were also observed to transport a range of other substrates including some pharmacologically active compounds. hPATs 3 and 4 were considered orphan transporters, with no information available except their mRNA localization in various tissues. Additionally there is no structural information available about the PAT family of transport proteins.

The thesis has two broad aims. The first aim was to study the orphan PAT transporters and to investigate whether these proteins are also amino acids transporters and, if so, what their substrates are. The second aim was to use transmembrane domain software to predict the secondary structure of the PAT transporters using hPAT1 as the model protein. Epitope tags, luminometry and functional assays were used to test these predictions, and homology modelling then employed to gain a better understanding of the structure-function relationship of this transporter family. The results of both these areas of investigation are reported in this thesis.

The orphan transporter hPAT4 was found to be a very high affinity electroneutral proline and tryptophan transporter when expressed in the *Xenopus laevis* oocyte system. Preliminary data for hPAT3 suggested uptake of proline at extracellular pH 7.4 in *Xenopus laevis* oocytes and in the mouse Sertoli cell line TM4. Membrane topology studies on hPAT1 provided experimental support for the prediction of 11 transmembrane domains for this transporter protein. Homology modelling indicates a region which might play a role in the substrate specificity of PAT proteins.

Scientific publications

SLC36A4 (hPAT4) is a high affinity amino acid transporter when expressed in *Xenopus laevis* oocytes.

Pillai S.M., Meredith D. (2011). *Journal of Biological Chemistry:*286(4): 2455-60

Scientific publications in abstract form

Functional characterization of human Proton Amino acid Transporter 4 (hPAT4) expressed in Xenopus oocytes.

Pillai S.M. and Meredith D. (2010). *Genes and Nutrition*: 5(Suppl 1):S25-S100 DOI 10.1007/s12263-010-0205-7

Structure-function studies on the human Proton-coupled Amino acid Transporter 1 (hPAT1). *Journal of Physiology, In Press*

Pillai S.M. and Meredith D. (2011).

Scientific publications under preparation or review

Structure-function studies of human Proton amino acid transporter hPAT1.

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List of Abbreviations used

ATP	Adenosine-5'-triphosphate
ADP	Adenosine-5'-diphosphate
BSA	Bovine serum albumin
Caco-2	Carcinoma colon 2 (cell line)
cDNA	Complementary DNA
cRNA	Complementary RNA
DNA	Deoxy-ribose nucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOPA	L-3,4-dihydroxyphenylalanine
E.coli	Escherichia coli
FCS	Foetal culf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT	Glucose transporter
HEK293	Human embryonic kidney cells 293
HEPES	N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
HS	Horse serum
hPAT	human proton coupled amino acid transporter
HRP	Horseradish peroxidase
IMS	Industrial Methylated Spirit
IPTG	Isopropyl-β-D-thio galactopyranoside
Ki	Half-saturation inhibition constant
Km	Half-saturation affinity constant
MES	2-(N-morpholino)ethanesulfonic acid
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RNA	Ribose nucleic acid
SLC	Solute carrier transporters
SOC	Super Optimal broth for Catobolite repression

TBE	Tris base, boric acid, EDTA
Tm	Melting temperature
TMD	Transmembrane domain
Vmax	Maximum transport rate
X-Gal	5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside

Chapter 1:
Introduction

1.1 The Plasma membrane

1.1.1 Features of the plasma membrane

The presence of a plasma membrane is a feature of all living cells from bacteria to mammals. The cell membrane defines shape and structure of the cell and holds the intracellular contents. It maintains the difference between the intracellular cytoplasm (and organelles) and the extracellular contents. The structure of the plasma membrane is explained by the fluid mosaic model (Figure 1.1). According to the model, the membrane consists of a lipid bilayer with phosphate groups i.e a phospholipid bilayer which has selective permeability. One of the most important characteristics of the fluid mosaic model is that the lipids are capable of movement within the membrane. It allows a variety of molecules such as oxygen and carbon dioxide to pass through readily while the movement of some other solutes like most nutrients and ions is regulated. In addition to this, the bilayer consists of a number of molecules, such as lipid rafts and protein-protein complexes. The fluidity of the bilayer is dependent on a number of factors including the type of cell and the contents it encloses. In the phospholipid bilayer, the hydrophobic (non polar) lipid tails of both layers face each other while the hydrophilic (polar) phosphate region faces either the cytoplasm or the outside of the cell. Within the lipid bilayer are a plethora of proteins that facilitate different reactions. Proteins are found to be embedded within the bilayer or found on the surface of the bilayer only partially attached at the hydrophilic regions. Proteins attached to the external side often tend to have conjugated carbohydrate groups (forming glycoprotein complexes). Also found in the plasma membrane are receptor molecules which participate in various signalling pathways. In case of some of these receptors the protein tends to form a pore within the membrane and has the binding site on the membrane surface. In addition to this embedded within the bilayer are channels, pumps, transportes and adhesion sites. Apart from enclosing the cell and being a component of the plasma membrane, lipid bilayers/membranes are also found around organelles like the nucleus, mitochondria, and chloroplast.

Figure 1.1: The Lipid bilayer. Figure(A) shows an micrograph of the human RBC, figures (B) and (C) show the two and three dimensional structures of the cell membrane (Alberts *et al.*, 2002).

1.1.2 Membrane potential of the plasma membrane and its maintenance

A resting membrane potential is a phenomenon of all cells across their cell membrane. It is the difference in electrical potential between the interior and exterior of the cell i.e the potential difference on either side of the membrane. This potential is maintained due to the uneven distribution of ions across the membrane. The difference in charge is maintained primarily by the sodium-potassium ATPase (Na^+-K^+ ATPase) and Na^+/K^+ channels of the membrane. The Na^+-K^+ ATPase or the Na^+ pump, which is one of the most well studied transporters, brings about transport of Na^+ and K^+ (Figure 1.2). 3 Na^+ and an ATP molecule first bind to the transporter intracellularly. The hydrolysis of the ATP molecule causes the phosphorylation of the transporter (at an apartate residue) and causes the release of ADP. A conformational change in the transporter takes place which drives the Na^+ ions outside the cell. In this conformation, the pump can now bind 2 K^+ ions externally. This causes dephosphorylation of the pump allowing it to revert back to its original conformation and moving the K^+ ions internally in the process. This process is in fact responsible for consuming $1/3^{rd}$ of the energy in animal cells. The exchange of the 3 Na^+ for 2 K^+ provides energy to facilitate movement of molecules by membrane transporters both through the membrane potential and the ionic electrochemical gradient. The membrane potential is maintained on account of the fact that that the membrane has a much greater permeability for K^+ ions and as a result of this a large number of K^+ ions move down the concentration gradient from within a cell to the extracellular region. At the same time, on account of the relatively lesser permeability of Na^+ a relatively small number of Na^+ ions are moving down their gradient from the extracellular region across the membrane. This causes the extracellular region of the membrane to have a (relative) positive charge and the intracellular region to have a negative charge. Together the channels and the Na^+/K^+ ATPase helps maintain the resting potential.

These mechanisms of transport will be discussed further in the next section (1.2).

Figure 1.2: Diagrammatic representation of the Na^+-K^+ pump. The pump actively transports Na^+ and K^+ against the electrochemical gradient. One complete reaction involving the hydrolysis of one ATP molecule results in the movement of 3 Na^+ outside the cell and 2 K^+ into the cell (Alberts *et al.*, 2002).

Membrane potential is critical for a number of reasons including indirectly being responsible for providing energy for the efficient working of transporters and channels and for bringing about the transmission of signals in excitable cells such as neurons.

1.2 Introduction to membrane transporters

As discussed in section 1.1.1, the lipid bilayer of the cell membrane helps maintain concentration of solutes within the cell at a level different from that outside. It allows water and non polar molecules to pass through without hindrance. It must be mentioned however that despite the relative free permeability of water molecules in through the membrane, there are still specialised integral membrane proteins that exist within the membrane viz. aquaporins that allow water molecules to rapidly pass through but prevent other molecules from doing so (Agre, 2003). In order for certain components such as polar molecules, ions, amino acids etc. to move across the bilayer transmembrane proteins are required. These proteins allow a specific component or a group of similar molecules to pass through. Halvor Christensen's group (Riggs *et al.*, 1958) showed that Na^+ coupled transport of Glycine takes place across the membrane. Around the same time Peter Mitchell (1961) had put forth the chemiosmotic hypothesis which suggested that the H^+ electrochemical gradient was capable of transporting carbohydrates across the membrane. This was first shown using the H^+ lactose co transporter in *E.coli* which had the LacY gene.

The vast genomic level studies undertaken over the past decade have unravelled a plethora of transporters. The two main classes of membrane transport proteins are 1) carrier proteins which can undergo a conformational change to move bound molecules across the membrane and 2) channel proteins which form a narrow hydrophilic pore which allows solutes to pass through. For the purpose of this thesis, the transporters which will be dealt with are carrier proteins. Due to the selective nature of their binding sites, these transport molecules generally only transport a certain class of molecules and in most cases only a few members of a particular class. Transporters regulate the influx and efflux of various components important to the

cell, thereby maintaining cellular homeostasis. Transporters generally have a fixed stoichiometry which means that at the end of one cycle, a fixed number of solute/ion molecules would have been transported across the membrane.

Amino acids are one such family of substrates that need transporters to aid in their movement across membranes. Amino acids are the building blocks of proteins and are very important in different metabolic processes. A number of different amino acid transporters exist which vary depending on the charge/size of the amino acid substrates.

Physiologically speaking the concentration of the different amino acids in the cells varies depending on a number of factors including cell type. For instance, the nonessential amino acid proline is found at a concentration of 0.17 mmol/l in the plasma and 0.83 mmol/l in the muscle. The essential amino acid valine on the other hand has similar concentrationsboth in the plasma and the muscle (0.22 and 0.26 mmol/l respectively) (Bergström *et al*.,1974). The concentrations of non essential amino acids are generally observed to be higher. The non essential amino acids are those which can be synthesised in the body in various metabolic processes. The non essential amino acids on the other hand cannot be synthesized in the body and is obtained through dietary sources.

1.3 Mechanism of transport across the lipid bilayer

The movement of solutes across the lipid bilayer can be brought about in two main ways viz., non-mediated and mediated (Figure 1.3). Each of the mechanisms are discussed in further detail in the following sections.

1.3.1 Non-mediated transport

Non-mediated transport/simple diffusion involves movement of solutes from a region of high concentration to a region of low solute concentration. Examples of molecules transported by this mechanism are non polar molecules such as steroids and oxygen which can easily pass through the membrane.

1.3.2 Mediated transport

Mediated transport can be further divided into 2 main categories, channels and transporters. Transporters can be further classified based on the mechanism as non-facilitated/powered and facilitated transport.

1.3.2.1 Channels

Channels allow movement of solutes/ions down the electrochemical gradient. They are different from transporters in that the amount of solute moving across the membrane is governed by how long the channel stays open i.e the gating of the channel. This is in contrast to transporters where the amount of substrate translocated per cycle is fixed. Molecules that are transported across channels include molecules such as sodium and chloride where the amount of movement across the membrane is dependent on how long the channel stays open, in other words the gating mechanisms, and the electrochemical gradient for that ion.

1.3.2.2 Transporters

A) Non-facilitated/Powered transport

These transporters are classified based on whether they use ATP directly or indirectly to bring about the movement of substrates across the membrane.

a) Pumps/ Primary active transport

Transporters that use this mechanism of transport bring about movement by using energy. Primary active transport is where the energy spent by the hydrolysis of ATP, causes a conformational change and brings about transport of the solute molecule. An example of primary active transport is the Na^+-K^+ ATPase which was discussed in section 1.1.2.

b) Secondary active transport

The other form of active transport is secondary active transport where the movement of ions down their electrochemical potential difference and across the membrane which helps bring about the conformational change of the transporter and causes movement of the solute across the membrane. The electrochemical gradient is generated by the movement of ions such as Na^+ or H^+ across the membrane. The solute molecules either move in the same direction as the ion (termed as symport) or in the opposite direction (called antiport). One example of secondary active transport is SGLT1. This transporter brings about symport of one glucose molecule and two sodium ions across the membrane. This movement is aided by the downhill Na^+ gradient created by the Na^+/K^+ ATPase.

c) Tertiary active transport

In tertiary active transport, the movement of solutes across the membrane is driven by a transporter is caused due to a gradient generated by a secondary active transporter. PepT1 is one such transporter that brings about movement of substrates across the membrane via tertiary active transport. PepT1 requires the H^+ gradient to bring about transport of di and tri peptides across the membrane. This gradient is supplied by NHE3. Both these transporters are present in the brush-border membrane of intestinal epithelium (Bookstein et al. 1994, Adibi, 2003). NHE3 brings about the movement of Na^+ and H^+ across the membrane via secondary active transport using the Na^+supplied by the Na^+/K^+ pump (primary transport). This region has an H^+ gradient which is generated by the NHE3. When the H^+ ions bind to the transporter a conformational change of the PepT1 transporter is brought about and this caused the transport of di and tri peptides across the membrane (Watanabe *et al.*, 2005).

B) Facilitated diffusion

Molecules that use this mechanism to move across the lipid bilayer do not require energy expenditure but still have specific molecules dedicated to their movement across the membrane. There are a number of factors however that affects transport of molecules by this mechanism such as the concentration and the electrochemical

gradient. Transporters which bring about movement of solutes by facilitated diffusion include GLUT1 (the erythrocyte glucose transporter) that transports glucose across the membrane by using the glucose binding sites which are present on both sides of the membrane (Nelson and Cox, 2008), in other words the transport of glucose can be brought about in either direction. Unlike for active transport, facilitated diffusion processes do not affect the equilibrium, just the rate at which equilibrium is reached.

1.3.2.3 Differences in the mechanism of transport by a transporter protein

A particular transporter protein does not necessarily bring about transport by a fixed mechanism in all cell types. There can be a variation depending on the cell type, the membrane where it is expressed and the other transporters that are expressed on the membrane. An example of this can be illustrated using the PAT1 (SLC36A1). This transporter has been localised to the brush border membrane of intestinal epithelium and the lysosomal membrane. In both these regions the transporter brings about a symport of a proton and an amino acid.

In the brush border membrane the primary active transport is brought about by the Na^+-K^+ pump which pumps out Na^+ and thereby increases the relative amounts of Na^+ in the extracellular region. The increase in Na^+ is used as a driving force by the secondary active transporter NHE3 which exchanges the Na^+ for an H^+ (thereby acting as a secondary active transporter). The H^+ rich microclimate is then utilised by PAT1 which uses this as a driving force to bring about a symport of an H^+ and an amino acid across the brush border membrane (figure 1.4).

Contrary to this, in the lysosomal membrane PAT1 brings about the movement of solutes by secondary active transport. In this case the driving force is provided by the H^+-ATPase (primary active transport) which causes an influx of H^+ into the lysosomes (figure 1.5).

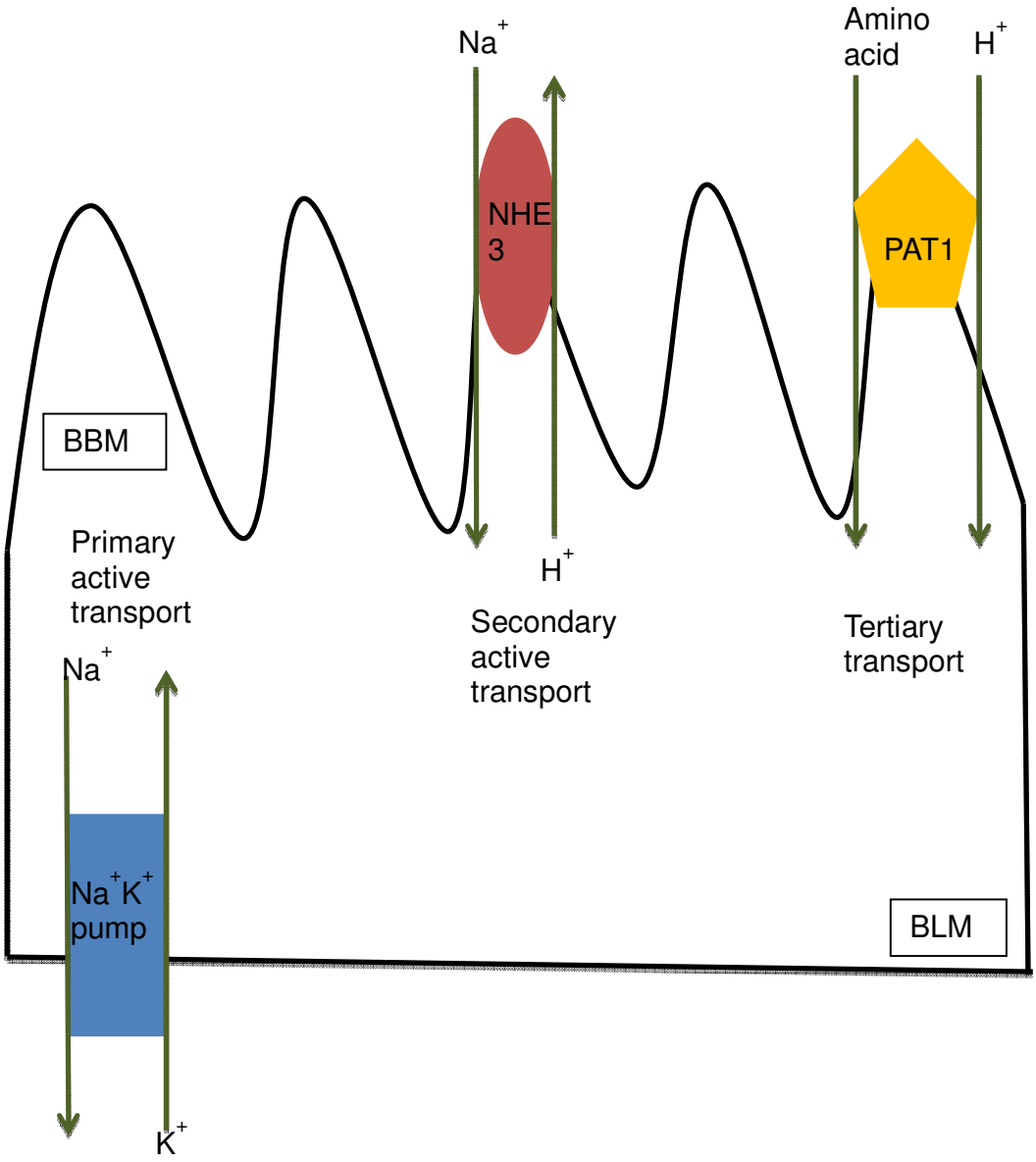

Figure 1.4: PAT1 as a tertiary active transporter.

Interactions between the Na+-K+ pump, NHE3 and PAT1 which bring about primary secondary and tertiary active transport respectively in the intestinal epithelium. BBM is the brush border membrane and BLM is the basolateral membrane.

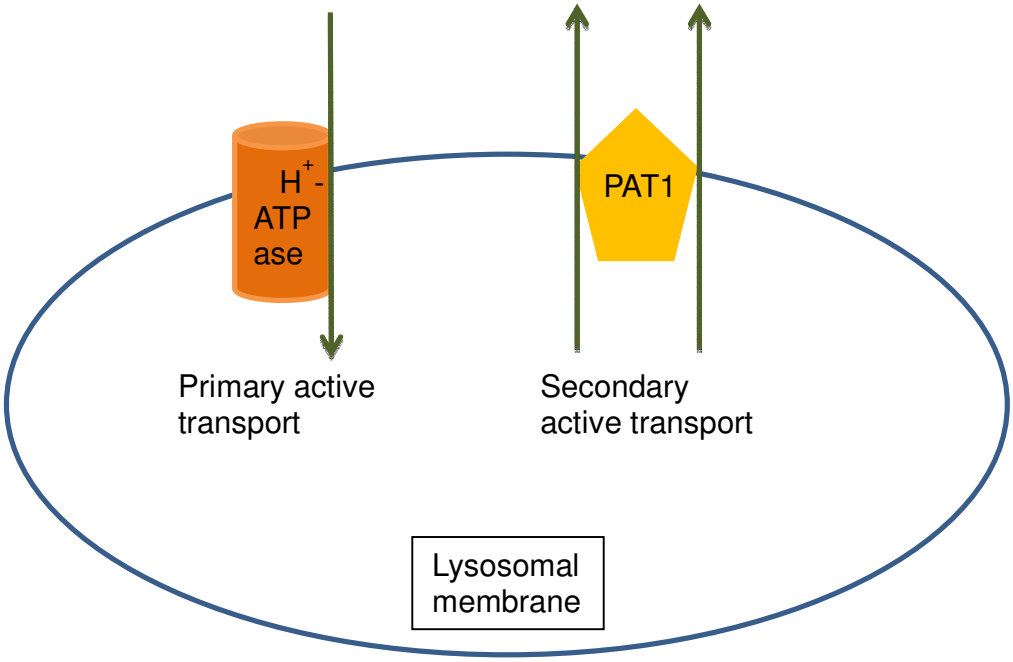

Figure 1.5: PAT1 as an ssecondary active transporter.

H^+-ATPase brings about primary active transport which supplies the energy for secondary active transport by PAT1.

1.4 The MFS and ABC superfamily

The wealth of genomic data and bioinformatics tools has resulted in the discovery of the MFS (major facilitator superfamily) and ABC (ATP-binding cassette superfamily) group of proteins, both of which are expressed in all organisms. The ABC permeases are observed to bring about primary active transport. The MFS on the other hand, forms one of the largest family of membrane transporters which generally bring about secondary active transport (Sigal, Molshanski-Mor and Bibi, 2006).

1.5 The SLC family of transporters

The solute linked carriers (SLCs) consist of 48 families containing over 300 genes which encode transporters (Hediger *et al.*, 2004). They are characterized to bring about transport by a variety of mechanisms including exchange, passive transport and coupled transport (Figure 1.6). Apart from the cell membrane they are found in the membrane of a variety of organelles such as vesicles and mitochondria (Hediger *et al.*, 2004).

Members present within the same SLC family have at least 20% identity to other members within the family. The nomenclature for members of the SLC family involves the abbreviation SLC (Slc in case of non human) followed by a number which is common to all the members of the SLC family under consideration, then the letter A which divides the family number from the number of the gene. A capital 'A' indicates a human gene whereas a small 'a' indicates a non-human gene. For instance, the proton coupled amino acid transporters PATs which are studied in this thesis belong to the SLC36 family. SLC36A1 indicates the solute carrier family 36 and the gene 1 (Thwaites and Anderson, 2007).

Figure 1.6: Diagrammatic representation showing the SLC transporters on the plasma membrane or membranes of different organelles in a cell and the other transport proteins that are expressed alongside (http://www.bioparadigms.org/slc/intro.htm).

1.6 The SLC36 (PAT) family

The SLC36 family is commonly grouped under the β-group of SLC transporters which bring about the transport of neutral amino acids (Sundberg *et al.*, 2008). The SLC36 family belongs to the amino acid auxin permease (AAAP) family. The SLC36 family of transporters was characterized based on homology based cloning to the H^+ coupled amino acid transporter families in yeast and plants (Boll, *et al.*, 2003). H^+-coupled transport plays a very important role in bacteria, plants, fungi and in recent years there has been considerable evidence to indicate its significance in mammals too (Metzner, *et al.*, 2006). Although most transporters in animals tend to use Na^+ as a driving force for the movement of solutes across membranes, there are cells in animals which still use H^+ as the ion which drives transport (Boll *et al.*, 2003).

There are three sub-families of the AAAP present in mammals: 1) the vesicular GABA transporter expressed in the brain and which brings about the transport of GABA and glycine, 2) a few representatives of the system A and N transporters which play a role in maintaining the balance of neutral amino acids in different tissues and 3) the Proton coupled Amino acid Transporters (PAT) transporters (Rubio-Aliaga *et al.*, 2004).

The members of this family are dependent on the H^+ electrochemical gradient to facilitate transport across the membrane (Thwaites and Anderson, 2007). In humans, the SLC36 (hPAT) family consists of four members SLC36A1 (hPAT1), SLC36A2 (hPAT2), SLC36A3 (hPAT3) and SLC36A4 (hPAT4).

SLC36A1 to A3 are located on the chromosome 5q33.1 whereas SLC36A4 is present on 11q14.3. These genes encode proteins containing about 500 amino acids. Different membrane prediction software predicts that the PATs contain 9-11 transmembrane domains.

As one of the major nutrition groups, proteins constitute an important part of our daily food intake. About 30% of our daily nutrition comes from proteins. These in turn are hydrolysed into peptides and amino acids in the stomach and the intestine and absorbed. In nature, amino acids play an essential role in the synthesis of proteins. Apart from this, amino acids also play a vital role in various biological functions such as cell growth, acting as carbon and nitrogen donors and as

components in various buffering systems. In the Central Nervous System they play an important role in neuro transmission. In order to be able to function efficiently these amino acids need to be transported across various biological membranes. This is an indispensible step in their metabolism. The SLC36 family of transporters are one such group which help in the movement of amino acids across the membrane. (Agulhon *et al.*, 2003).

The epithelial layer of the small intestine plays an important role in absorbing peptides and amino acids. The importance of intestinal transporters is further emphasized by the fact that they also play an important role in mediating the absorption of certain drugs and it is believed that the hPATs can play a significant role in this case (Brandsch, 2006).

1.7 PAT1

Initial evidence for the existence of PAT1 was in the 1960s when Newey and Smyth (Newey and Smyth 1964) reported a glycine-proline carrier which was later modified to an imino acid transport system by Lerner and Karcher in the 1970s (Lerner and Karcher 1978). By the 1980s two groups had shown the presence of an H^+-L-proline co-transporter (Roigaard-Petersen, *et al.*, 1987) and an H^+-glycine co-transporter (Rajendran *et al.*, 1987). Sagné *et al.* (2001) established the existence of a PAT transporter in the rat brain (LYAAT1, the lysosomal amino acid transporter 1), it was then cloned from mouse intestine (and named PAT1) and eventually the human form was isolated from Caco2 cells (Boll *et al.*, 2002). hPAT1 shows 85% similarity to the rat PAT1 ie LYAAT1 (Chen *et al.*, 2003).

1.7.1 Function

PAT1 acts as a proton coupled symporter of small neutral amino acids. The transport activity is pH dependent and Cl^- independent. Its activity was initially thought to be partially Na^+dependent but it has since been shown that this is only an artefact of its initial studies *in vivo* (Boll *et al.*, 2004). In this case it was observed that PAT1 shows

a dependence on the Na^+/H^+ exchanger 3 (NHE3) and was considered as partially Na^+dependent. The NHE3 exchanger helps maintain an acidic microclimate thereby facilitating transport via the PATs. NHE3 is involved in the inward movement of Na^+ using the downward electrochemical gradient in exchange for an outward movement of H^+ thereby creating a proton motive force available for transporters such as PATs to utilise (Thwaites and Anderson, 2007). However it was noted that in the lysosomes, H^+-ATPase helps drive the H^+/amino acid symport (Sagné *et al.*, 2001) and this showed that there is no Na^+ dependence in this case and that the NHE exchanger was only aiding in driving the protons thereby creating a gradient which could then be utilised by hPAT1 for the movement of amino acids across the membrane. Furthermore, when these studies are performed in Xenopus laevis oocytes and cell lines in controlled Na^+ free conditions at pH5.5, they are still observed to bring about transport similar to that *in vivo* (Frølund *et al.* 2010).

1.7.2 Substrate Affinity

The Km of an enzyme denotes the concentration at which the rate of the reaction is half the maximum. Similarly for a transporter the Km value is a measure of the affinity that a particular substrate has for that transporter, and is defined in the same way i.e. the concentration of substrate that causes half of the maximum transport rate.
The Michaelis-Menten equation is the basis for calculating the Km of an enzyme/transporter. The equation is simplified to

$$v = \frac{V_{max}[S]}{\{K_m + [S]\}}$$

Where v is the initial rate of the reaction, Vmax is the maximum rate of the reaction, Km is the Michaelis constant which is the substrate concentration when the reaction rate is half of Vmax and S is the substrate concentration.
Lineweaver and Burk eventually used a double-reciprocal plot to better estimate the Km of the enzyme for graphical representations.

$$\frac{1}{v} = \left[\frac{K_m(1)}{V_{max}[S]} + \frac{1}{V_{max}}\right]$$

In recent years various software have been observed to modified this equation taking into account various parameters to be able to fit differnet parameters required to correctly assess the Km value.

If a substrate is also capable of causing inhibition then the Ki value and Km value will be the same. For experiments performed to determine the Km of a transporter for a substrate, it usually is not the true Km but the apparent Km. This is explained by the fact that there is always an amount (usually small amounts in the micromolar range) of substrate present in the radiolabelled tracer used for these measurements. hPAT1 has Km values in the mM range (1-10mM) (Boll *et al.*, 2004). As discussed in the previous section, the physiological concentration of proline is less than 1 mM. Thus there is no possibility of the transporter being saturated and being unable to bring about transport of substrates across the membrane. SLC36A1 acts as a transporter for a number of amino (beta and gamma) and imino (both D- and L-) acids in addition to certain drugs (Thwaites *et al.*, 1994, Thwaites and Anderson, 2011). The main substrates for PAT1 are D and L proline, glycine and L alanine (Metzner *et al.*, 2006). Proline has a higher affinity than glycine or alanine for PAT1 (Bröer *et al.*, 2006). PAT1 is able to distinguish between D and L amino acids. In fact for some amino acids the affinity for some D amino acids is greater than that for L form (Bröer *et al.*, 2006) (Boll *et al.*, 2002). For instance it cannot transport the L form of serine and cysteine but can transport the D form. PAT1 loses the ability to transport the L form of the amino acid if there is more than one -CH_2 group in the side chain (Foltz *et al.*, 2004) (Boll *et al.*, 2003).

PAT1 is also capable of transporting small unbranched, apolar, zwitterionic (alpha, beta and gamma) amino and imino acids, D and L amino acids, heterocyclic amino/imino acids with 4-6 membered rings and N-methylated amino and imino acids (Hediger *et al.*, 2004). The drugs that PAT1 can transport include L-azetidine-2-carboxylic acid (which is an anti-angiogenic) and δ-aminolevulinic acid (which is

used in photodynamic therapy) (Frølund *et al*., 2010). The aliphatic amino acids have a higher affinity than aromatic ones (Metzner *et al.*, 2006). Methylation of the amino group in case of the aliphatic amino acids enhances the affinity further (Boll *et al.*, 2003). PAT1 is also observed to be one of the transporters responsible for the uptake across the membrane Anderson *et al.*, 2009). Analysis of PAT1 kinetics indicates that the proton binds to the transporter first followed by the substrate. The H^+ and substrate then move across the membrane simultaneously (Foltz *et al.*, 2005).

1.7.3 Localisation

The PAT1 protein is found in a variety of tissues ranging from the intestinal epithelium to neurons. Even the sub-cellular localisation in these tissues is found to vary. In the intestinal epithelia PAT1 is isolated to the brush border whereas in the neurons it is localised mainly in the lysosomal membrane and to a smaller extent in the plasma membrane (Hediger *et al.*, 2004).

PAT1 transport in both the neurons and the intestinal epithelium is driven by the H^+ (Boll *et al.*, 2004). Lysosomes are an important organelle in eukaryotic cells which help bring about the degradation of macromolecules. The products of this degradation are then transported out via specific membrane transporters and are reused. Defects in the working of these transporters can result in lysosomal storage diseases; such as sialic acid storage disease and nephropathic cystinosis (Sagné *et al.*, 2001). PAT1 in the lysosomal membrane helps transport amino acids which are a formed as a result of proteolysis into the cytoplasm. The H^+ rich conditions are provided by the V-type ATPases that cause the movement of H^+ into the lysosome by using the energy derived by the hydrolysis of an ATP molecule. In the brush border membrane of the intestinal epithelium which is another region from where PAT1 was isolated, the acidic micro environment is provided by NHE3 (Boll *et al.*, 2004). LYAAT1 was found to localise to lysosomes in some neurons. This seemed to be independent of the type of neuron it was (Agulhon *et al.*, 2003).

1.8 PAT2

1.8.1 Function

PAT2 has been shown to bring about 1:1 amino acid: H^+ symport. While PAT2 transports amino acids, it is also suggested that it might play a role in myelination of neurons (Boll *et al.*, 2004). PAT2 is also believed to be a regulating factor in myelin differentiation (Chen *et al.*, 2003). Experiments involving the giant patch clamp technique using glycine as a substrate on oocytes showed that it is capable of transporting in both directions.

1.8.2 Substrate affinity

PAT2 shows higher substrate selectivity and affinity than PAT1 with Km values in the micromolar range (100-600 μM) (Boll *et al.*, 2004). It is also observed to be pH-dependent like PAT1 and is also dependent on the membrane potential.

The small neutral amino acids glycine, alanine and proline are the main substrates for PAT2 (Boll *et al.*, 2002). Sarcosine is found to inhibit the transport activity. PAT2 has narrower substrate specificity than PAT1 (Rubio-Aliaga *et al.*, 2004). PAT2 is Na^+ independent similar to PAT1 (Chen *et al.*, 2003). While both β and γ-ABAs (amino butyric acids) can inhibit PAT1 the ABAs seem to have no effect on PAT2 (Kennedy *et al.*, 2005). PAT2 has a lower substrate affinity for GABA and β alanine.

1.8.3 Localisation

hPAT2 mRNA shows a high abundance in organs such as the kidneys, lungs and the brain (Foltz *et al.*, 2004). In recent years immuno histochemistry studies have indicated the presence of the PAT2 protein in very specific regions of the spinal cord and the brain. The sub-cellular localisation of PAT2 is still to be determined, but in HeLa cells it does not localize to lysosomes but seems to be expressed in the plasma membrane (Boll *et al.*, 2004). PAT2 (endogenous) is shown to localise at least in part to the plasma membrane of neurons (Rubio-Aliaga *et al.*, 2004). However studies have also shown that PAT2 localised to the endoplasmic reticulum and also on the surface of the recycling endosomes present in the neurons (Rubio-Aliaga *et al.*,

2004). In the kidneys hPAT2 is expressed in the apical surface. Its presence appears to be restricted to the proximal tubule in the kidneys (Vanslambrouck *et al.*, 2010)
The PATs could play an important role in causing iminoglycinuria and hPAT2 has always appeared to be the strongest contender for this on account of its substrate specificity and lower affinity (Broer *et al.*, 2008). This will be further discussed in section 1.11.

1.9 PAT3 and PAT4

PAT3 and 4 are considered as orphan transporters. PAT3 was first cloned from mice (Boll *et al*,. 2003). The PAT3 mRNA expression was found to be very high in the testis and negligible elsewhere (Bermingham and Pennington, 2004). There are two variants of PAT3 in humans variant 1 which is 123 bp longer than variant 2. Throughout the thesis variant 1 will be referred to as hPAT3.1 and variant 2 will be referred to as hPAT3.2. PAT4 was first identified based on sequence similarity to the remaining members of the SLC36 family. It seems to have a more ubiquitous expression (Foltz *et al.*, 2004).

1.10 Transporters and diseases

The importance of these transporters is further highlighted by the fact that mutations in these transporters can often lead to diseases.
For instance cystinuria which is the inability to transport cystine and can result in the accumulation of this product in the urine and lead to kidney stones is caused by a mutation in either the cationic amino acid transporter (SLC7A9) or in the cystine, dibasic and neutral amino acid transporter (SLC3A1) genes (Harnevik, Hoppe and Söderkvis, 2006). The fatal disease cystic fibrosis is caused by a mutation in the Cystic fibrosis transmembrane conductance regulator (CFTR) gene. Another disease which is caused by a defect in membrane transporter is Wilson's disease which is the

result of a mutation in the ATP7B gene which results in a defect in the transportation of copper across the membrane (Orquin, 2011).

1.11 PATs – Diseases and mutations

Iminoglycinuria involves the excretion of proline and glycine in the urine. It is an autosomal recessive disorder and is of particular interest due to the complex phenotypes that are observed and the pedigree studies involved. There are three phenotypes observed. Two of these forms show normal heterozygotes who have renal iminoglycinuria but either with or without a defect in renal absorption. In the third type, heterozygotes show renal iminoglycinuria but with a normal intestinal phenotype (Chesney, 2001, Scriver, 1968). Mutations in the SLC36A2 gene have been shown to be the major gene responsible for iminoglycinuria. Two mutations have been found in the gene, the first one is found to introduce a stop codon which causes translation of a non functional protein. The other mutation is a G87V point mutation which is a missense mutation which only partially affects transport function (Bröer *et al.*, 2008).

Another PAT related mutation which has been observed is the champagne mutation in horses. This point mutation causes a C to G mutation in exon 2 of PAT1 which changes the amino acid bein encoded from threonine to arginine. This point mutation is observed to be responsible for the champagne coat colour which is observed in horses (Cook *et al.*, 2008).

1.12 Research in the transport field

Studies on characterizing transporter systems were undertaken initially in the 1960s. Most research was focussed on studying the substrates for the different transport systems and how they interact in intact tissues. Transport proteins (rather than systems) as we know them today were started in the 1980s with the cloning of SGLT1 (Ikeda *et al.* 1989).

Studies in the transport systems field prior to molecular and genomic techniques were made particularly difficult by the fact that different transporters were capable of transporting the same substrates. This overlapping substrate specificity is also why defects in transporters are generally not lethal. For example one of the first families of amino acid transport systems existence was established was the IMINO system. Although the IMINO system is considered as a classical transport system for amino acids in mammals it has yet to be well characterized at the molecular level. The vast genomic level studies undertaken over the past decade have unravelled a plethora of transporters, many of which have not been well characterized. Many of these were until now undiscovered mainly due to their low levels or tissue/cell/ sub-cellular specificity. In many cases these transporters are considered important for drug delivery. Transporters showing cellular specificity and high affinity for a drug that needs to target the region where it expresses it will be indispensible to the medical world. For this purpose, extensive studies are now being undertaken on these transporters to understand their substrate affinities so that they can then influence drug design (Kennedy *et al.*, 2005).

Intestinal transporters have always been considered important for nutrient absorption. However, in recent years they have gained a lot of importance as mediators of drug uptake. A variety of drugs have been designed and used which rely on the proton coupled transporters to aid in drug transport. The H^+ proton gradient especially in the small intestine has been found to be responsible in aiding absorption of a variety of substrates including amino acids, peptides, vitamins and short chain fatty acids.

1.13 Membrane topology studies of transport proteins

1.13.1 Commonly used techniques

The tertiary structure of transport proteins are generally very difficult to study due to the difficulties associated in extraction of the transport protein from the bilayer using detergent in addition to the conformational changes they undergo. Therefore when a

large amount of protein is isolated, which is one of the requirements of crystallography, the odds that most or all of them are in the same conformation are slim. Since there are very few crystal structure available for membrane transporters, different sratergies are used instead. Indirect methods are used to determine the membrane topology secondary structure) and then to predict the tertiary structure. Epitope tags have previously been used to characterize transporters for instance the EYMPE epitope tag has been used to characterize the H^+ dipeptide transporter PepT1 (Covitz *et al.*, 1998).

Determining how many transmembrane domains (TMDs) exist and what their relative orientation is the first step in trying to decipher the structure of these molecules. Various techniques have been developed by groups the world over for this purpose. In some cases, people generated fusion proteins using sections of phoA (Ehrmann *et al.*, 1990) or lacZ (Wu *et al.*, 1992). However, this is often found to result in a protein with an incorrect conformation due to its bulkiness. In the past few years, with increased development of software to predict the protein structure, the study of protein conformation has undergone a drastic change. The first step used in trying to understand the structure of a membrane transporter is to use prediction software to try and determine the various regions of the protein. The software analyses the primary structure of the protein and predicts the TMDs and N-C termini orientation (intra- or extra-cellular).

Some of the commonly used membrane protein prediction software includes MEMSAT3, TMHMM, TopPred, SVMtm and TMpred. These were used for determining the various possible TMD regions of the hPATs. The results obtained based on these are discussed in the results chapter 4. Hydropathy plots of AAAP family members (to which PATs also belong) indicate that its members tend to have 11 TMDs unlike the more common 12 TMD structure (Young *et al.*, 1998). While similar considerations are taken into account by each of these softwares the level of stringency applied varies. This means that for the same transporter, depending on the software used the number of transmembrane domains predicted can vary making it necessary for experimental data to back up the membrane predictions.

Apart from crystallography, epitope insertion can also be used to study the conformation of the protein and the location of the TMDs. One of the methods commonly used to check the structure predicted using different programs is to use a combination of site directed mutagenesis and luminometry. Both these techniques are described in greater detail in the following section. In the initial stages, an epitope tag to which a commercial antibody is available is inserted into different regions of the gene via site directed mutagenesis.

1.13.2 Site directed mutagenesis

Site directed mutagenesis is a commonly used technique in molecular biology to help better understand the structure or function of genes. These mutations may be insertions or deletions; of single nucleotide mutations or larger regions such as epitope tags and multiple amino acids. The primary technique involved in site directed mutagenesis (SDM) is the Polymerase Chain Reaction (PCR) developed by Kary Mullis (Saiki *et al*., 1985). Primers designed for the PCR reaction are designed to contain the mutations that are to be introduced.

The gene is then converted into a form suitable for introduction into the orgamism, (such as cDNA or cRNA injections). It is then to be expressed in the organism of choice. It is expected that unless the insertion has caused a loss of function, the functional transporter will be expressed on the membrane of the cells. The presence of the epitope, i.e whether external or internal, can be determined by luminometry. In intact cells, if the region where the epitope is inserted is present in TMD or within the intracellular region, then the region will not be available for the antibody to bind to and consequently it will remain undetected. However if the cells are permeabilised, then these sites will become accessible and can be detected. On the other hand, if the epitope is present on the extracellular region, then the antibody can interact and its presence can be detected even when the cell is unpermeabilised. FLAG is one such commonly used epitope, consisting of 8 amino acids (DYKDDDDK) (Hopp *et al*., 1988). Antibodies are commercially available to this epitope already conjugated to the enzyme horseradish peroxidise (HRP). When the HRP conjugate is subjected to a high efficiency ELISA substrate it emits photons which can be detected by a

luminometer. In order to determine that the epitope tagged protein is functional, transport assays are performed. This involves comparing the transport activity of the protein containing the FLAG and one without it to check that the epitope tagging has not affected the function. However in case of proteins whose function is unknown (such as the orphan transporters), this technique of using a transport assay might not prove conclusive to ascertain whether the protein is functional. In these cases, the epitope tagging technique will have to be well established using members of the same family whose function is known before moving on to one which may not necessarily have known transport activity.

1.14 Studies of SLC36 homologs in *Drosophila*

Apart from mice, homologs of the PATs have also been studied in *Drosophila*. There are eleven PAT-related transporters in *Drosophila* (Boll *et al.*, 2004). These studies have proved especially useful in case of the orphan PATs whose functions have not been established. Due to the ease with which the organism can be maintained and the short time required to observe the changes when a gene is over/under expressed, *Drosophila* allow the researcher to study if there are other functions which the protein could be participating in.

It is known that signals from the insulin receptors (InR) and local nutrients help regulate cellular growth. These signals in turn help modulate the TOR (Target of Rapamycin) kinase activity. Nutrient signals are in part modulated by amino acid levels which in turn are modulated by amino acid transporters. In Drosophila InR pathway plays an important role in regulating local nutrient levels especially amino acid levels. These levels in turn are dependent on amino acid transporters. Goberdhan *et al.*, 2005 showed that the PAT family can regulate growth in tissues in vivo. The components of InR/TOR also interact at a molecular level with genes such as PATH (pathetic) which is a member of the *Drosophila* PAT family.

This is an experimental tool, over-expressing genes in these particulat ‘reporter tissues’. Path is expressed during various developmental stages and is important for both local and overall growth. CG1139 which is functionally similar to PAT1 has

been characterized in *Drosophila* melanogaster and is found to be associated with growth. Defects in eye and wing, size and development were found to be associated with mutations in this gene. CG1139 can regulate growth similar to PATH which has different transporter properties indicating that members of this family which have sequence similarity but different substrates can have a conserved function of regulating growth (Goberdhan *et al.*, 2005).

1.15 Choice of model system

1.15.1 *Xenopus* oocytes

Oocytes from *Xenopus laevis*, the South African clawed frog are commonly used as a popular system to study the expression of proteins (Gurdon and Melton, 1981). They were first developed as a model system in the University of Oxford by Gurdon as a graduate student. He had observed that *Xenopus laevis* oocytes which had their own nuclei removed/destroyed and replaced by one from another cell could express the non-nature proteins from the cell type of the donor nucleus.

The oocytes have the ability to translate RNA that they are injected with into proteins (Laskey *et al.*, 1972) using their own translation machinery. The oocyte system is commonly used to study ion channels and transporters since the translated protein is then directed to the membrane and its functions can be observed (Brown, 2004). The *Xenopus* oocytes have a fairly large diameter (1.1-1.3 mm) and are well equipped to bring about translation of a heterologous protein. In addition to this, they express a rather small range of endogenous membrane proteins, which in turn implies that the heterologous proteins which are expressed will have a fairly low background. This makes the *Xenopus laevis* oocyte a favoured model system in which to study transporters (Wagner *et al.*, 2000). They are also extremely useful in electrophysiological studies. In addition to this the membrane proteins that the oocyte expresses such as receptors and channels have been well characterized and documented over time. For instance, the *Xenopus* ooccyte expresses endogenous muscarinic acetylcholine receptors but not nicotinic ones (Herrera *et al.*, 1993).

Perhaps the most popular application of the Xenopus laevis oocyte was in the discovery of aquaporins by Peter Agre for which he won the Nobel Prize in 2003.

1.15.2 TM4 cell line

Currently, there is no evidence to indicate the presence of PAT3 in any tissues except in the early developmental stages of the mouse testis. TM4 is a mouse Sertoli cell line and it appears to be a good candidate to investigate for the endogenous expression of PAT3. In case there was no endogenous expression of PAT3, it could then be a good system to study PAT3 using transfections to express the PAT3 gene. On the other hand, if there was expression this cell line could then be used to test transport function directly and interactions that it may have with the other PATs could then be studied using RNAi. As a Sertoli cell line, it is hoped that TM4 cells will provide an environment similar to that in the testis thereby facilitating expression of PAT3.

1.15.3 HEK 293 cell line

The Human Embryonic Kidney 293 cell line as the name suggests was derived from primary embryonic kidney cells and is one of the most commonly used cell lines. They are used extensively on account of the ease of culturing them and performing transfections. They tend tend to give very good transfection efficiencies in the range of >80 % with most reagents. HEK293 cells are therefore used extensively to study recombinant proteins.

There is a lot of debate about whether the origin of these cells is epithelial or endothelial with new research even suggesting that these cells could have properties of neuronal cells (Shaw *et al.*, 2002). Due to these reasons, the HEK 293 cell line is not used to study a particular cell type but to get a better idea about what the roles of recombinant proteins in a cell culture.

Aims of the Thesis

The general aim of the project was to better understand the hPAT transporter family. hPAT1 and hPAT2 had been functionally characterized as proton coupled amino acid transporters. hPAT3 and hPAT4 on the other hand were categorised as orphan transporters and their functions unknown. The main aims of the project were as follows

- To elucidate the function of hPAT4 (using the oocyte expression system)
- To characterize substrate affinities, specificity and other features of the transporter
- To predict the Trans Membrane Domain structure using different membrane prediction software
- To confirm the predicted structure using the techniques of site directed mutagenesis, epitope tagging and luminometry
- To study the champagne mutation using expression in *Xenopus* laevis oocytes and to study how the mutation might produce effects.
- To use *Xenopus laevis* oocytes and cell culture techniques to study the functions of hPAT3

Chapter 2:

Materials and methods

A General materials and methods

2.1 General materials

The table summarises where the products used for the different experiments were purchased from. Antibodies etc are mentioned in the relevant sections

	Product/ Material	**Supplier**
	General chemicals	Sigma Aldrich
Molecular biology- Subcloning/ Mutations	Easy A polymerase	Stratagene
	GoTaq Flexi polymerase/ GoTaq polymerase	Promega
	Plasmid extraction Miniprep, midiprep, maxiprep	Promega
	PCR purification Gel extraction	Qiagen
	RNA extraction	Qiagen
	Subcloning grade competent cells	Stratagene
	Quickchange Pfu polymerase	Stratagene
	Antibiotics- Kanamycin, Ampicillin	Sigma Aldrich
	LB agar, LB broth	Sigma Aldrich
	Primers for cloning / mutating genes into the pXT7 vector	Sigma Aldrich
	Primers for cloning genes into the mammalian vector pcDNA3.1(-)	Fisher Scientific
Oocyte work	[^{3}H] L- amino acids	Perkin Elmer
	Scintillation cocktail	Perkin Elmer
Cell culture	Confocal compatable dishes (zero thickness)	IBIDI
	6 well plates	BD Biosciences
	75 ml flasks	Nunc
	Bradfords reagent	Bio-rad

2.2 General methods

2.2.1 Plasmid DNA extraction

a) For minipreps 5 ml culture was grown overnight in a 50 ml tube at 37 ºC in LB broth containing antibiotic

b) For midipreps 50 ml culture was grown overnight in a 50 ml tube at 37 ºC in LB broth containing antibiotic

c) For maxipreps 250 ml culture was grown overnight in a 500 ml tube at 37 ºC LB broth containing antibiotic

In all three cases even though the initial volumes were different from the manufacturer's recommendations, the protocol followed was still the same (Promega UK, PureYield Plasmid Mini-Prep system, PureYield Plasmid Midiprep System and Pureyield Plasmid Maxiprep System). Mini preps were eluted in 50 µl, midipreps in 600 µl and maxi preps were generally eluted in 1.5 ml nuclease free water.

2.2.2 Transformation of ligation/PCR products/plasmids into competent cells

a) using heat shock

1. 100 µl (in case of ligation and 50 µl for general plasmid transformations) subcloning grade competent cells were transferred into 15 ml tubes placed on ice.
2. 4 µl of the ligation product or 1 µl of the plasmid was added to the cells and mixed.
3. The tubes were then placed on ice for 30 mins
4. They were then subjected to heat shock for 45 seconds at 42 ºC and then placed on ice for 2 minutes.
5. 450 µl SOC medium was then added to the tube and placed in the incubator for 2 hours.
6. 200 µl of the medium was then placed on LB agar plates with antibiotic overnight at 37 ºC.

b) using electroporation (XL1 blue electroporation competent cells)

1. 30 µl of cells are placed in a 0.5 ml tube placed on ice.

2. 0.5 µl plasmid DNA or 2 µl ligation product was then added to the cells and mixed.
3. This mixture was then transferred to the cuvettes specially designed for electroporation (Equibio-ECU102, 0.2-cm gap).
4. A pulse was then given and the cuvettes were placed on ice for 2 minutes (200W resistance, 2500Volts using a Gene pulser from Bio-Rad, UK).
5. After this time, the cells were transferred into 15 ml tubes with 950 µl SOC medium and placed in the shaker at 37 ºC for 2 hours.
6. 50 µl – 100 µl was then plated on LB agar plates (with antibiotic) and placed in the 37 ºC incubator overnight.

2.2.3 PCR purification and gel extraction

PCR purifications and gel extractions were performed as per manufacturer's instructions (Qiagen, UK) but were then eluted in 25 µl nuclease free water.

B Molecular biology of transporter encoding genes

2.3 Obtaining the clones

Clones of hPATs 2, 3 and 4 were purchased from Source BioScience, UK. hPAT1 (clone no. 9020426) was obtained from Deborah Goberdan (personal communication) in the insect vector pUAST. hPAT2 (clone number 40010274) was in the pCR BluntII TOPO vector, while PAT4 (clone number 5313230) was in the pBluescriptR vector. Two clones of hPAT3 i.e the two different splice variants (clone numbers 40009826 and 400009832) were also in the pCR BluntII TOPO vector.

2.4 Preparation of miniprep stocks and glycerol stocks

The most common technique for making stocks of DNA plasmids is to use minipreps or midepreps. Minipreps generally tend to usually give smaller amounts (approx. 75

µl of 0.3-0.5 µg/ µl) of DNA whereas midipreps tend to give about 1 ml of 1 µg/µl DNA. Overnight cultures were set up and the plasmid DNA was extracted using either a miniprep or a midiprep. The quality of the plasmid obtained was observed to be better using minipreps as compared to midipreps. Multiple sequential minipreps were observed to give better quality DNA which was more useful for cRNA synthesis as opposed to midipreps. For both the midi prep and mini prep the manufacturer's instructions were followed (Promega).

The colonies which were confirmed by sequencing were transformed as described earlier and plated on LB agar plates containing ampicillin. Colonies were selected the following morning and incubated in 5 ml LB broth with ampicillin. 100 µl of the sample was then added to six 50 ml Corning tubes containing 10 ml of LB medium each (with 50 mM Amp) in the evening and incubated overnight at 37°C. The following day, 1000 µl of the sample was taken from one of the tubes to prepare glycerol stocks. Glycerol stocks were prepared by adding 800 µl of cell culture and 200 µl glycerol , mixed and stored at -80°C The tubes were spun at 13000 rpm and the pellet was resuspended in 500 µl resuspension solution (promega) and divided into two tubes and the mini prep (Promega) was performed as per the manufacturers instructions. The DNA was then eluted in 50 µl each and pooled together at the end giving a final volume of approximately 600 µl. The concentration of the DNA was measured and a sample sent for sequencing to confirm the sequence

2.5 Subcloning of hPATs into the oocyte vector

2.5.1 Introduction

Preliminary experiments were set up to express the hPATs in Xenopus using the vectors that the clones were purchased in. The results obtained however showed very low transport activity even for the controls. It was therefore decided to subclone the hPATs into an oocyte expression vector. Figure 2.1 shows a schematic representation of the stratergy followed for subcloning the hPATs into the Xenopus vector.

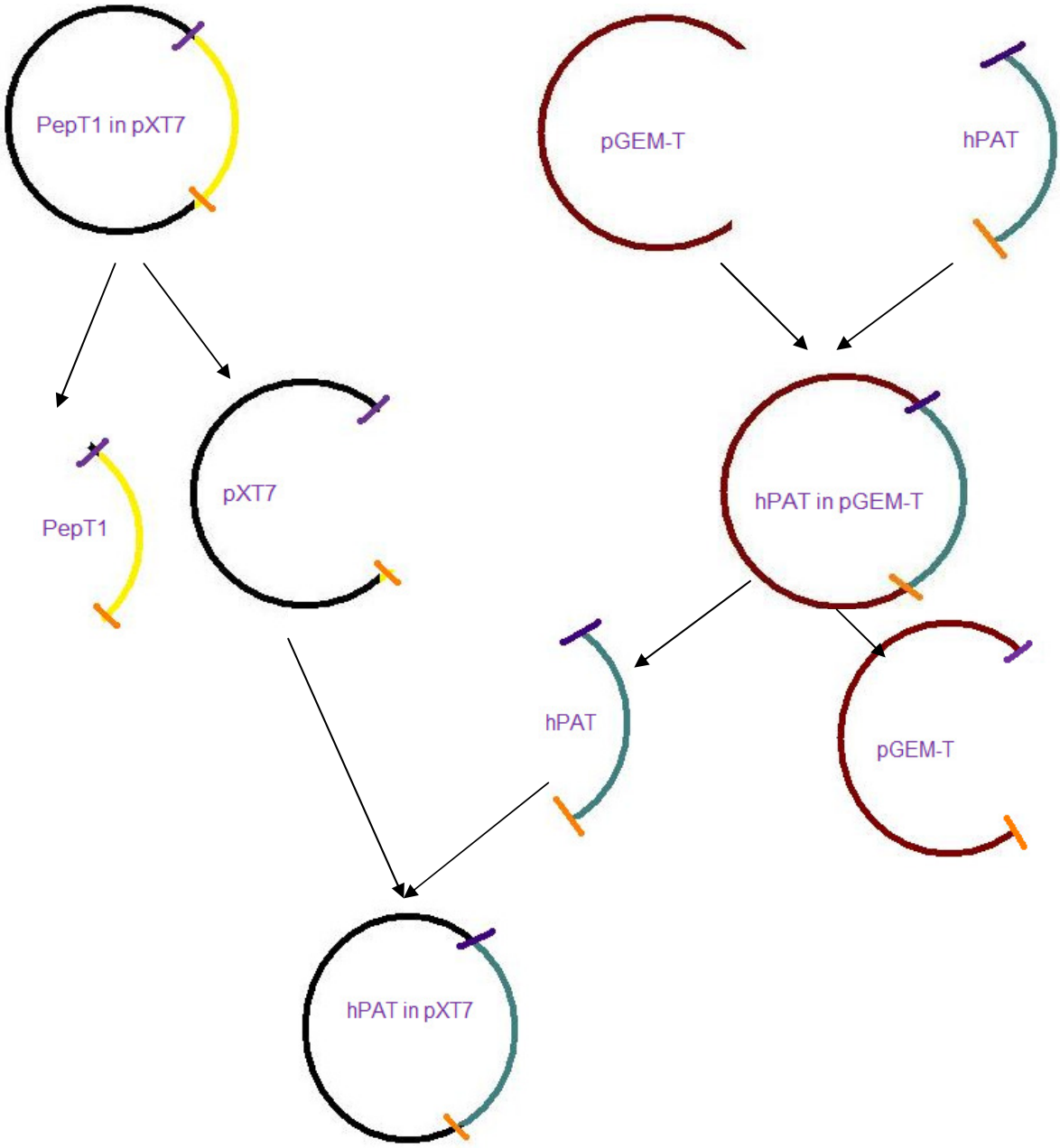

Figure 2.1: Schematic representation showing the subcloning stratergy adopted to clone the hPATs into the oocyte expression vector. The hPAT gene was first subcloned onto the pGEM-T Easy. The gene was then digested out of pGEM-T Easy and introduced into pXT7 which had also been digested using the same restriction endonucleases.

Subcloning is a technique that involves removing a gene out of the vector, which might not be suitable for expressing the gene in the model system and cloning it into a vector which expresses well in the system of choice (in this case Xenopus laevis). The preliminary step in subcloning is choosing restriction sites which are unique to the final vector that the gene is to be introduced into.

2.5.2 Subcloning of hPATs into pGEM-T Easy

Prior to cloning the genes into the final vector (pXT7 which will be explained further in the next section), the genes were first cloned into the pGEM-T Easy (Promega) system. This was primarily so that we can grow the gene in pGEM-T Easy as much as required and could have a large stock of it. This would be useful if the direct ligation were to fail we would have to start with the initial PCR step again. In addition to this the pGEM-T Easy step also ascertained that a single clone was used to grow up the final template.

2.5.2.1 PCR

As described in section 2.5.1 primers unique to the final vector are required to insert the gene of choice into it. Since none of the hPATs have KpnI or EagI sites present in the genes or the vectors, these sites were introduced via PCR into them. The primers used for subcloning hPATs 1-4 are enlisted in table 2.1

Primer	Sequence
PAT1 Forward	GCTGGTACCCCATGTCCACGCAGAGACTTCG
PAT1 Reverse	AGCCGGCCGTCCCTATATGAAGGCACAGGTGGAATTG
PAT2 Forward	GCTGGTACCGCTCCTTGTTTCTTAAGCAGTCATGTCTGTG
PAT2 Reverse	AGCCGGCCGTCGGGTGCTGGTAGGCAAGGA
PAT3 Forward	GCTGGTACCGTTGAAGATGTCATTGCTTGGAAGGGACTAC
PAT3 Reverse	AGCCGGCCGGAATAAAAACAGATAATTATGCATGGACACCTG TGG
PAT4 Forward	GCTGGTACCGGCGGCGTCCTGGAGACC
PAT4 Reverse	AGCCGGCCGGAAGACTCATGATTCTGCTTTTACTATTTCAAAC CAG

Table 2.1: Primers used in the subcloning of the hPATs into pGEM-T Easy and eventually the pXT7 vector

KEY

Alaline linker

Res site

Forward: KpnI

Reverse: EagI

Untranslated region

The PCR was set up using Easy A polymerase which adds aditional A nucleotides which will facilitate ligation with the vector pGEM-T Easy which is an open vector that has T nucleotides flanking the ends (table 2.2)

Reagent	Amount
10x easyA buffer	1x
Forward primer (10 µM)	1.25 µl
Reverse primer (10 µM)	1.25 µl
dNTPs (25 mM)	1 µl
Template (50ng)	1 µl
H2O	To bring up the volume to 50 µl
Enzyme	1 µl

Table 2.2: PCR set up using Easy A

The PCR conditions were set up with an initial denaturation step 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 65 °C for 1 minute and extension at 72 °C for 2 minutes. This was followed by a final extension step for 10 minutes. The reaction was then allowed to soak at 4
An annealing temperature of 65°C was selected since each of the primers had a Tm of about 70 °C.

2.5.2.2 DpnI treatment and ligation of the PCR product with pGEM-T Easy

The PCR product was DpnI (New England Biolabs) treated for thirty minutes at 37 ºC and then PCR purified. DpnI digests only the methylated bacterial DNA template leaving behind only the PCR product. 5 µl of the sample was run on the gel to check that the product was the correct size. The concentration of the product was determined and an overnight ligation was set up using the pGEM-T Easy vector. Ligations were set up using 1:3, 1:1 and 3:1 molar ratios of insert:vevctor. The reaction was set up to contain reagents as enlisted in table 2.3. Control reactions were also set up alongside.

	1:1	3:1	Negative control (without vector)	Negative control (without insert)	Positive control
Insert	1 µl at 25 ng/µl	1 µl at 75 ng/µl	1 µl at 75 ng/µl	-	1 µl (supplied with the kit)
Vector (50ng/µl)	1 µl	1 µl	-	1 µl	1 µl
Buffer	2 µl	2 µl	2 µl	2 µl	2 µl
ddH2O	2 µl	2 µl	3 µl	3 µl	2 µl
T4 ligase	1 µl	1 µl	1 µl	1 µl	1 µl
Total volume	10 µl	10 µl	10 µl	10 µl	10 µl

Table 2.3: Table showing pGEM-T Easy ligation set up

The tubes were then placed in the 17 ºC incubator overnight. On the following day, 2 µl of the ligation product was transformed into electrocompetent cells and placed in the 37°C for an hour. LB agar plates containing ampicillin were prepared. When the transformants were being incubated for an hour, 100 µl X-Gal IPTG was spread on the ampicillin plates. The plates were allowed to dry for 2 mins and placed in the 37°C incubator till the cells were ready to be plated. After the 1 hour incubation in SOC, 100 µl of cells was then plated onto LB agar plates with Ampicillin and X-Gal-IPTG to help select positive colonies. Colonies which are white are possible positive clones. The principle of Blue-white screening involves genetic engineering of the lac operon. Disruption of the subunit of the lacZα gene by the insert prevents the lacZα gene from being encoded . If the vector had religated in the absence of the insert then the lacZα subunit from the vector and the Ω subunit encoded by the bacterial gene can complement to form the functional β-galactosidase enzyme. β-galactosidase enzyme is capable of breaking down X-Gal to form an insoluble blue product, 5-bromo-4 chloroindole. IPTG is used to induce the synthesis of Lac operon. The plates were then incubated overnight at 37 °C. On the next day 5-10 positive colonies (white

colonies) were selected and incubated overnight in LB medium containing Ampicillin. The cultures were then purified (promega miniprep).

2.5.2.3 Analysing positive clones

The possible clones were then checked for the presence of the insert by using restriction digests (sites which were present within the gene but not in the vector), and those that appeared to be positive were send for sequencing to confirm (GeneService). Primer T7F was used for sequencing unless specified otherwise.

2.5.3 Ligation of hPATs with pXT7

2.5.3.1 Introduction

hPATs which were cloned into pGEM-T Easy were then used as a template to perform the ligation of the genes into pXT7.

The pXT7 vector is a modified pGEM-4Z vector. PepT1 was cloned into the pXT7 vector by Dr. David Meredith (personal communication). This vector had previously been used by the lab to study transporters such as PepT1 and PepT2 and is found to be a good expression vector for the oocyte system. pXT7 contains an Ampicillin resistance gene in addition to two strong promoters T7F and SF6. pXT7 has untranslated regions (UTR) of the rabbit beta globin gene flanking the multiple cloning site; 50 bp at the 5' end and 290 bp at the 3' end. These UTR regions increase the efficiency of translation. Figure 2.2 gives a diagrammatic representation of the pXT7 oocyte expression vector.

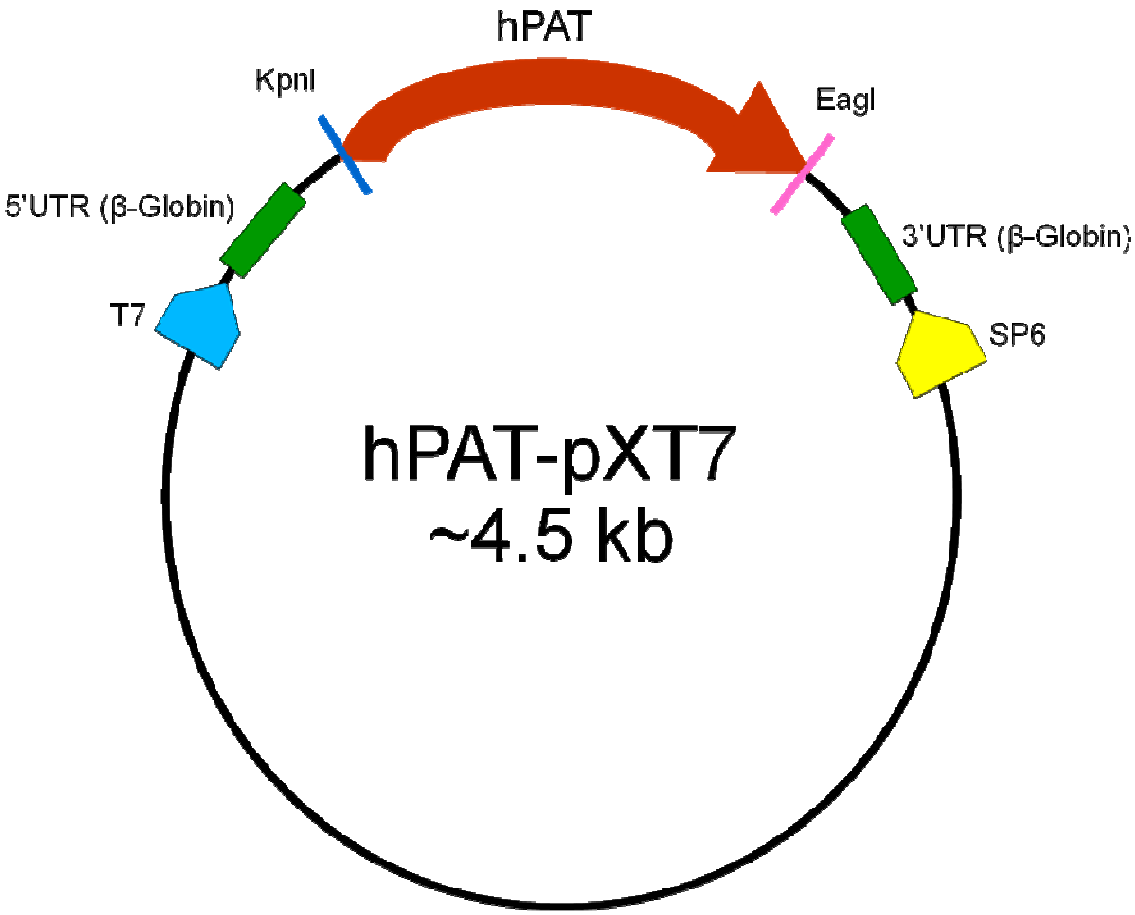

Figure 2.2 : Diagrammatic representation of the pXT7 vector with the hPAT gene. Flanking the hPAT gene are the *Xenopus* Beta globin untranslated regions. The vector has both T7 and SP6 promoters; however for the purpose of cRNA synthesis the T7 promoter was used. The gene was subcloned into the vector using 5' KpnI and 3' EagI sites.

Prior to subcloning the hPAT genes into the pXT7 vector, it was necessary to remove the PepT1 gene from it. For this purpose it was necessary to chose two restriction enzymes which were absent in the genes (hPATs) and the pGEM-T Easy vector system (which will be explained further in the next section). The two restriction enzymes selected were KpnI and EagI. KpnI was found upstream of the PepT1 gene within the pXT7 vector. EagI on the other hand is found close to the stop codon within the PepT1 gene. PepT1 in pXT7 was digested with KpnI (New England Biolabs) and purified (table 2.4). PCR purification was done after all the restriction digestion steps using the manufacturer's instructions (Quiagen).

Reagent	Final concentration
pXT7-PepT1	15 µg
10x Buffer (NEB3)	1x
EagI (NEB)	15 units
Nuclease free water	To bring up the final volume to 50 µl

Table 2.4: Linearization of pXT7-PepT1. Sequential digest Part 1

Following this the EagI digest was performed (table 2.5), PCR purified and then run on the gel. The vector was then cut out of the gel, purified and its concentration measured.

Reagent	Final concentration
pXT7-PepT1 linearised with EagI	10 µg
10x Buffer (NEB3)	1x
BSA	100 µg/ml
KpnI (NEB)	10 units
Nuclease free water	To bring up the final volume to 50 µl

Table 2.5: Digesting the linearised pXT7-PepT1 to obtain an open pXT7 vector. Sequential digest Part 2

2.5.3.2 Restriction digests

The genes were sequentially restriction digested using EagI (table 2.6) for three hours and KpnI (table 2.7) for an hour. The restriction digest was purified both in between

the two digests and after the second digest. The products were then run on the gel to check for the fragment of the correct size. The fragments of the expected size (approximately 1.5 kb) were then cut out and purified; the concentration of the products was measured.

Reagent	Final concentration
Template (hPAT in pGEM-T Easy)	5 µg
10x Buffer (NEB3)	1x
EagI (NEB)	10 units
Nuclease free water	To bring up the final volume to 50 µl

Table 2.6: Linearisation of hPAT in pGEM-T Easy with EagI (PartI of the 2 step sequential digest)

Reagent	Final concentration
Template (hPAT in pGEM-T Easy) linearised with EagI	5 µg
10x Buffer (NEB1)	1x
BSA	100 µg/ml
KpnI (NEB)	10 units
Nuclease free water	To bring up the final volume to 50 µl

Table 2.7: Restriction digest of hPAT (linearised with EagI and purified) using KpnI (Part 2 of the sequential digest)

2.5.3.3 Ligation of hPAT genes with pXT7

	3:1	Negative control (without vector)	Negative control (without insert)	Positive control
Insert x	75 ng	75 ng	-	PepT1 75ng
Vector y (50ng/µl)	50 ng	-	50 ng	50 ng
Buffer	2 µl	2 µl	2 µl	2 µl
ddH2O	10-(x+y+2) µl			
T4 ligase	1 µl	1 µl	1 µl	1 µl
Total volume	10 µl	10 µl	10 µl	10 µl

Table 2.8: Ligation of hPAT with pXT7

Overnight ligations were set up using molar ratios of insert:vector of 3:1, 1:1 and 1:3 as shown in table 2.8.

The next day transformations were set up using electrocompetent cells as described in section on general methods. Positive clones were detected as described earlier in section 2.3.4.

2.5.3.4 Confirming positive ligations

Colonies were selected the following day and incubated in LB Broth with ampicillin overnight. The following day plasmid DNA was extracted from the cultures and concentration measured. Potential positive clones were checked by restriction digest using NdeI which is present in pXT7 but not in hPATs 2, 3 (both isoforms) and 4. For hPAT1 restriction digest was done using SalI which is present in the pXT7 vector but absent from hPAT1. Clones that were digested and appeared to be positive were then sent for sequencing to confirm.

2.6 Site directed mutaganesis for introduction of the FLAG epitope tags into hPAT1

2.6.1 Primer design for the introduction of epitope tags in the hPAT1 gene

Based on the MEMSAT3 (Jones, Taylor and Thronton, 1994) prediction results which will be discussed in the results chapter, twelve separate FLAG tags were introduced into the PAT1 gene (in the pXT7 vector). FLAG epitope contains the amino acid sequence DYKDDDDK which as a nucleotide sequence can be written as 5’ gac tac aaa gac gac gac gac aaa 3’. The primer sequences used to insert the FLAG are in the table below (2.9 and 2.10). Two sets of FLAGs were inserted, one set of potential external FLAGs and one set of potential internal FLAGs.

Primer name	Sequence
1F	CTC CCT CTG GCG GTG GAC TAC AAA GAC GAC GAC GAC AAA AAT GCA GGC ATC
1R	GAT GCC TGC ATT TTT GTC GTC GTC GTC TTT GTA GTC CAC CGC CAG AGG GAG
2F	GCT GAC AAC TTT GAC TAC AAA GAC GAC GAC GAC AAA CAG GTG ATA GAA GCG
2R	CGC TTC TAT CAC CTG TTT GTC GTC GTC GTC TTT GTA GTC AAA GTT GTC AGC
3F	AGG ATC CCA GAC TAC AAA GAC GAC GAC GAC AAA CCC AGC CAC CTC CCC
3R	GGG GAG GTG GCT GGG TTT GTC GTC GTC GTC TTT GTA GTC TGG GAT CCT
4F	GCT AAT ATC CAA GGC AGC GAC TAC AAA GAC GAC GAC GAC AAA ATA ACC CTC AAC
4R	GTT GAG GGT TAT TTT GTC GTC GTC GTC TTT GTA GTC GCT GCC TTG GAT ATT AGC
5F	CCC CGC CTG GAC CTG GAC TAC AAA GAC GAC GAC GAC AAA GTC ATC TCC CTG
5R	CAG GGA GAT GAC TTT GTC GTC GTC GTC TTT GTA GTC CAG GTC CAG GCG GGG
6F	CCC ATC TTC ATC AAT TCC GAC TAC AAA GAC GAC GAC GAC AAA ACC TGT GCC TTC
6R	GAA GGC ACA GGT TTT GTC GTC GTC GTC TTT GTA GTC GGA ATT GAT GAA GAT GGG

Table 2.9: Primers for introducing the putative external FLAGs in hPAT1

Primer name	Sequence
i1F	GAC TAC CAC GAC TAC AAA GAC GAC GAC GAC AAA GAC TAC AGC TCC
i1R	GGA GCT GTA GTC TTT GTC GTC GTC GTC TTT GTA GTC GTG GTA GTC
i2F	AGG CTG AAT AAA GAC TAC AAA GAC GAC GAC GAC AAA TCC TTT GTG
i2R	TCC GAC TTA TTT CTG ATG TTT CTG CTG CTG CTG TTT AGG AAA CAC
i3F	CCT CCG AGC CCT GAC TAC AAA GAC GAC GAC GAC AAA GTC CAT CTT CTC
i3R	GAG AAG ATG GAC TTT GTC GTC GTC GTC TTT GTA GTC AGG GCT CGG AGG
i4F	GAT CCT CGG AAG GAC TAC AAA GAC GAC GAC GAC AAA TTC CCA CTC ATC
i4R	GAT GAG TGG GAA TTT GTC GTC GTC GTC TTT GTA GTC CTT CCG AGG ATC
i5F	GAG CAC TGT GAG GAC TAC AAA GAC GAC GAC GAC AAA TTA GTG GTG GAC
i5R	GTC CAC CAC TAA TTT GTC GTC GTC GTC TTT GTA GTC CTC ACA GTG CTC
i6F	GGC ATG AGC CCC GAC TAC AAA GAC GAC GAC GAC AAA CTC ACC ATC TTT AAG
i6R	CTT AAA GAT GGT GAG TTT GTC GTC GTC GTC TTT GTA GTC GGG GCT CAT GCC

Table 2.10: Primers for introducing the putative internal FLAGs in hPAT1

2.6.2 Introduction of the epitope tag using PCR

For the intrroduction of the FLAG epitope either the quick change lightning site directed mutagenesis kit (Agilent/Stratagene) was used. The PCR reaction was set up to contain reagents as shown in table 2.11

Component	Volume
Template (50ng)	1 µl
Forward primer (10 µM)	1.25 µl
Reverse primer (10 µM)	1.25 µl
Quickchange solution	1.5 µl
dNTP mix	1 µl
H2O	To a final volume of 50 µl
Enzyme	1 µl

Table 2.11: PCR set up for site directed mutagenesis to introduce the FLAG epitope into the hPAT gene

The PCR reaction was set up using initial denaturing at 95°C for 2 minutes, followed by 18 cycles of denaturation at 95°C for 20 seconds, 60°C for 10 seconds and 68°C for 2.5 minutes. A final extension step at 68°C was performed for 5 minutes before storing at 4°C.

2.6.3 DpnI treatment and transformation of the PCR products into bacterial cells

The PCR product was then treated with DpnI for 30 minutes and purified before transforming it into XL-10 Gold competent cells. To 40 µl of cells on ice, 2 µl of beta mercaptoethanol was added and mixed. After 2 minutes, 2 µl of the PCR product was added and mixed and placed on ice for 30 mins. The mixture was then heat-shocked for 40 seconds and placed on ice for 2 minutes before adding 200 µl of SOC medium to it and incubating it for 1 hour. 200 µl of the mixture was then plated on LB agar plates containing Ampicillin.

2.6.4 Analysis of positive clones

The following day positive clones were selected and confirmed by sequencing. T7F was used for sequencing FLAGS 1-4 and internal FLAGSi1-i4, the sequencing primer seq1.4 (TCTGGGGTACCTGCAATTTG) was used to confirm the sequences FLAGS 5, 6, i5 and i6.

2.7 Introduction of champagne mutation (using SDM)

2.7.1 Primer Design

The champagne mutation was introduced using the primers enlisted in table 2.12. The champagne mutation is a C → G point mutation which causes a change in the amino acid from a threonine → arginine.

Primer name	Sequence
champF	GGC AAC ATT GGC AGA GGA CTC CTG
champR	CAG GAG TCC TCT GCC AAT GTT GCC

Table 2.12: Primers to introduce the champagne mutation in the hPAT1 gene

2.7.2 PCR reaction to introduce the mutation

The mutation was introduced using both the PAT1 and the FLAG1 PAT1 templates. The PCR set up using the same conditions as for the FLAG insertions. The PCR reaction was set up using initial denaturing at 95°C for 2 minutes, followed by 18 cycles of denaturation at 95°C for 20 seconds, 60°C for 10 seconds and 68°C for 2.5 minutes. A final extension step at 68°C was performed for 5 minutes before storing at 4°C.

Component	Volume
Template (50ng)	1 µl
Forward primer (10 µM)	1.25 µl
Reverse primer (10 µM)	1.25 µl
Quickchange solution	1.5 µl
dNTP mix	1 µl
H2O	To a final volume of 50 µl
Enzyme	1 µl

Table 2.13: PCR mix for the SDM of hPAT to introduce the champagne mutation

2.7.3 DpnI treatment, transformation and analysis

The product was DpnI treated for 30 minutes, PCR purified and transformed into XL-10 Gold cells (as described in section 2.1.4.3) and plated on LB agar plates as described. Clones were selected at random and grown in LB medium with Ampicillin and analysed by sequencing.

2.8 Subcloning of hPATs into the mammalian vector

pCDNA3.1(-) (Invitrogen) was the mammalian vector which was used to subclone the hPAT3. Since there weren't any restriction sites that were unique and common to both pCDNA3.1(-) and hPATs in pXT7 and in the same orientation as required it was necessary once again to introduce restriction sites via PCR as used in the subcloning into the pXT7 vector. This time both the 5' and 3' ends were designed to have KpnI sites.

Figure 2.3 gives a diagrammatic representation of the subcloning strategy followed.

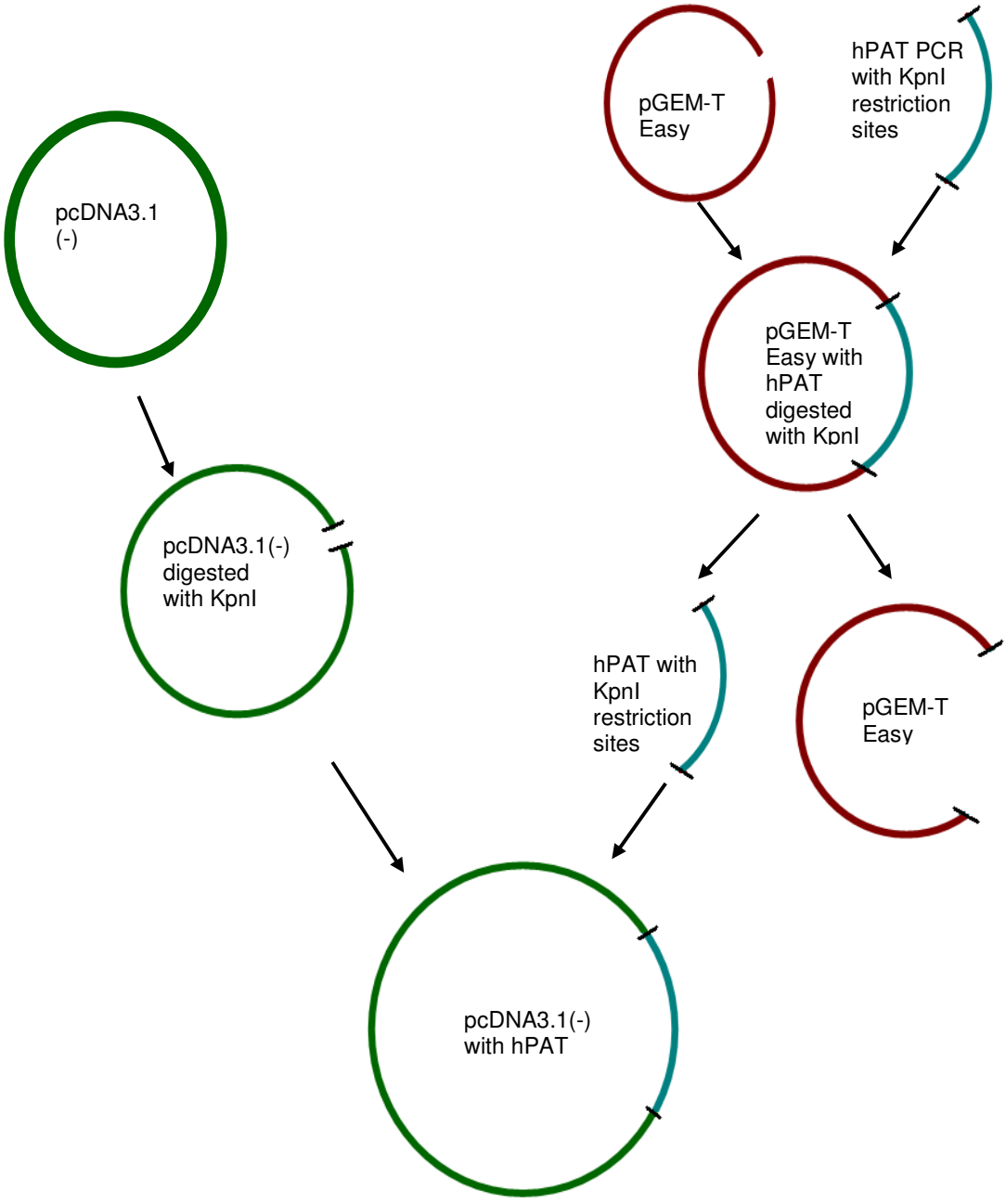

Figure 2.3: Cloning strategy to subclone hPATs 2, 3.1 and 3.2 into the mammalian expression vector pcDNA3.1(-). The pcDNA3.1(-) was lineared using KpnI and ligated with the hPAT genes (which which were restriction digested out of pGEM-T Easy vector using the same restriction site.

The same forward primer was used as the one for subcloning hPAT2 and 3 into pXT7 but the reverse primer was designed to contain a KpnI site instead of an EagI site as used in case of the pXT7 subcloning.

Primer	Sequence
PAT2 Reverse	AGCGGTACCTCGGGTGCTGGTAGGCAAGGA
PAT3 Reverse	AGCGGTACCGAATAAAAACAGATAATTATGCATGGACACCTG TGG

KEY Alanine linker Res site Reverse: KpnI Untranslated region

Table 2.14: Primers used to subclone hPATs 2, 3.1 and 3.2 into the mammalian vector

The hPAT genes was first subcloned into the pGEM-T Easy vector and then digested out using the KpnI site and inserted into the pcDNA3.1(-) vector (which was also digested with KpnI).

The vector was digested using KpnI (high Fidelity) table 2.15

Reagent	Final concentration
pcDNA3.1(-)	10 µg
Buffer J (Promega)	1x
BSA	100 µg/ml
Alkaline phosphatase	5 µl
KpnI (Promega)	10 units
Nuclease free water	To bring up the final volume to 50 µl

Table 2.15: Linearistion of pcDNA3.1(-) using KpnI

The template was PCR purified and its concentration measured and the digested vector stored at -20 °C

The PCR was set up under the following conditions

Reagent	Amount
10x easyA buffer	1x
Forward primer (10 µM)	1.25 µl
Reverse primer (10 µM)	1.25 µl
dNTPs (25 mM)	1 µl
Template (50ng)	1 µl
H2O	To bring up the volume to 50 µl
Enzyme	1 µl

Table 2.16: PCR set up for the amplification of hPAT2, 3.1 and 3.2 genes to introduce KpnI sites at the 5' and 3' ends

The product was PCR purified and run on a gel to confirm the size. The PCR product was then ligated with the digested vector using a molar ratio of 3:1 (insert:vector) and 50 ng of Vector DNA and adjusting the volume of the insert to adjust for the molar ratio. 10 µl of the solution was set up to contain a final concentration of 1x buffer and 2 µl T4 DNA ligase (Promega) and kept in the 17 degree incubator overnight. 4 µl of the ligation product was transformed using subcloning grade heat shock cells as described in section (2.4.1) and plating it on Ampicillin plates followed by overnight incubation at 37 degrees.

The presence of hPAT genes with KpnI sites in pGEM was confirmed by sequencing and this was used as the template to set up restriction reactions with KpnI.

Reagent	Final concentration
hPATs	5 µg
Buffer J (Promega)	1x
BSA	100 µg/ml
KpnI (Promega)	10 units
Nuclease free water	To bring up the final volume to 50 µl

Table 2.17: Restriction digest of hPATS with KpnI

The digestion products corresponding to the correct size (approx 1.5 kb) was gel extracted, purified and used to set up ligation reactions with 100 ng vector. The

strategy followed was the same as the subcloning of the genes into pXT7. The positive clones were confirmed for the presence of the gene using restriction digest and sequencing.

2.9 cRNA synthesis

In vitro cRNA synthesis was performed using either the Ribomax T7 RNA synthesis kit (Promega) or T7 RNA synthesis kit (Ambion).

2.9.1 Linearisation of the plasmid DNA

For either kit, the first step was linearising the DNA template (table 2.18). For hPATs 2, 3 and 4 the restriction enzyme NdeI (NEB) was used.

Reagent	Final concentration
Template (hPAT in pGEM-T Easy)	10 µg
10x Buffer (NEB3)	1x
NdeI (NEB)	10 units
Nuclease free water	To bring up the final volume to 50 µl

Table 2.18: Linearisation of hPATs 2, 3 and 4 using NdeI

For linearising hPAT1 and its mutants/FLAG (table 2.19) variants the restriction enzyme SalI was used

Reagent	Final concentration
Template (hPAT in pGEM-T Easy)	5 µg
10x Buffer (NEB3)	1x
SalI (NEB)	10 units
BSA	100 µg/ml
Nuclease free water	To bring up the final volume to 50 µl

Table 2.19: Linearisation of hPAT1 using SalI

The digested template was then purified and eluted in 30 µl. 5 µl was run on the gel to confirm the linearisation.

2.9.2 Invitro transcription of cRNA

The synthesis of RNA was performed depending on the kit used

For the Ribomax kit, a 20 µl reaction was set up to contain components as described in the table 2.20

Component	Final amount	Volume
Template (+ nuclease free water)	1-5 µg	6.5 µl
Buffer	1 x	4 µl
RNTP mix (25mM CTP, UTP, ATP and 2 mM GTP)		6 µl
Cap Analog		1.5 µl
Enzyme		2 µl
	Total volume	20 µl

Table 2.20: Invitro synthesis of cRNA (Promega Ribomax T7 kit)

For the Ambion kit, a 20 µl reaction was set up as shown in table 2.21

Component	Final amount	Volume
Template	1 µg	6 µl
Buffer	1x	2 µl
NTP mix		10 µl
Enzyme		2 µl
	Total volume	20 µl

Table 2.21: Invitro synthesis of cRNA (Ambion T7 RNA synthesis kit)

In both cases the tubes were mixed and incubated for 4 hours at 37°C.

2.9.3 Dnase treatment and purification

After 4 hours, Dnase (5 for the Promega kit and 2 µl for the Ambion kit) was added to the mixture and incubated at 37°C for 15 minutes. After the incubation, the RNA was purified using the RNA clean up kit (Quiagen). The RNA was then eluted in 25 µl.

2.9.4 Confirmation of the size and quality of the RNA obtained

The concentration and quality of the RNA was determined using a spectrophotometer (NanoDrop 1000 Spectrophotometer) and was generally found to be around 1 µg/µl. A 260/280 ratio of 2.0 (+/-0.2) and a 260/230 ratio of 1.8-2.2 was considered acceptable. The integrity of the RNA was checked by running it on a 1% agarose gel for 20 mins at 80 volts and checking for distict bands which corresponded to the expected size. If a smear was observed then the RNA was discarded and fresh RNA synthesized.

C) Exogenous expression of transporters

2.10 The Xenopus laevis expression system

As discusses in the introduction, the Xenopus laevis oocyte system provides a very good model system to study transporters. Before preparing the oocytes for cRNA injection, Barth's and Calcium free solution were prepared prior to harvesting the eggs.

2.10.1 Solutions for preparation and maintenance of oocytes

2.10.1.1 Barth's Solution

This solution has a composition similar to that found in the extracellular fluid of Xenopus laevis and was used to store to oocyte at all stages except during collagenase treatment and during uptake experiments. The composition of this solution is shown in the table below (Table 2.22)

Compound	Concentration (mM)	g/litre
Sodium chloride (NaCl)	88	5.14
Potassium chloride (KCl)	1	0.07
Magnesium sulphate (MgSO4)	0.82	0.2
Calcium nitrate (CaNO3)	0.33	0.08
Sodium carbonate (NaHCO3)	2.4	0.2
Calcium chloride (CaCl2)	0.41	0.06
HEPES	15	3.57

After adjusting the pH of the solution to 7.6 using sterile 1 M NaOH, the following compounds are then added to the solution

Compound	Concentration (mM)	g/litre
Sodium pyruvate	5	0.55
Gentamycin	50 µg/ml	0.05

Table 2.22: Composition of Barth's solution. This solution is the medium used to store oocytes in

The solution was stored at 4 degrees. Before using the solution it was always filtered using a 10uM filter

2.10.1.2 Calcium free solution

This solution is used during the collagenase treatment of oocytes and while cleaning the ovaries after harvesting them (table 2.23). This solution contains

Compound	Concentration (mM)	g/litre
Sodium chloride (NaCl)	80	4.68
Potassium chloride (KCl)	2	0.15
Magnesium chloride (MgCl2)	1	0..095
HEPES	5	1.19

Table 2.23: Composition of the Calcium free solution

The pH of the solution was adjusted to 7.6 using sterile 1 M NaOH

The Ca^{2+} free solution was stored at 4 degrees. Before using the solution it was always filtered using a 10uM filter.

2.10.2 Preparation of the oocytes

Oocytes were harvested from the female *Xenopus* laevis. The frogs were anaesthetized in regulation with home office regulations. They were placed on ice for 30 mins following which an incision was made and the spinal cord broken. Following this an incision is made on the ventral region below the abdomen and the ovaries removed using a pair of foreceps. The follicles (containing the oocytes) were then placed in 50 ml petri dish containing Barth's solution. They were then washed to remove any debris or blood with Barth's solution. The oocytes were then ready to be collagenased.

2.10.3 Collagenase treatment of oocytes

The final stage in the treatment of oocytes prior to cRNA injection is to remove the follicular layer that envelops the oocytes. The removal of the follicular layer is essential to aide the cRNA injection and protein expression in oocytes. The oocytes were initially manually separated into groups of 30-50 oocyte using 2 pairs of foreceps under the light microscope (Leica). The oocytes were then transferred into a 50 ml Corning tube containing Ca^{2+} free solution and washed. The oocytes were then incubated in a 50 ml tube with Ca^{2+} free solution containing 1 mg/ml Collagenase (Sigma Aldrich) on a roller mixture. After 45 minutes, the Ca^{2+} free solution containing collagenase was discarded and replaced with fresh Ca^{2+} free solution containing 1 mg/ml Collagenase and incubaetd for a futher 45 minutes (total collagenase treatment 1.5 hours). Following this incubation, the oocytes were repeatedly washed with Ca^{2+} free solution till the solution until the supernatant poured off was clear. The oocytes were then repeatedly washed with Barth's solution and transferred to a 90 mm petri dish with Barth's for future use.

2.10.4 Selection of oocytes for microinjection

Defolliculated oocytes which appeared healthy and were blemish free were selected. Oocytes which were in the V^{th}-VI^{th} stage of development and were about 1 mm in diameter were chosen and placed in a 20 mm petri dishes containing Barth's solution and stored in the incubator at 17°C overnight.

2.10.5 Injection of oocytes

2.10.5.1 Preparation of needles

Fine glass needles were prepared using glass capillaries (Drummand Sciences, Biohit) that were pulled. The glass needles were observed under the light microscope (Leica) and the tip was manually cut using a pair of forceps. The needle was then back filled with mineral oil prior to attaching it to the semi automatic Drummond Nanoject injector and filling with cRNA.

2.10.5.2 Injecting cRNA into the oocytes

Any unhealthy oocytes were removed from the petri dish prior to injection. The oocytes ready to be injected were transferred into fresh 25 mm petri dishes. 25 oocytes were selected at a time and transferred onto Perspex slides with a groove. This helps facilitate injection rather than using a pair of forceps to hold the oocytes while injecting.

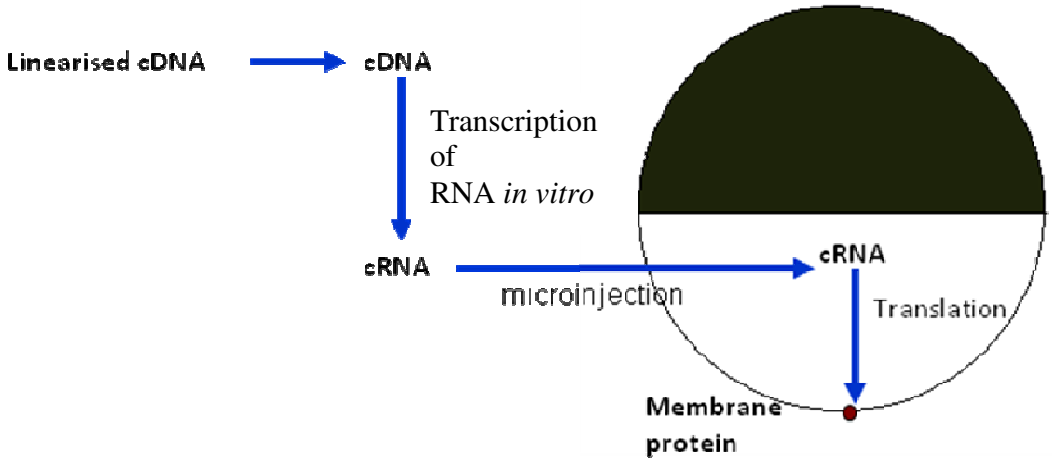

Figure 2.4: Diagrammatic representation showing the injection of cRNA into *Xenopus laevis* oocytes

cRNA was filled into the glass micro pipette needle attached to the Nanoject system. It is possible to arrange the range of the volume injected from 2-69 nl. The Nanoject needle was adjusted manually so that the glass capillary tip was very close to the slide and then adjusted with the controls so that the tip of the needle entered the vegetal

pole of the oocyte. A conscious effort was made not to enter the animal pole which contains the nucleus, which might be damaged with the needle. 27 ng of the cRNA was injected into the oocyte while adjusting the volume accordingly. Since water injected oocyes were observed to be similar to non injected ones, non injected oocytes were generally used as control throughout the thesis unless stated otherwise.

2.10.6 Incubation of oocytes

Post injection the oocytes were transferred into petri dishes containing Barth's solution. 2-3 hours post injection, the oocytes which were undamaged and had no blemishes were transferred into fresh petri plates (25 mm) containing fresh Barth's solution. The oocytes were stored in an incubator at 17 degree for at least 72 hours which is the time that it takes to bring about efficient translation of proteins and cell surface expression of transporters. The oocytes were transferred into fresh Barth's solution every day. In this period the oocyte translation machinery, translates the cRNA into proteins and trafficks it to the oocyte membrane.

D) Functional assay of transporters in Xenopus oocytes

2.11 Assays to characterize function of hPAT4

2.11.1 Basic transport assay

2.11.1.1 Preparation of uptake solution

Uptake assays were performed at three different pHs, 5.5, 7.4 and 8.4 were prepared as shown in table 2.22

Compound	Concentration
Choline chloride	100 mM
Potassium chloride	2 mM
Magnesium chloride	1 mM
Calcium chloride	1 mM
HEPES-Tris/ MES-Tris*	10 mM

Table 2.24: Uptake medium for transport assay

MES-Tris solution was adjusted to 5.5 and the HEPES-Tris solution was adjusted to pH 7.4 or 8.4. The pH of the solution was adjusted by adding 200 ul of this solution to a total 10 ml

2.11.1.2 Influx Assay

72 hours after injection the oocytes were tested for transport function. The oocytes each were incubated in uptake medium without substrate for 2 minutes prior to performing uptake experiments. After the preliminary 2 minute incubation 10 oocytes for each data point, 5 oocytes each were transferred into 100 µl/well of uptake medium each containing radiolabelled substrate [^{3}H] L-Proline unless specified otherwise. experimental details). The radio-labelledamino acids used were as follows (final concentration, µM): [^{3}H] L-Pro (0.10), [^{3}H] L-Trp (0.25), [^{3}H] L-Ala (0.11) and [^{14}C] L-Cys(50.0), [^{14}C] L-Ser (6.21) and [^{14}C] Gly (10.1). The specific activities of the amino acids were [^{3}H] L-Pro 25 Ci/mmol, [^{3}H] L-Trp 20 Ci/mmol, [^{3}H] L-Ala, [^{3}H] 47 Ci/mmol, L-Leu 69 Ci/mmol, [^{14}C] L-Cys 20 Ci/mmol, [^{14}C] L-Ser 164 Ci/mmol and [^{14}C] Gly 102 Ci/mmol. The [^{3}H] amino acids (L-Pro, L-Trp, L-Leu and L-Ala) were supplied at a concentration of 1µCi/µl. The [^{14}C] amino acids L-Cys, L-Ser and L-Gly were supplied at 50 µCi/µl, 51 µCi/µl and 52 µCi/µl respectively. The radioactivity was always supplied with >97% purity and in a 2:98 solution of ethanol:water. The uptake medium was at a pH of 5.5, 7.4 or 8.4 as per the requirements of the experiment. The oocytes were incubated in the uptake medium for 1 hour (with the exception of time course experiments).

For Na+ dependence experiments, the same uptake medium was used as described in table 2.22, except that the choline chloride was replaced with sodium chloride and the transport assay performed as usual.

2.11.1.3 Wash and measurement of uptake

Oocytes for each point were washed 5 times sequentially with cold NaCl (0.12 M) before transferring one oocyte into one of the wells of the 96 well scintillation plate. Any excess NaCl was removed before adding 50 µl 2 % SDS into each of the wells and placing it on a shaker for 1 hour or till no visible particles of the oocyte could be seen. After one hour incubation, 250 ul scintillant was added to each well. In addition to this 10 ul of each of the standards was added to the scintillation plates and 250 ul scintillant (Optiphase Scintillation cocktail, Perkin Elmer) was added to it. The plate was then shaken for at least an hour before counting the plate. Cpm readings were recorded.

2.11.2 Time course

For the time course experiments, the preliminary wash in the uptake medium without radiolabelled substrate was performed. This was followed by incubation of oocytes (5 oocytes in 100 ul) as described in the section on influx assays in the uptake medium at pH7.4. After the required time period the oocytes were washed, lysed with SDS and counted as described in section 2.3.1.3

2.11.3 Ki determination

2.11.3.1 Experimental setup

Ki determination experiments were set up using varing amounts of non radiolabelled substrate in the uptake medium with the same amount of [^{3}H] L-Proline to adjust the final concentration of the test substrate as required. Oocytes were incubated (10 oocytes per concentration) 5 in each well containing 100 ul uptake medium with the substrate at the required concentration and the radiolabelled tracer. Depending on the transporter and the substrate varying concentrations were used.

2.11.3.1 Calculation of Ki

Affinity calculation was calculated with Sigma Plot using the method of Deves and Boyd (1989). In all cases [^{3}H] L-Proline was used as a tracer. This means that even when L-Proline (unlabelled is used as a substrate) there is already some L-Proline (contributed by the tracer), present in the medium making it an apparent Km as opposed to a true Km. In case of other substrates, we are measuring the inhibition of L-Proline transport due to competitive inhibition. Once again in this case there is a component of the recorded transport which is contributed by L-Proline while the majority is the effect of the unlabelled excess.

2.11.4 Electrophysiology

Oocytes were collagenased and injected in the same way as for the transport assays. 72 hours post injection the oocytes were then used for two electrode voltage clamp experiments. Barth's solution without any substrate was used as a perfusion solution and Barth's solution with substrates was used to test the oocytes. Different concentrations of substrates were used to test whether 1) the transporter was electrogenic, as in the case of hPAT4 and 2) to test whether a compound is a substrate for the electrogenic transporter hPAT1.

The needles for electrophysiology were prepared by a two stage electrode puller. To perform the two voltage electrode clamp, electrodes were prepared by back-filling (approximately 5 mm from the top) with a 1 mg/ml solution of agarose in 3 M KCl solution. Prior to use the needles were then filled with 3 M KCl and electrodes with a resistance between 0.5 to 1 MΩ were used. The oocytes were placed in a 0.1 ml chamber and were then impaled by the two electrodes. Oocytes were clamped at -60 mV (Geneclamp 500B amplifier was used to measure the recordings). Continuous perfusion was performed at a rate of 10 ml/min. The different solutions were selected manually. Data were recorded on a paper chart and the numbers were noted manually.

E) Surface expression in *Xenopus* oocytes

2.12 Luminometry to determine the topology of the membrane protein

As discussed in the introduction, epitope tagging is a technique by which FLAG tags can be inserted into different regions of the gene. The process relies on chemiluminescence to help determine the topology of the membrane protein.

2.12.1 Solutions required for the luminometry analysis

2.12.1.1 Barth's-BSA

Barth's-BSA solution was prepared to contain 1% w/v of BSA in a Barth's solution and placed on the roller to dissolve. The solution was then filtered through a 0.1μM filter and stored at 4 degrees till required.

2.12.1.2 Primary Antibody

The primary antibody (Anti-FLAG M2 monoclonal Antibody-HorseRadish Peroxidase conjugate, C-terminus HRP Sigma Aldrich) was prepared by making a 1:500 solution of using Barth's-BSA.

2.12.1.3 Secondary Antibody

High sensitivity ELISA femto substrate conjugated to the HRP (SuperSignal ELISA Femto substrate) was used for detection of the primary antibody.

2.12.2 Methodology

All the steps leading upto the final measurement were performed on 24 well plates which were placed on ice using chilled solutions of Barth's-BSA, Barth's and primary antibody.

2.12.2.1 Blocking of the oocytes

At least 12 oocytes per data point were incubated in 400 ul Barth's-BSA for 30

minutes.

2.12.2.2 Incubation of the oocytes in primary antibody

The oocytes were incubated in 300 ul of the primary antibody (1:500 v/v with Barth's-BSA as described earlier) for 1 hour.

2.12.2.3 Washes with Barth's-BSA and Barth's

The oocytes were washes sequentially with 400 ul Barth's-BSA six times for 1 and a half hours (once every 15 minutes). The oocytes were then transferred to Barth's solution (400 ul) and sequentially washed 5 times for 30 minutes (once every 3 minutes).

2.12.2.4 Preparation of luminometry plates

100 ul of filtered Barth's is added to each well of the Luminometry plate (black and white isoplate-96, Perkin Elmer). The oocytes which were washed were ready for use. One oocyte was placed into each well of the plate. Equal volumes of the two solutions (ELISA high sensitivity HRP-conjugated substrate) were added to a 15 ml Corning tube wrapped in aluminium foil (since the substrate is light sensitive). The plate was then placed into a rigid plate holder which fits into the plate counter. Just prior to use 20 ul of the substrate was added into each well rapidly while covering the wells into which substrate had been added by constantly covering it with a opaque cover to prevent light from initiating the reaction. The plates were then manually shaken and placed in the counter ready for counting.

In order to eliminate variation in techniques as much as possible, the luminometry readings were started within 10 minutes after the ELISA substrate was added to the first well. If more time was taken, the counts were not performed. Luminometry readings for the internal and external FLAGs were done at the same time as much as possible. The controls used were non injected oocytes and hPAT1 (without a FLAG epitope) injected oocytes to confirm that the FLAG antibody was binding only to the epitope and not to an endogenous protein of the oocyte or the nonFLAGed hPAT1

protein. There were no tagged hPAT available so a tagged PepT1 was used as a positive control in the initial experiment to ensure that the technique worked.

F) Cell culture

2.13 TM4 cell culture

2.13.1 Growth and maintenance of TM4 cells

TM4 cells were grown in 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium with 1.2g/litre sodium bicarbonate, 15 mM HEPES, 5 % horse serum (HS), fetal bovine serum (FBS) and 2 mM glutamine. Penicillin-streptomcin was used as an antibiotic.

The flasks were kept at 37 ºC in an incubator with 95 % air and 5 % CO_2.

When the cells had reached confluence they were subultured. The medium was removed and the cells were washed using 3 ml PBS and the PBS removed. 3 ml 1x trypsin was then added to the flask and placed in the incubator for 5 minutes. Fresh medium was then added and the cells resuspended to avoid any clumping. Subculturing was carried out in a ratio of 1:25.

2.13.2 Endogenous expression of mPATs

2.13.2.1 Primers

For endogenous expression of mPATs the primers used are shown in table 2.25. With the exception of GAPDH, the mPATs were exon-exon spanning

Primer name	Sequence
mPAT1 Forward	GTCCCGAGTGCCTGAGC
mPAT1 Reverse	CTCTACATGCATAACAAGTGGTTTG
mPAT2 Forward	CAACTGCTGGCTGTACCAATC
mPAT2 Reverse	CACTGAATAAACATGGTGGAGTTG
mPAT3 Forward	GCCTGACCTGTTTCTCAGCC
mPAT3 Reverse	CCATGTAAAAGTGGGTCTGC
mPAT4 Forward	GGACATGTGGTTGTATCAGTCAGTG
mPAT4 Reverse	CAAACCATTGTTATGCATGTAGAG
mGAPDH Forward	AGGCCGGTGCTGAGTATGTC
mGAPDH Reverse	TGCCAGTGAGCTTCCCGTTCA

Table 2.25: Primers used to check for the expression of mPATs

2.13.2.2 Cell culture preparation

To check for the endogenous expression of mPATs in the mouse Sertoli cell line TM4 the principle of reverse transcriptase PCR was used. Two days after the passage when the TM4 cells showed about 90% confluence, the cells were washed with PBS and harvested using 2ml trypsin. 10 ml DMEM:F12 medium was then added to the flask, the cells mixed by pipetting and transferred to a 15 ml tube. 0.1 ml of the cell suspension was taken and kept aside to count the number of cells. The cells were then spun at 1000 rpm for 10 mins at 4 ºC. The supernatant was discarded and the cells resuspended in 2 ml PBS and transferred to a 1.5 ml tube. The cells were spun again and the supernatant discarded. Total RNA was then extracted from the pellet (Quiagen RNA clean up kit).

2.13.2.3 cDNA synthesis

cDNA was synthesized using the RNA which was collected from the cells. The RNA was treated with Dnase for 30 mins at 37 ºC. Reverse transcriptase reaction for cDNA synthesis was set up using the GoScript Reverse Transcription system (Promega) as shown in table 2.26.

Component	Volume (sample)
RNA from cells	8 µl
Primer oligo (dT)15	2 µl
Final volume	10 µl

Table 2.26: Components for cDNA synthesis in TM4 cells

The tubes were then placed in a pre heated heat block at 70 ºC for 5 minutes. The tubes were then immediately placed on ice until ready to be used for the reverse transcriptase reaction.

A mastermix was prepared to allow 14 µl of the reaction mix for each the control and the sample. The reverse transcription reaction mixture was prepared to contain

Component	Volume
5X GoScript reaction buffer	4 µl
$MgCl_2$ (25 mM)	2 µl
PCR nucleotide mix	1 µl
H2O	7 µl To bring the final volume to 14 µl

Table 2.27: Reverse transcription reaction set up (TM4 cells)

The reaction buffer contained RNAse inhibitors thereby eliminating the need to add them separately. The cRNA was aliquoted into two tubes containing 5 µl each, labelled sample and the other control. 14 µl of the reaction mixture was added to both the control and sample reaction tubes which were placed on ice and mixed gently. 1 µl of reverse transcriptase was then added to the sample and 1 µl of nuclease free water was added to the control reaction and the contents of the tube were mixed by pipetting. The tubes were then incubated on a heating block for 25 minutes for 5 minutes. The reaction tubes were then incubated at 42 ºC for 60 minutes. The reverse transcriptase was inactivated by heating at 70 ºC for 15 minutes. The concentration of the DNA (single stranded) was measured and stored at -20 ºC or used directly for the PCR.

2.13.2.4 PCR amplification of mPATs and controls

PCR amplification reactions were set up using two sets of primers, one set designed specifically for mPATs and another set designed of GAPDH as shown in table 2.28.

Reagent	Amount
Go Taq Flexi buffer	5 µl
Forward primer (10 µM)	1.25 µl
Reverse primer (10 µM)	1.25 µl
dNTPs (25 mM)	1 µl
Template (100ng)	1 µl
H2O	To bring up the volume to 50 µl
Enzyme	0.5 µl

Table 2.28: PCR set up to check for endogenous expression of mPATs in TM4 cells

Thermal cycling was set up using initial denaturing at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1.5 minutes. A final extension step at 72°C was performed for 5 minutes before storing at 4°C.

2.14 HEK cell culture – growth and maintenance

HEK cells were grown in Dulbecco's modified Eagle's medium with 1g/litre glucose and sodium bicarbonate, pyridoxine, 5 % fetal bovine serum (FBS) and 2 mM glutamine. Penicillin-streptomycin was used as an antibiotic.

The flasks were kept at 37 ºC in an incubator with 95 % air and 5 % CO_2.

When the cells had reached confluence they were subcultured. The media was removed and the cells were washed using 3 ml PBS and the PBS removed. 1 ml 1x trypsin was then added to the flask and placed in the incubator for 5 minutes. Fresh medium was then added and the cells resuspended to avoid any clumping. Subculturing was carried out in a ratio of 1:10.

G) Transfections and transport studies in cell culture

2.15 Transfections using TM4/HEK293 Cells

TM4/HEK293 cells which had reached confluence were trypsinised, resuspended and then resuspended in medium. The cells were then spun for 5 mins at 1200 rpm and the supernatant removed. Fresh antibiotic free medium was then added to the pellet and resuspended. 50 µl of the cell suspension was mixed with 50 µl trypan blue and loaded onto a haemocytometer.

The cells were counted and the cells plated at the appropriate density. For 6 well plates, 5 x 10^5 cells were plated per well in 2 ml medium. The cells were placed in the incubator overnight. By the next day the cells had reached about 70 – 80 % confluence.

The medium was removed and 1.5 ml OPTIMEM (Invitrogen) was added to each well. 2.25 µg plasmid DNA (1.125 µg E-GFP C and 1.125 µg pcDNA3.1(-)/ hPAT2/ hPAT3.1/ hPAT3.2) was added 250 µl OPTIMEM per reaction. 6 µl Lipofectamine LTX was added to 250 µl OPTIMEM per reaction. Both these (OPTIMEM-Ltx and OPTIMEM-DNA) were incubated for 5 min and then mixed and left at room temperature for 30 min.

500 µl of the mixture was then added to each well. The plates were rocked from side to side a few times to mix the medium and placed in the incubatr.

The following day, the OPTIMEM was removed and fresh DMEM:F12 was added to the wells. Confocal analysis were done 48 hours after transfection and transport assays were performed

2.15 Transport assays in cell culture

Krebs Ringer solutions at different pH were prepared to study the hPATs in cell lines. Three different pH were used 5.5, 7.4 and 8.4. The medium used for studying transport assays is described in table 2.27

Component	Final concentration (mM)	g/litre
NaCl	137	8.01
KCl	5.4	0.40
CaCl2	2.8	0.41
MgSO4	1.0	0.25
NaH2PO4	0.3	0.04
KH2PO4	0.3	0.04
Glucose	10	1.8
HEPES-Tris (each 0.5 M adjusted to a final pH of 7.4 or 8.4)	10	20 ml
MES-Tris (each 0.5 M adjusted to a final pH of 5.5)	10	20 ml

Table 2.39: Composition of the Krebs Ringers solution

The cell culture medium was aspirated from the wells. The cells were then washed with warm Kreb's solution at the appropriate pH using 2 ml solution per well. The solution was then aspitated and this process repeated again. After the wash solution had been removed completely, the incubation mixture (containing [^{3}H] L-Proline) was added to each well. The proline tracer was diluted 5 µl/ml of medium

H) Data analysis

2.16 Software used

The programs which were used to help with the statistical analysis were Excel, GraphPad and SigmaPlot. Excel was used to tabulate the data from different experiments. GraphPad Prism 5 was used to analyse the data and determine if it was significant (student t-test, paired/unpaired $p < 0.05$). SigmaPlot was use to analyse substrate affinity experiments and to plot graphs.

2.17 Statistical analysis

2.17.1 Transport and luminometry assays

The data was tested for outliers and removed by using the Grubbs' test for detection of outliers (GraphPad Software, Quick calcs online). For each experiment the values were calculated as mean +/- SEM.

2.17.2 fmol/oocyte/hour calculations

Students unpaired t-test with $p < 0.05$ was used to determine significance except in the case of cell culture experiments where a one sample t-test was used (with $p < 0.05$).

In each case, the uptake for the non injected oocytes were subtracted from the mean values, to give the hPAT mediated amino acid transport. The values are then expressed in terms of fmol/oocyte/ hour or as normalized data.

Normalised data was presented after subtracting the non injected from the injected oocytes and presented as a fraction of a control transporter (for instance SLC36A2 which has been characterised as a transporter). The sample to which it was normalized is specified in the figure legend/text. The rates of uptake for these oocytes are also mentioned.

2.17.3 Ki measurements

Ki measurements were done using SigmaPlot. The equations used were as described by Deves and Boyd (1989).

This equation is a derivative from the Michaelis-Menten equation which was discussed earlier ,

$$v_1 = \frac{V_{max}[S]}{\{K_m + [S]\}}$$

The Deves and Boyd method was initially used to help determine the kinetic parameters for carrier protein mediated transport in placental membrane vesicles.

This was done by using an excess of unlabelled substrate (or a substrate analog) which would competitively bind to the transporter thereby decreasing the amount of labelled transporter that can bind to it.

The schematic representation of the interaction between the different factors can be summaried as shown below

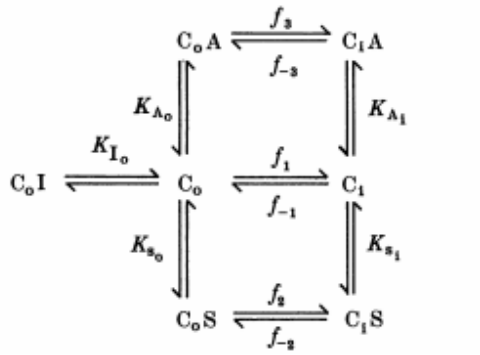

Figure 2.5: Transport scheme for a substrate and an analog with a competitive inhibitor. S represents the substrate, A the analog, I the competitive inhibitor, C_I and C_O are the inward and outward facing conformations which can bind to ligands in the medium depending on the dissociation constants K and rate constant f. (Deves and Boyd, 1989).

The final calculations for substrate affinity calculations were made using SigmaPlot, by plotting normalised uptake and the corresponding errors against the substrate concentrations and using a curve to fit in the data. Following this the Ki or apparent Km could then be obtained.

Chapter 3
Molecular Biology

3.1 Subcloning of hPATs into the pXT7 vector

For checking heterologous protein expression in *Xenopus* oocytes the genes of interest first needed to be introduced into the oocyte expression vector, pXT7. Similarly for transfecting the genes of interest into cell lines, they were first subcloned into the mammalian expression vector pcDNA3.1(-). In this section the different results obtained during each stage will be discussed. As discussed

3.1.1 PCR amplification of the hPATs to introduce KpnI and EagI sites

The hPAT genes were amplified using specific primers (materials and methods) to introduce KpnI and EagI at the 5' and 3' ends respectively. The choice of restriction sites was based on the presence of both these sites in the pXT7 vector and its absence in the cloning vector pGEM-T Easy. In addition to these both these sites were absent from hPATs 2, 3 and 4 which was ideal. There was however a KpnI site within the gene however this problem was solved using partial digests. Figure 3.1 shows the PCR products obtained by amplification of the hPAT genes. PCRs were set up in the presence of Easy-A polymerase which has proof-reading activity in addition to adding A overhangs at the end of the PCR product.

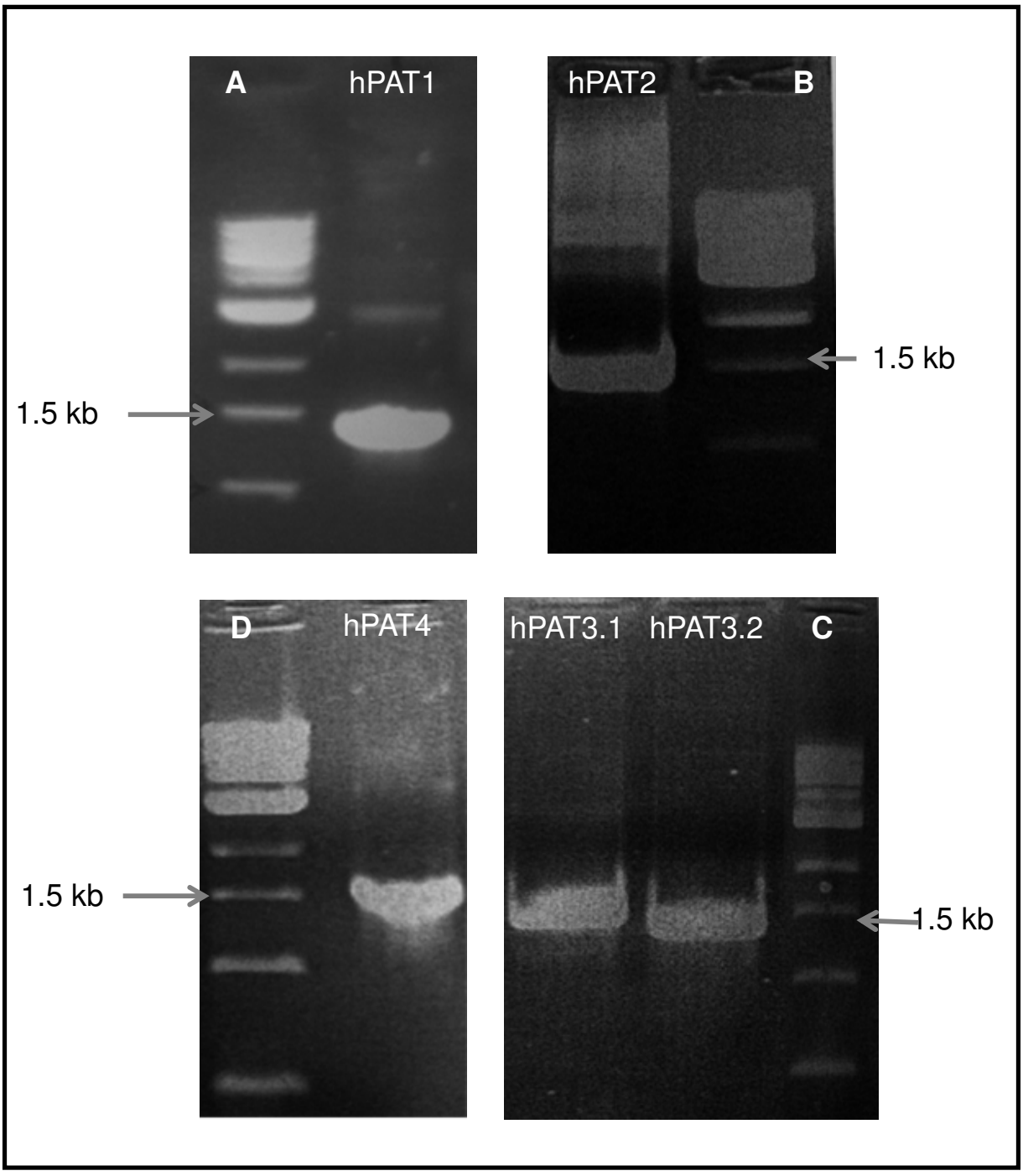

Figure 3.1: PCR amplification of hPATs1,2,3.1,3.2 and 4. Panels A, B, C and D show the products obtained by PCR amplification of hPATs 1, 2, 3 and 4 respectively using primers with 5' KpnI and 3' EagI sites. In each image 1.5 kb is marked on the DNA ladder (marker).

3.1.2 Ligation of the hPATs with pGEM-T Easy

Ligation of the PCR product with A-overhangs was performed with pGEM-T easy which has complementary T overhangs thereby facilitating ligation. Positive clones which were selected by blue white screening, restriction digests and sequencing were then grown up as stock for the next stage of introducing the genes into the pXT7 vector.

3.1.3 Restriction digests of hPATs

The hPATs were sequentially digested with PCR purification after each digestion step (materials and methods) and run on the gel to confirm complete digest (partial digest in case of hPAT1) had taken place. Figure 3.2 shows the restriction digests and an indication of the fragments which were cut out, purified and used to set up ligation reactions.

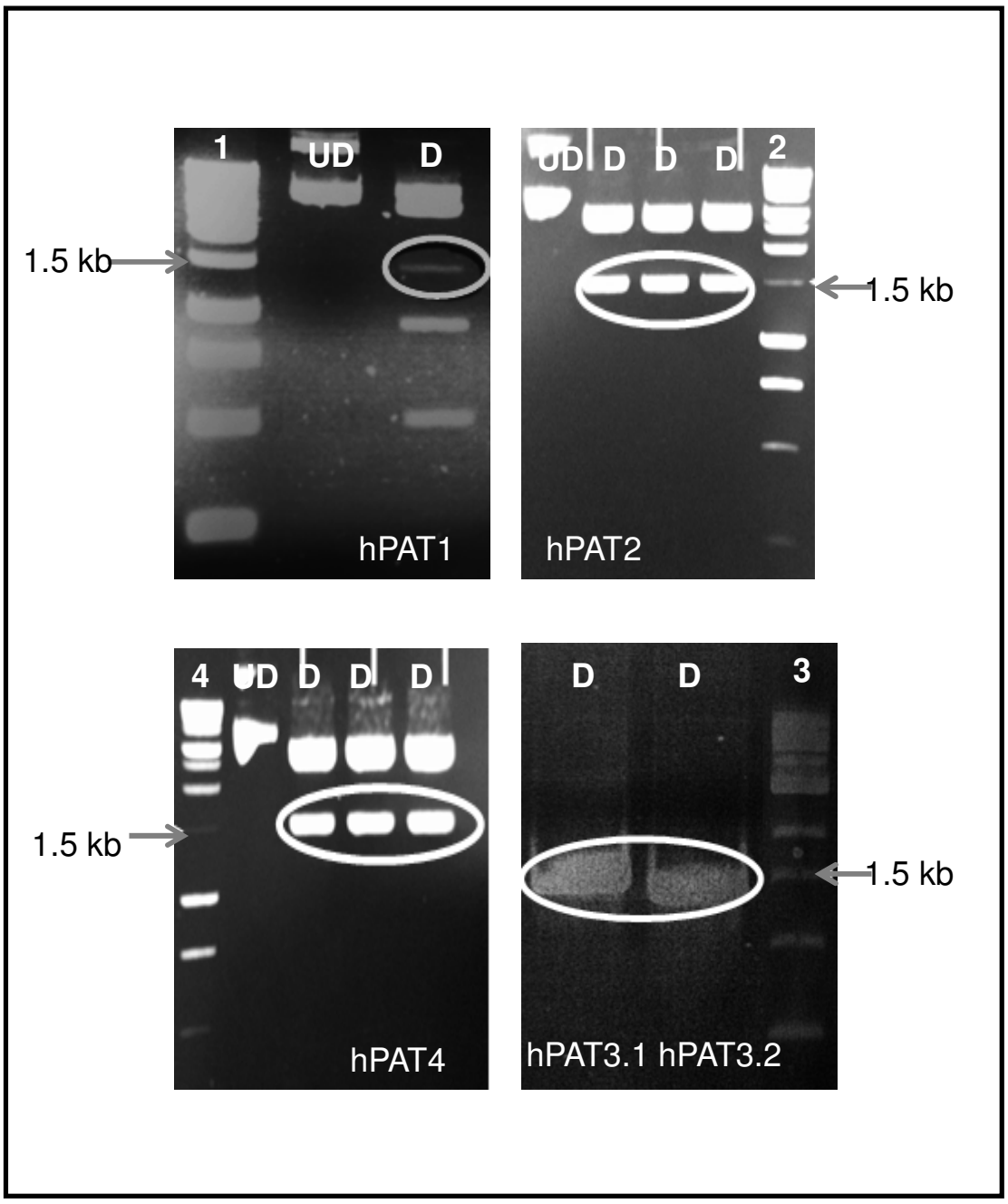

Figure 3.2: Restriction digestion of hPATs with KpnI and EagI. Panels 1, 2, 3 and 4 show the products obtained by performing restriction digestion of hPATs 1, 2, 3 and 4 respectively with KpnI and EagI. For panels 1, 2 and 4 both the digested (D) and undigested template (UD) are shown. In panel 3 only the digestion products are shown. In each image 1.5 kb is marked on the DNA ladder (marker).

3.1.4 Ligation of hPATs with pXT7 and confirmation of insert

Ligation reactions were set up using vector (pXT7) and insert (hPATs) as described in the materials and methods. The ligation products were transformed, plated on LB agar and eventually grown on LB broth and plasmid DNA extracted. To confirm the presence of the insert hPATs 2,3 and 4 were digested with NdeI and hPAT1 was digested with SalI. Colonies which appeared positive were then sent for sequencing. The results were analyzed to check for the genes and to confirm that the orientation was correct i.e. T7 5→3'. This was to facilitate T7 RNA synthesis since that was the only usable one in pXT7.

3.2 Introduction of FLAG epitope tags into the hPAT1 gene

The FLAG tag was introduced into different regions of the hPAT1 gene and will be discussed in detail in chapter 5. Eleven epitope tags were designed and inserted using site directed mutagenesis. F1, F2, F3, F4, F5 and F6 were the presumptive external FLAGs inserted while iF1, iF2, iF3, iF5 and iF6 were the presumptive internal FLAGs. The colonies obtained by transforming DpnI treated ligation products were incubated in LB (+Amp) overnight and plasmid DNA extracted from them. The inserts were confirmed by restriction digest and sequencing.

3.3 Introduction of the champagne mutation in the hPAT1 gene and the FLAG labeled hPAT1 gene

The G to C champagne mutation which is present in the SLC36A1 gene causes a change in the amino acid being coded from Threonine to Arginine. This mutation has been shown to be responsible for champagne coat colour in horses. This mutation was introduced into two variants of the hPAT1 gene; the wildtype hPAT1 gene and the hPAT1 gene with an external FLAG epitope tag. The reason for doing this was to

check that if the champagne mutation caused a loss of function mutation then the expression of the gene could be confirmed using luminometry techniques.

3.4 Invitro cRNA synthesis and confirmation

cRNA was synthesized invitro as described in the introduction. Following the 3-4 hour incubation and the RNA clean up, the concentration of RNA was measured. To confirm that the RNA was intact the RNA was then run on a gel at low voltage. Figure 3.3 shows an example of a typical gel which is run following RNA synthesis. Uncut plasmid DNA, linearised DNA and cRNA are run on the gel for 30 mins on a 1% agarose gel (TBE). The bands observed for the RNA are single standed RNA (which appears to be half the size of the ladder) and complex bound RNA. Since the marker used is for double stranded DNA therefore cRNA which is about 1.5 kb will typically be present close to 750 kb; the bound double stranded one would be 1.5 kb.

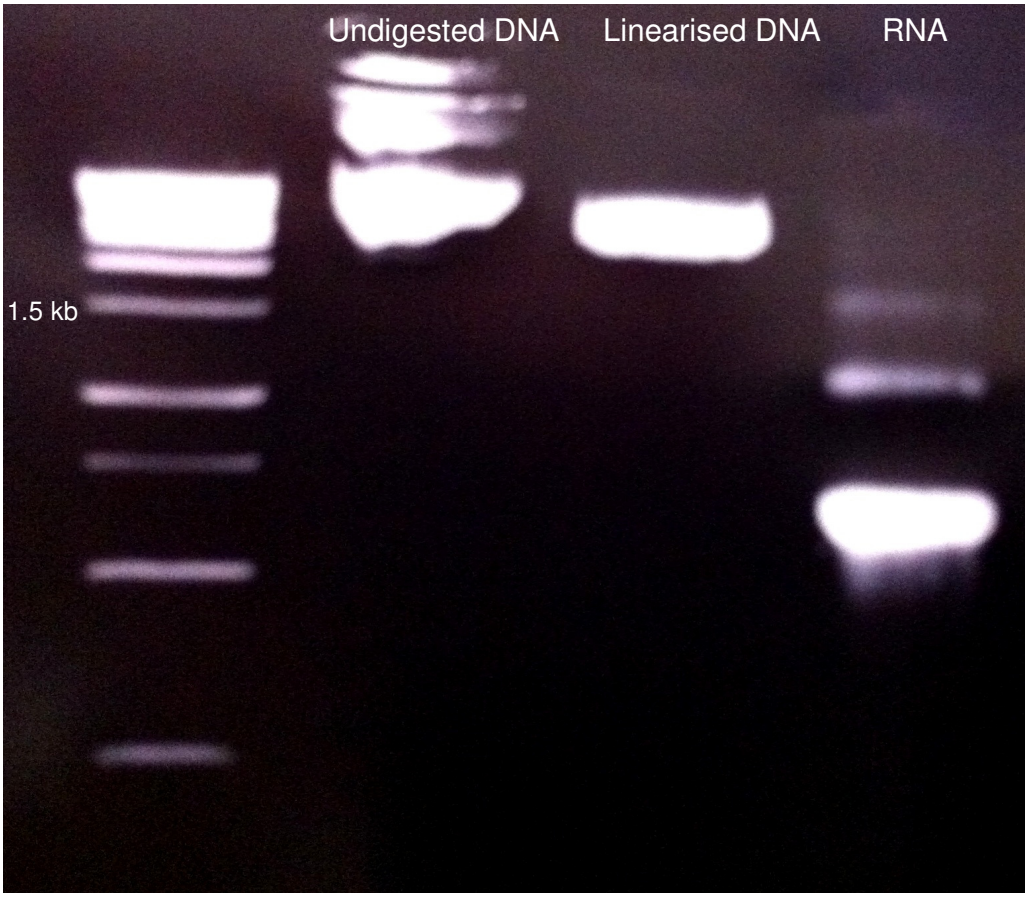

Figure 3.3: An example of a RNA gel. The undigested (circular plasmid), linearised plasmid and cRNA which has been synthesized in vitro are shown. The cRNA synthesized is smaller than the size of the gene (~1.5 kb) on account of it being single stranded.

3.5 Subcloning of hPATs into the pcDNA3.1(-) vector

For cell culture purposes, it was essential to subclone the hPATs into a mammalian expression vector. The vector used for this purpose was pcDNA3.1(-) which has a CMV promoter.

3.5.1 PCR amplification of the hPATs to introduce KpnI sites at both the 5' and 3' ends

hPATs 2, 3.1 and 3.2 were subcloned into the mammalian expression vector for cell culture experiments. hPATs were PCR amplified using specific primers with both 5' and 3' KpnI sites. The choice of the KpnI site was based on the fact that there was very little choice of restriction sites which were present in the vector multiple cloning site but absent from the gene. Figure3.4 shows the products obtained by PCR amplification of the hPATs when run on a 1% agarose gel.

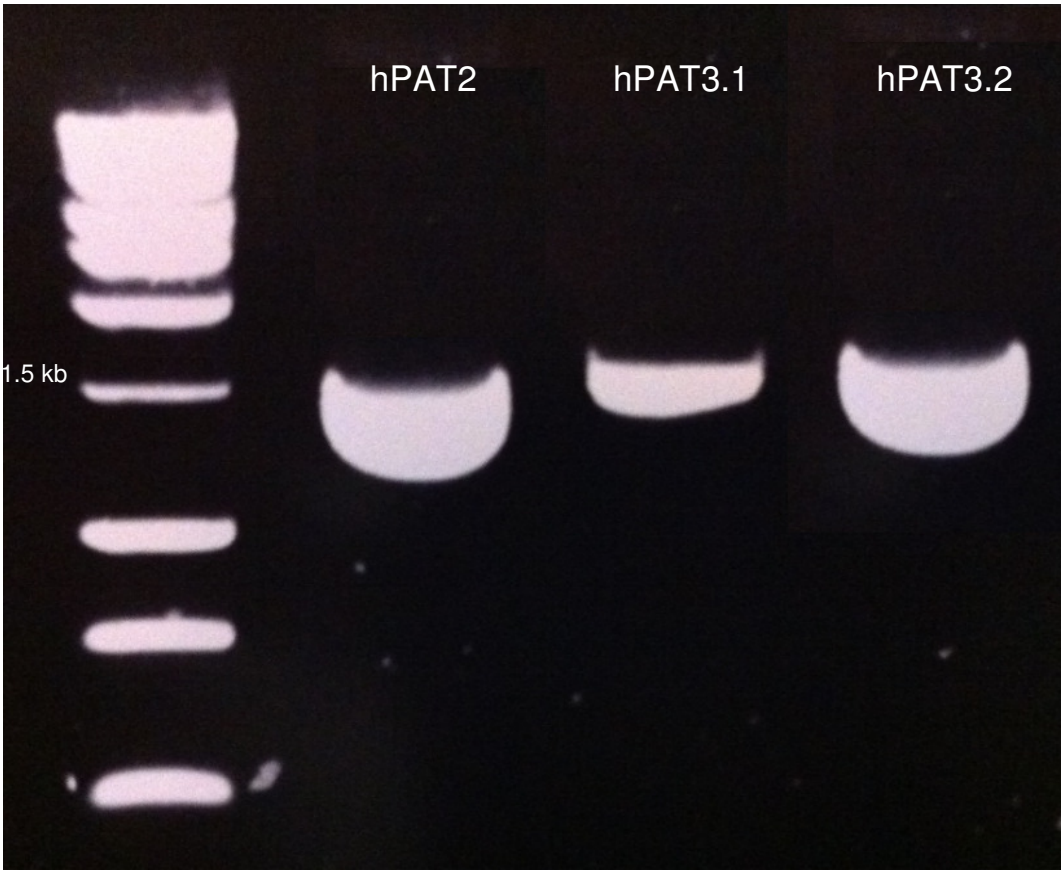

Figure 3.4: Products of PCR amplification of hPAT2, hPAT3.1 and hPAT3.2 using primers containing KpnI sites.

3.5.2 Ligation of the hPATs with pGEM-T Easy

The PCR products were purified and ligated into the pGEM-T Easy vector. The colonies which were selected using blue white screening were then digested using KpnI to check for the presence of the insert. Sequencing was then carried out to confirm the sequence and to ensure that no mutations were introduced.

3.5.3 Restriction digests of hPATs and pcDNA3.1(-)

Plasmids which confirmed to contain the hPAT genes in pGEM-T Easy vector were used as stock to set up restriction digests with KpnI. Figure 3.5 shows the products obtained by digestion of hPATs and pcDNA3.1(-). These products were then gel extracted to use for ligation reactions.

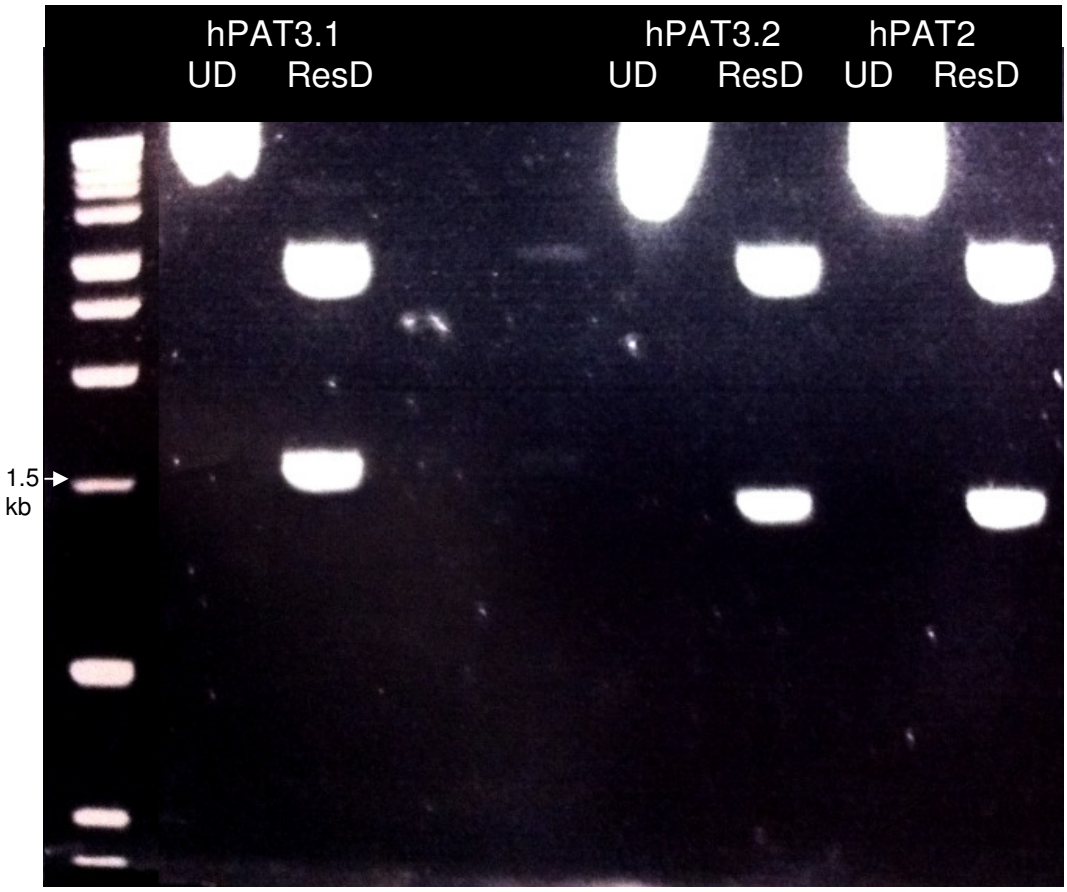

Figure 3.5: Restriction digestion of hPAT2, hPAT3.1 and hPAT3.2 with KpnI

3.5.4 Ligation of hPATs with pcDNA3.1(-) and confirmation of insert

Ligation reactions were set up with alkaline phosphatase treated vector and hPATs. The ligation products were transformed, plated on LB agar and eventually grown on LB broth and plasmid DNA extracted. To confirm the presence of the hPATs restriction digests were set up using KpnI. Plasmids which appeared to have the insert were then confirmed using sequencing. The orientation of the insert was also required to be 5'→3' in the direction of the T7 promoter (which is also the same orientation as the CMV promoter).

3.6 Chapter summary

- 5' → 3' KpnI and EagI sites were introduced into hPATs 1, 2, 3.1, 3.2 and 4 which were then subcloned into the cloning vector pGEM-T.
- Sequential restriction digestion was then carried out and the products purified before introducing it into the restriction digested pXT7 oocyte expression vector.
- The sequence was then confirmed by restriction digestion and sequencing.
- 5' → 3' KpnI sites were introduced into hPATs 2, 3.1 and 3.2 which were then subcloned into the cloning vector pGEM-T
- KpnI digestion was then performed on the genes in pGEM-T and the mammalian expression vector pcDNA3.1(-) and purified.
- The genes were then ligated to pcDNA3.1(-).
- To confirm that the orientation of the genes was in the direction of the CMV promoter, sequencing reaction were performed for confirmation.

Chapter 4:
Functional Characterization of hPAT4

This chapter deals with the characterization of the orphan transporter hPAT4. The data are generally expressed in two ways either in fmol/oocyte/hour or as normalized values i.e. a fraction of the control (in this case the value for 1, is mentioned in fmol/oocyte/hour). In each case they have been corrected for the endogenous uptake (non injected) values. The fmol/oocte/hour data for different experiments are observed to show variability on account of variations in the oocytes observed seasonally. The results were calculated for each experiment by comparing it to the non injected oocytes from the same set. This practice also extends to normalised data where each experiment was normalised v/s the non injected and the means were then calculated for n number of experiments to overcome any variations due to seasonal changes.

4.1 hPAT2 as a positive control

As discussed in the introduction, hPAT4 is a member of the Proton coupled Amino acid Transporter family which had not been previously characterized but was placed into the PAT family based on sequence homology to the other members of the family. Before establishing an assay to test for the functionality of hPAT4 it was necessary to have suitable controls. Since both hPAT1 and hPAT2 had been well established to be amino acid transporters at an acidic pH, either of these could then be used as a control for hPAT4 experiments

When hPAT2 and hPAT4 was expressed in the oocyte system without subcloning it into the pXT7 vector no significant uptake was observed compared to non injected oocytes (fig4.1).

After subcloning into oocyte expression vectors, hPAT2 showed a significantly higher uptake of [^{3}H] L-Proline compared to the non injected oocytes (described in detail in section 4.2).

Similar results were also obtained when uptake assays were performed using hPAT1, therefore either of these could be used as a control to assist in the characterization of hPAT4.

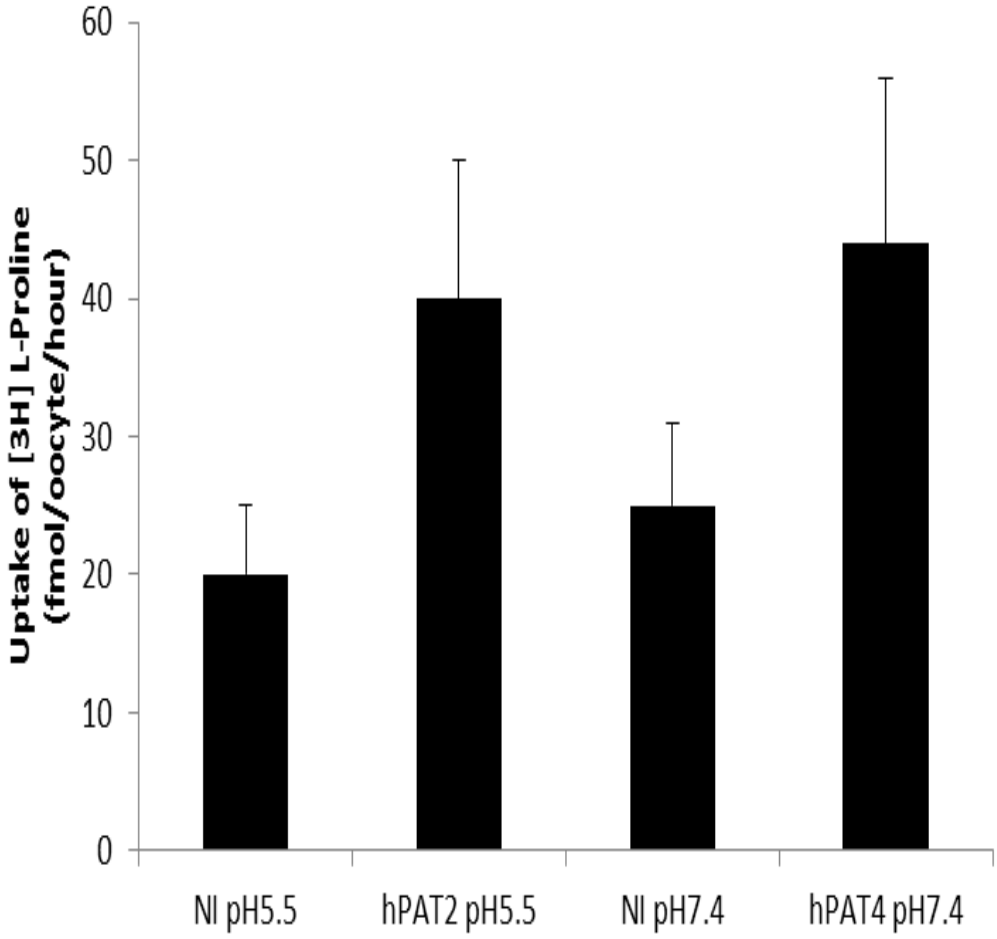

Figure 4.1: pH dependence of hPAT2 and hPAT4 when expressed in the bacterial expression vector. The graph shows that there was no significant uptake for the hPAT2 positive control at pH5.5 or the hPAT4 test sample at pH 7.4 ($p>0.05$). This was perhaps due to the fact that the vector was not an oocyte expression system. Proline present in the radiolabelled tracer which is 0.25 μM.

4.2 pH dependence

It had been shown by previous groups that both hPAT1 and hPAT2 are pH dependent transporters that showed an increase in transport activity when the pH is decreased to 5.5. Similar results were obtained when the experiments were repeated during this project.

Since the SLC36 family of transporters was presumed to be proton dependent with a maximal transport activity at pH 5.5, the first step was to check for transport function of hPAT4 at this pH. hPAT2 was used as a control for these experiments.

[^{3}H] L-Proline was used as the tracer and uptake assay was performed as described in the material and methods. hPAT4 showed no significant uptake at pH5.5 whereas the hPAT2 control showed significant uptake as expected (fig4.2).

Since hPAT4 did not show any [^{3}H] L-Proline uptake at pH 5.5 it was decided to try and check for the uptake at a higher pH. When the pH of the uptake medium was increased to 7.4 hPAT4 showed a significant uptake compared to the non injected.

However, the transport activity was found to decrease when the pH was increased further to pH 8.4 (figure 4.2).

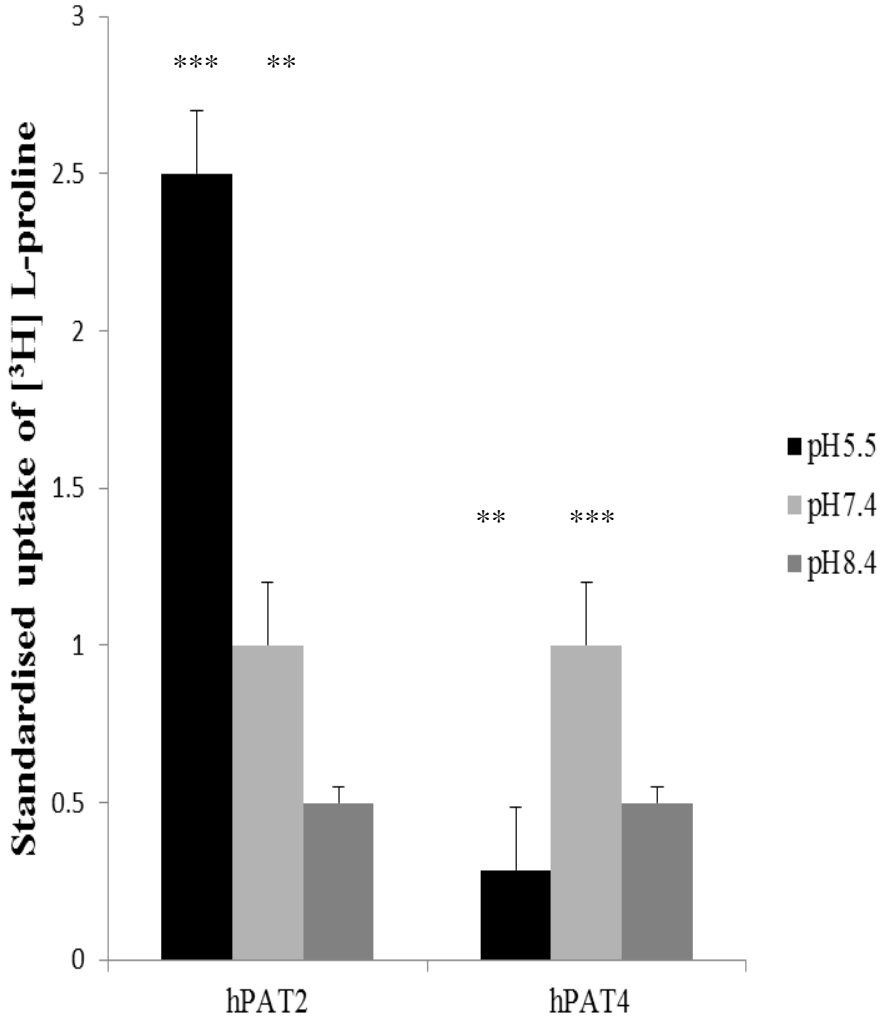

Figure 4.2: Dependence of hPAT4-mediated [^{3}H] L-proline uptake on extracellular pH in *Xenopus* oocytes expressing either hPAT2 or hPAT4. Data are standardised to uptake at extracellular pH of 7.4, which for hPAT2 was 10.96 +/- 2.04 and for hPAT4 was 14.91 +/- 2.14 fmoles/oocyte/hour, n≥3 oocyte preparations. The significance shown is that when compared to the non injected oocytes of the same data set (** $p < 0.01$, *** $p < 0.001$). Concentration of L-Proline in the tracer 0.25 µM.

4.3 Time course

Rates of transport vary from one transporter to another. While uptake experiments for hPAT1 and 2 in *Xenopus* oocytes was traditionally done for an hour, it was possible that hPAT4 reaches saturation by this time (especially if it were electrogenic). Time course experiments were set up in uptake medium at pH7.4. The results obtained are shown in figure 4.3.

All transporters go through an incease in uptake followed by a plateau. The fraction of the substrate is taken up i.e. concentration of substrate in the extracellular medium v/s intracellular can vary depending on the type of transporter. For a transporter that relies only on facilitated diffusion, the plateau that is reached is at equilibrium where the external and internal concentrations of the substrate are the same. On the other hand, for a powered transporter, the stage at which saturation is reached is when there is a much higher amount of substrate which has been moved across the membrane so that intracellular substrate>>extracellular substrate.

For instance : If the standard values are 50000 cpm/10 µl (which is the same amount used in the uptake medium), then approximately 6 hours after incubation the corrected uptake values (after the non injected values were subtracted from the injected ones) for the transporter which has facililated diffusion would be about 5000 cpm. This is based on the assumption that the oocyte has a diameter of 1.2 mm. If the transport was driven then the count would increase above 5000 cpm and eventually plateau.

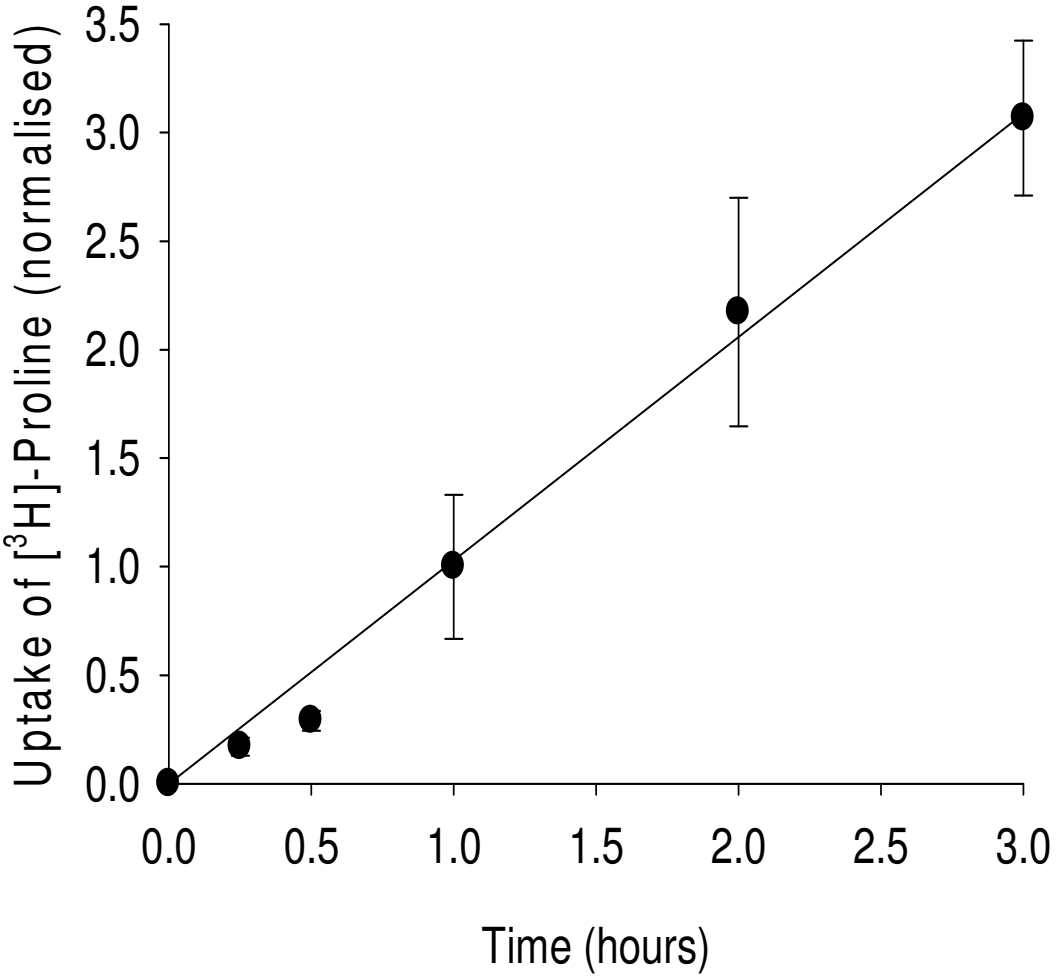

Figure 4.3 : Time dependence of hPAT4-mediated [^{3}H] L-proline uptake into *Xenopus* oocytes. Data are standardised to uptake at 1 hour, which was 2.96 ± 0.98 fmoles/oocyte/hour, n=4 oocyte preparations. Concentration of L-Proline in the tracer 0.25 µM.

As shown in the figure, the uptake shows a linear path indicating that one hour is a sensible time point to perform infflux experiments.

4.4 Na^+ dependence

hPAT4 does not appear to be H^+ dependent since its activity is higher at external pH 7.4 than at a pH 5.5 environment.

Majority of the trasporters in higher organisms are Na^+ driven; exceptions include PAT1 and 2. It thus seemed possible that hPAT4 was Na^+ driven. Since the four members of the hPAT family were placed in the same family based on sequence homology and not functional studies it was possible that they were not all H^+ coupled. One of the first tests to check for was whether hPAT4 was Na^+ dependent. While both hPAT1 and hPAT2 were shown to be Na^+ independent by previous groups (Boll *et al.*, 2004), it is likely, by extension to presume that the other members of the family must also be Na^+ independent. However since hPAT4 was shown to be different from the other known members in terms of pH dependence, it was entirely possible that hPAT4 was Na^+ dependent. When choline chloride (Na^+ free environment) was replaced by sodium chloride, then the uptake of tracer [^{3}H] L-Proline was found not to be significantly different (Figure 4.4).

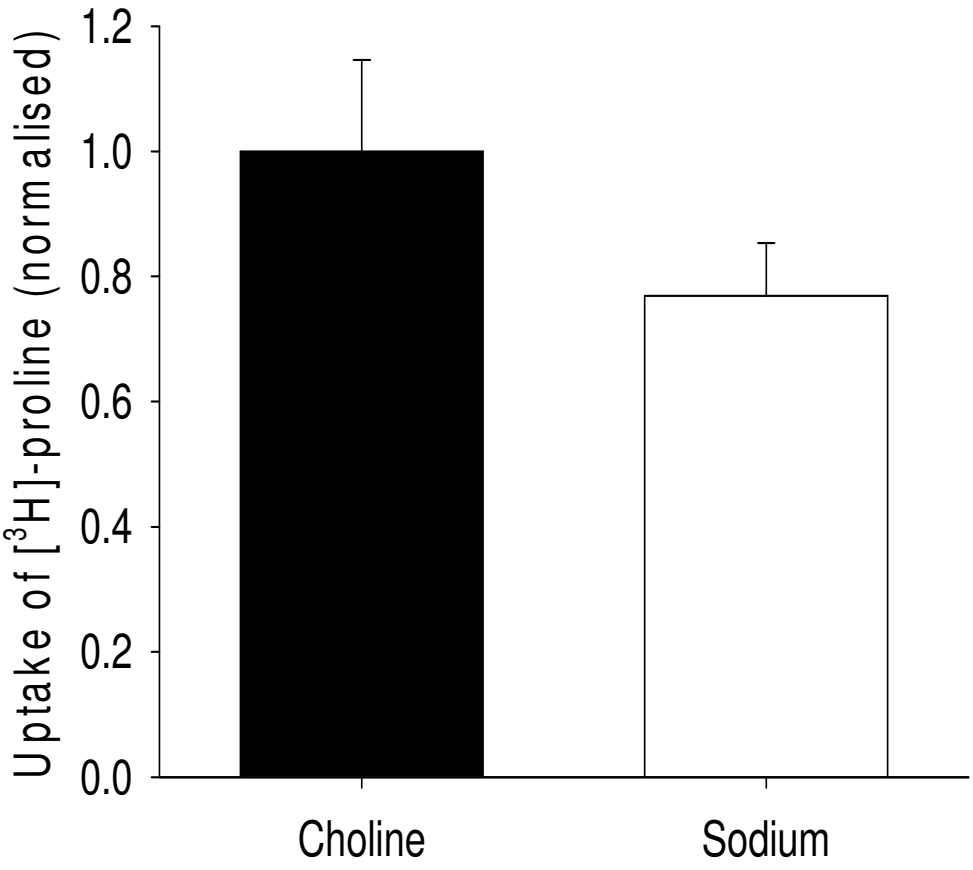

Figure 4.4: Uptake of hPAT4-mediated [^{3}H] L-Proline in the absence or presence of sodium, normalised to that seen in the absence of sodium (replaced by choline, actual uptake 27.8 ± 1.52 fmoles/oocyte/hour). $p>0.05$ Student's paired t-test n=4 oocyte preparations. Concentration of L-Proline in the tracer 0.25 µM.

4.5 Ki of L-Proline

Since hPAT4 was observed to be a transporter for L-Proline, it was important to determine what the affinity of the transporter for this substrate was. It was observed that the transport of [^{3}H] L-Proline was completely inhibited in the presence of 5 mM unlabelled L-Proline. This clearly showed that the affinity (the amount of substrate required to completely inhibit the transport of a substrate) of the transporter must be lower than 5 mM. Inhibition of transport was checked at lower concentrations 1 mM and 0.5 mM, and these concentrations were also found to completely inhibit uptake of [^{3}H] L-Proline uptake indicating that hPAT4 was a high affinity transporter. Substrate affinity experiments were thus set up using a range of concentrations from 0 (which is the [^{3}H] L-Proline alone) to 1000 µM (which is the [^{3}H] L-Proline and 1 mM cold unlabelled L-Proline). The transport activity measured and oocytes were lysed. The values were corrected for non injected and normalized to tracer only hPAT uptake. The values were then plotted using Sigma Plot (figure 4.5).The Ki of hPAT for L-Proline was observed to be 3.13 µM +/- 1.13 µM.

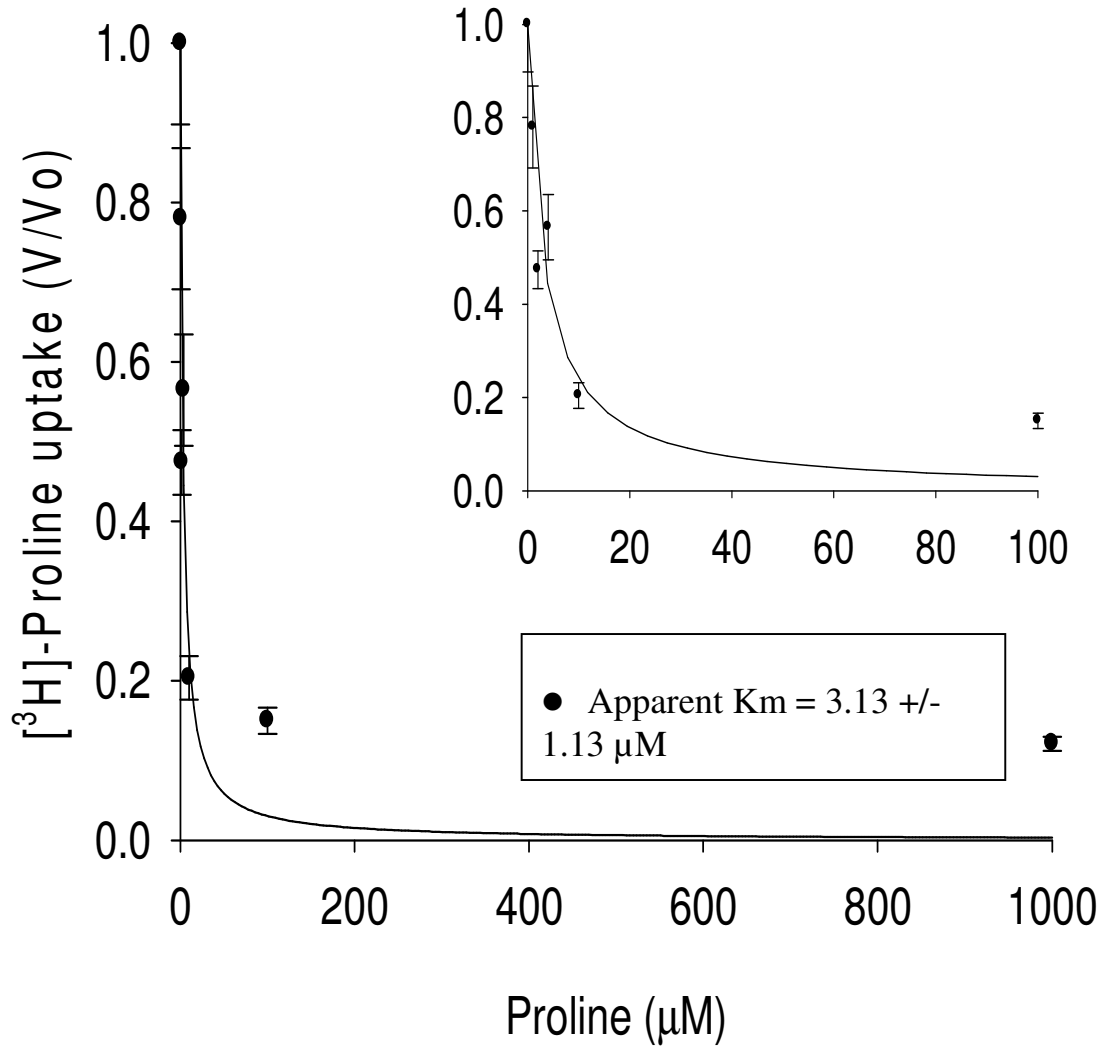

Figure 4.5: Concentration dependence of hPAT4-mediated proline uptake, as determined by inhibition of [^{3}H] L-Proline by increasing concentrations of unlabelled proline. Inset shows 0-100µM proline at higher magnification for clarity. Apparent Km was 3.1 ± 1.1 µM, n = 3 oocyte preparations. V/V0 at 1 is 40.3 +/- 5.03 fmol/oocyte/hour. The non-inhibitable component is in part contributed by the L-Proline present in the radiolabelled tracer which is 0.25 µM.

4.6 Inhibition of hPAT4 L-Proline uptake

In the presence of an excess of unlabelled amino acids, competitive inhibition takes place. This means that a lower quantity of tracer [^{3}H] L-Proline will enter oocytes incubated in medium containing other substrates.

Figure 4.6 provides a summary of the results obtained when [^{3}H] L-Proline uptake was inhibited by each of the 20 naturally occurring amino acids. For the inhibition experiment the non injected values were subtracted from injected values. The value for each of the amino acids was then normalized to the [^{3}H] L-Proline (tracer only) uptake.

With the exception (for reasons of solubility) of L-Tryptophan (1 mM), L-Tyrosine (2 mM) and L-Phenylalanine (1 mM) all other amino acids were tested at 5 mM. The first two amino acids tested Glycine and L-Alanine were known to be substrates of hPAT1 and hPAT2. However for hPAT4 while L-Alanine shows significant inhibition, Glycine did not cause significant inhibition at 5 mM. Amino acids which inhibited tracer Proline uptake were L-Alanine, L-Isoleucine, L-Methionine, L-Tryptophan, L-Threonine, L-Glutamine and L-Cysteine.

Thus amino acids which were not “typical” substrates for hPATs 1 and 2 were found to inhibit hPAT4, these amino acids are L-Methionine, L-Isoleucine, L-Threonine and L-Glutamine. Neither D nor L- Serine was found to inibit hPAT4 significantly. However inhibition by D-Serine though non-significant at 5 mM does seem to be more than that of L-Serine.

Inhibition assays generally give a good indication of the affinity of the transporter for different groups of molecules. For instance PAT1 and PAT2 have always been grouped together as relatively low affinity transporters of small neutral amino acids, hPAT4 appears to have a more diverse range of amino acids that can inhibit uptake of [^{3}H] L-Proline.

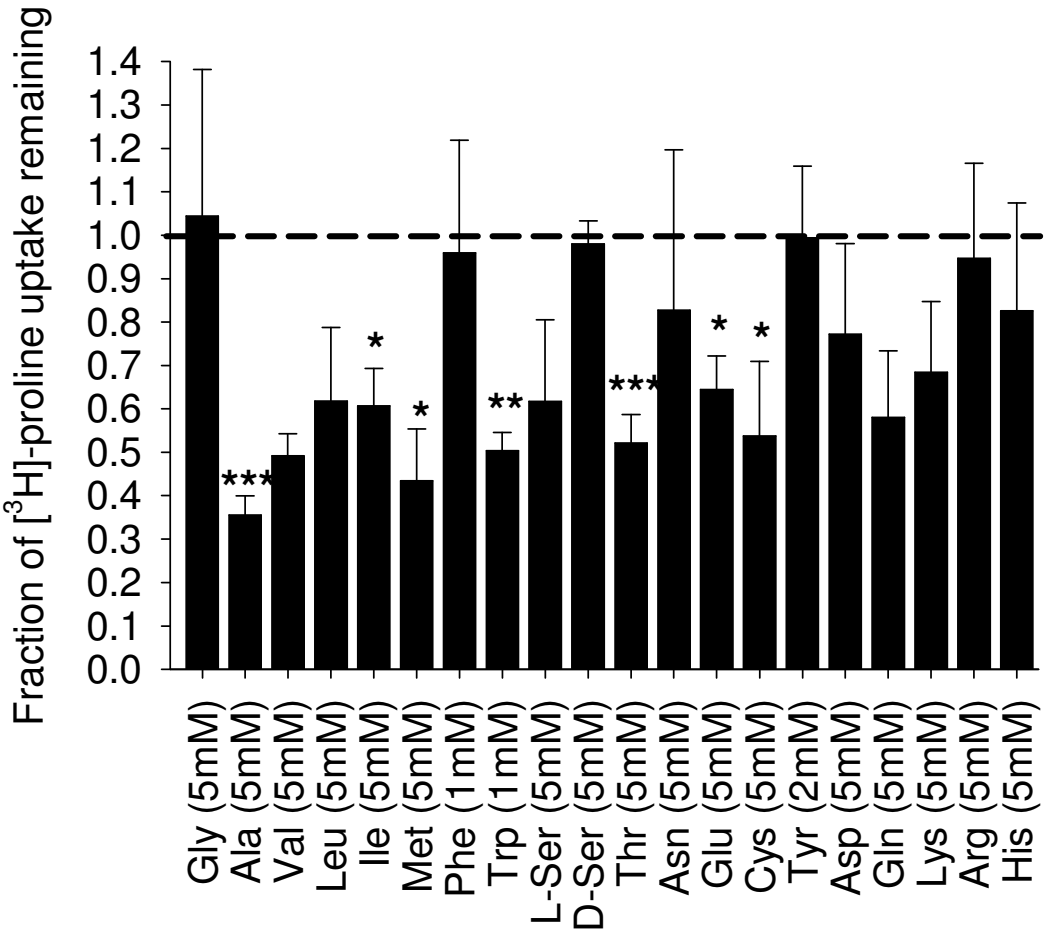

Figure 4.6: Inhibition of hPAT4-mediated [^{3}H] L-Proline uptake into *Xenopus* oocytes by the proteogenic amino acids. Uptake has been normalised to that seen in the absence of inhibitor i.e. [^{3}H] L-Proline alone which was 35.65 +/- 3.44 fmol/oocyte/ hour. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one sample t-test, $n \geq 3$ oocyte preparations. Concentration of L-Proline in the tracer 0.25 µM.

4.7 Substrate affinity of amino acids which inhibited tracer uptake

Substrate affinities were calculated for some of the amino acids which successfully inhibited L-proline uptake of hPAT4. The Ki values were calculated for these using Sigma plot and are summarized in figure 4.9. It is interesting to note that hPAT4 appears to have a higher affinity for L-Glutamine, L-Isoleucine and L-Methionine compared to L-Alanine which until now has been characterized as one of the main substrates for hPATs.

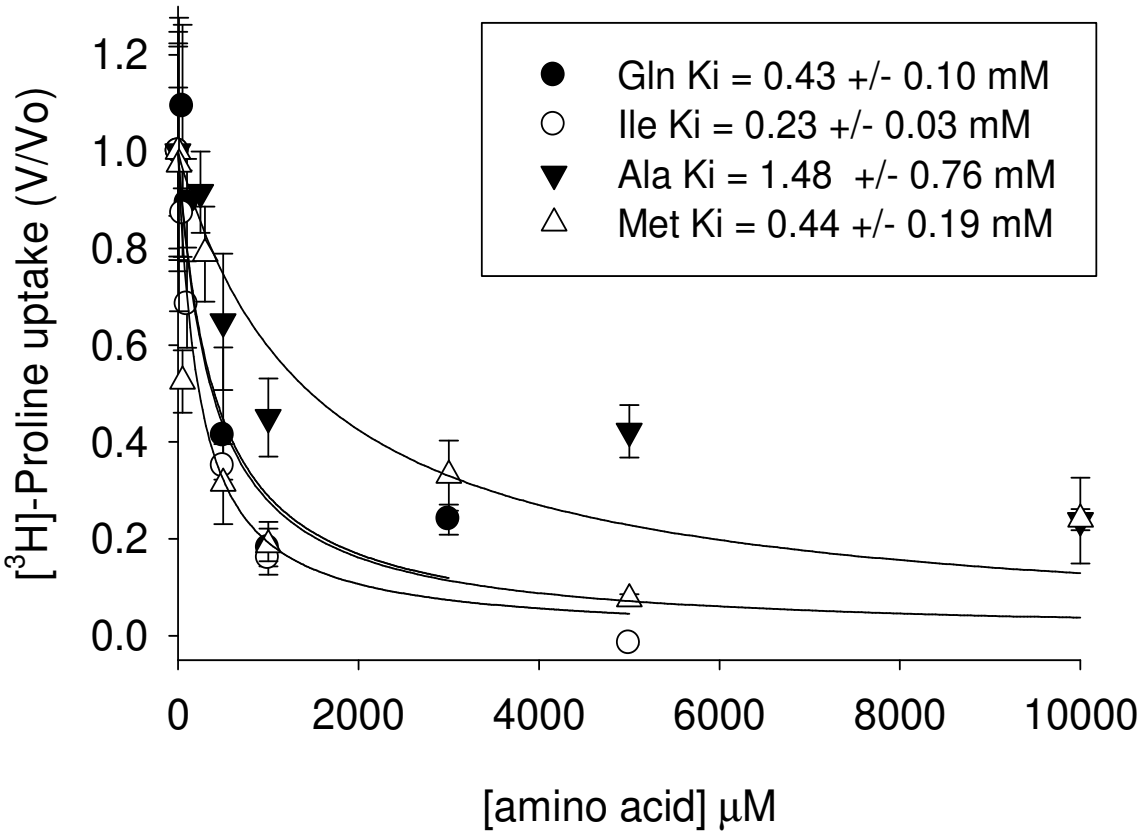

Figure 4.7: Concentration dependence of hPAT4-mediated isoleucine, methionine, glutamine and alanine inhibition of [^{3}H] L-Proline uptake: the Ki values were L-Isoleucine 0.23 ± 0.03 mM, L-Methionine 0.44 ± 0.19 mM, L-Glutamine 0.43 ± 0.10 mM and L-Alanine 1.48 ± 0.76 mM, n=2-4 oocyte preparations. V/V_0 at 1 is 45.34 +/- 2.65 fmol/oocyte/hour. The non inhibitable component is contributed by the tracer present within the system. Concentration of L-Proline in the tracer 0.25 µM.

4.8 The exceptional affinity of L-Tryptophan for hPAT4

L-Tryptophan and some of its derivates have been shown to bind PAT1 (Metzner et al., 2005) and PAT2 (Edwards *et al.,* 2011); however no transport activity for L-Tryptophan has been shown in either of them. Inhibition assays with unlabelled L-Tryptophan (containing only [^{3}H] L-Proline) showed that 1 mM L-Tryptophan could inhibit tracer uptake.

Affinity inhibition experiments were set up in varying concentrations (0 mM to 1 mM L-Tryptophan) with constant amount of [^{3}H] L-Proline tracer. The results obtained are shown in figure 4.8.

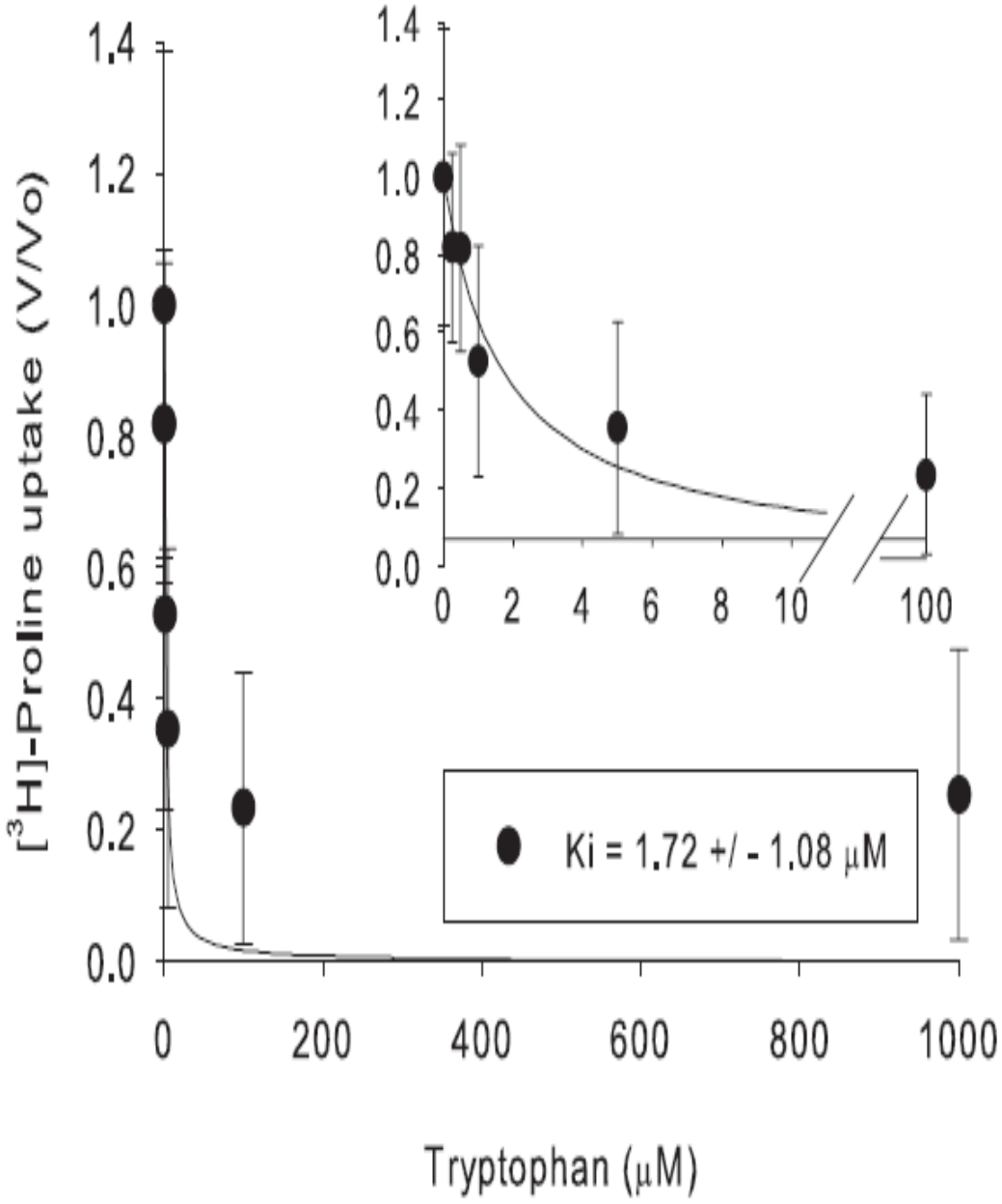

Figure 4.8: Concentration dependence of hPAT4-mediated tryptophan inhibition of [^{3}H] L-Proline uptake. Km was 1.7 ± 1.1 µM, n=2 oocyte preparations. V/V_0 at 1 is 43.56 +/- 3.43 fmol/oocyte/hour. Concentration of L-Proline in the tracer 0.25 µM.

4.9 Transport of other radiolabelled amino acids

Radiolabelled amino acids were tested for transport activity in *Xenopus* oocytes. The radiolabelled amino acids tested were either ^{3}H or ^{14}C labeled. The ^{14}C amino acids tested were L-Serine, L-Cysteine and Glycine. The ^{3}H ones tested were L-Tyrosine, L-Tryptophan, L-Lysine, L-Leucine and L-Alanine. Even though some of these were not found to inhibit [^{3}H] L-Proline uptake in hPAT4, they were tested nevertheless since they were available and there was always the possibility that hPAT4 might be a very low affinity transporter for the amino acid.

Of the amino acids tested (figure 4.9), the amino acids other than L-Proline which showed significant uptake compared to the non injected in influx assays were L-Alanine and L-Tryptophan. Glycine was neither transported nor inhibited. L-Tyrosine, L-Cysteine, L-Serine and L-Tyrosine were also not found to be transported (data not shown).

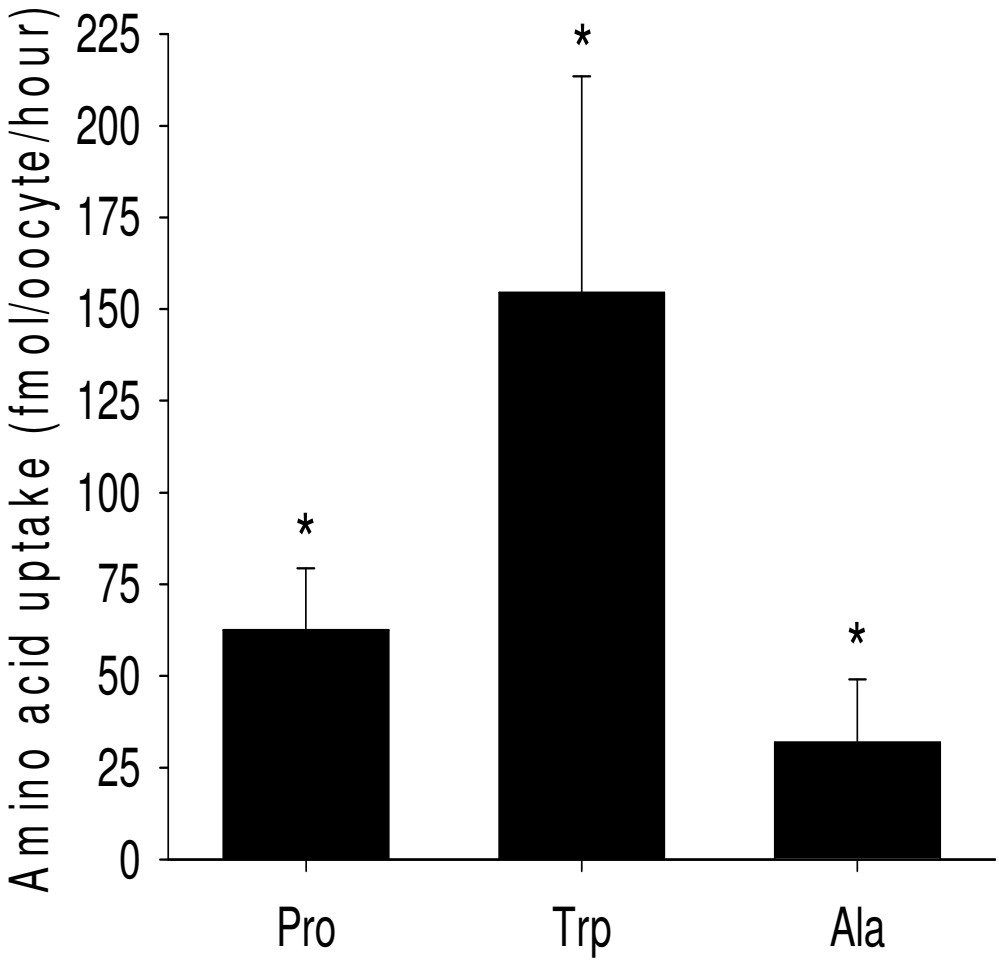

Figure 4.9: hPAT4-mediated uptake of amino acids into *Xenopus* oocytes (fmol/oocte/hour). *$p < 0.05$, Student's t-test, compared to non injected oocytes, $n \geq 3$ oocyte preparations. Concentration of amino acid in the tracer 0.25 µM – 1 µM.

4.10 Electrogenecity

Two approaches were taken to answer the question of electrogenecity of hPAT4. The first was to perform electrophysiology experiments by inserting electrodes into the oocyte, and performing voltage clamp experiments to measure any current flow associated with substrate flux thrugh transport. The second was to change the membrane potential by biochemical methods and observe if any changes took place in the transport properties.

4.10.1 Electrophysiology

Changes in currents are measured when the wash medium was replaced by medium containing high concentrations (at least 5 times the Ki) of the substrate. The results obtained indicated a very small current for the hPAT4 injected oocytes which was not however specific to hPAT4 since it was also observed in the non injected oocytes. hPAT1 which was run as a control was found to show a current which was specific to it (i.e after correcting for non injected). The results obtained are summarized in table 4.1

	hPAT1 (nA)	hPAT4 (nA)	Non injected (nA)
pH5.5 20µM Pro	NMS	NMS	NMS
pH5.5 10mM Pro	16.8 ± 2.09* (n=17)	4.70 ± 0.60 (n=10)	7.60 ± 0.87 (n=5)
pH7.4 20µM Pro	NMS	NMS	NMS
pH7.4 10mM Pro	13.5 ± 1.18# (n=6)	9.00 ± 0.93 (n=12)	8.67 ± 1.17 (n=6)

Table 4.1: Characterization of hPAT4-mediated transport.

Experiments were conducted by two electrode voltage clamp electrophysiology, as compared to hPAT1-expressing and control (non injected) oocytes ($n \geq 3$ oocytes per condition).

* $p < 0.05$ vs pH5.5 non injected, # $p < 0.05$ vs pH7.4 non injected,

NMS – no measurable signal

4.10.2 Biochemical methods

The second approach used was a biochemical one, manipulating membrane potential by altering external potassium. Influx assays were set up to contain varying concentrations of external potassium or choline chloride.

hPAT1 was run as a control alongside for each point and each experiment along with hPAT4. Since hPAT1 is an electrogenic transporter driven by H^+ co-transport comparison with hPAT4 will indicate whether hPAT4 is electroneutral.

For the coupled transporter hPAT1, the uptake decreases when the external potassium concentration is decreased. In contrast for hPAT4, there appears to be very little change in uptake following a change in K^+ levels.

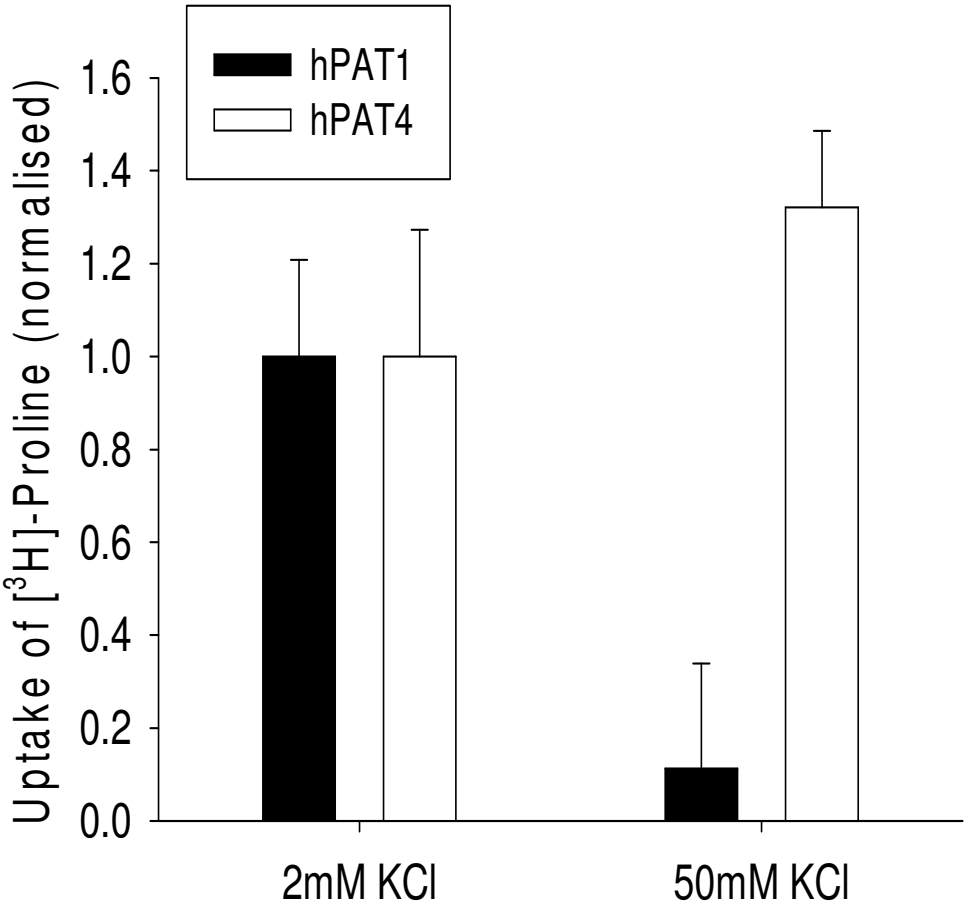

Figure 4.10: Uptake of hPAT4-mediated [^{3}H] L-proline in normal (2mM) and raised (50mM) extracellular potassium, normalised to that seen in the normal potassium (mean uptake of 25.3 ± 12.2 and 31.8 ± 24.9 fmoles/oocyte/hour, respectively, n=3 oocyte preparations with $p < 0.05$ for 2 mM versus 50 mM for hPAT1 and $p > 0.05$ for hPAT4 in each). For hPAT1 the normalised uptake at 1 corresponds to 63.63 +/- 2.22 and 6.36 +/- 1.56 fmol/oocyte/hour for 2 mM and 50 mM potassium respectively). Concentration of L-Proline in the tracer 0.25 µM.

4.11 The inhibition of hPAT4 L-Proline uptake by non-proteogenic amino acids

A few non proteogenic amino acids were tested to check if they caused inhibition of radiolabelled proline uptake. In recent years there has been much attention focussed on PAT1 and PAT2 on account of the observation that these transporters are capable of bringing about the movement of various therapeutic drugs and non-amino acid substrates across the membrane (Thwaites and Anderson, 2011).

A variety of non-proteogenic amino acids and drugs were tested including L-Dopa, β-alanine, γ-aminobutyric acid (GABA), δ-aminolevulinic acid and sarcosine. None of these substrates caused inhibition except sarcosine. Sarcosine was observed to cause significant inhibition ($p < 0.05$, one-sample t-test) with an affinity of 1.09 ± 0.35 mM.

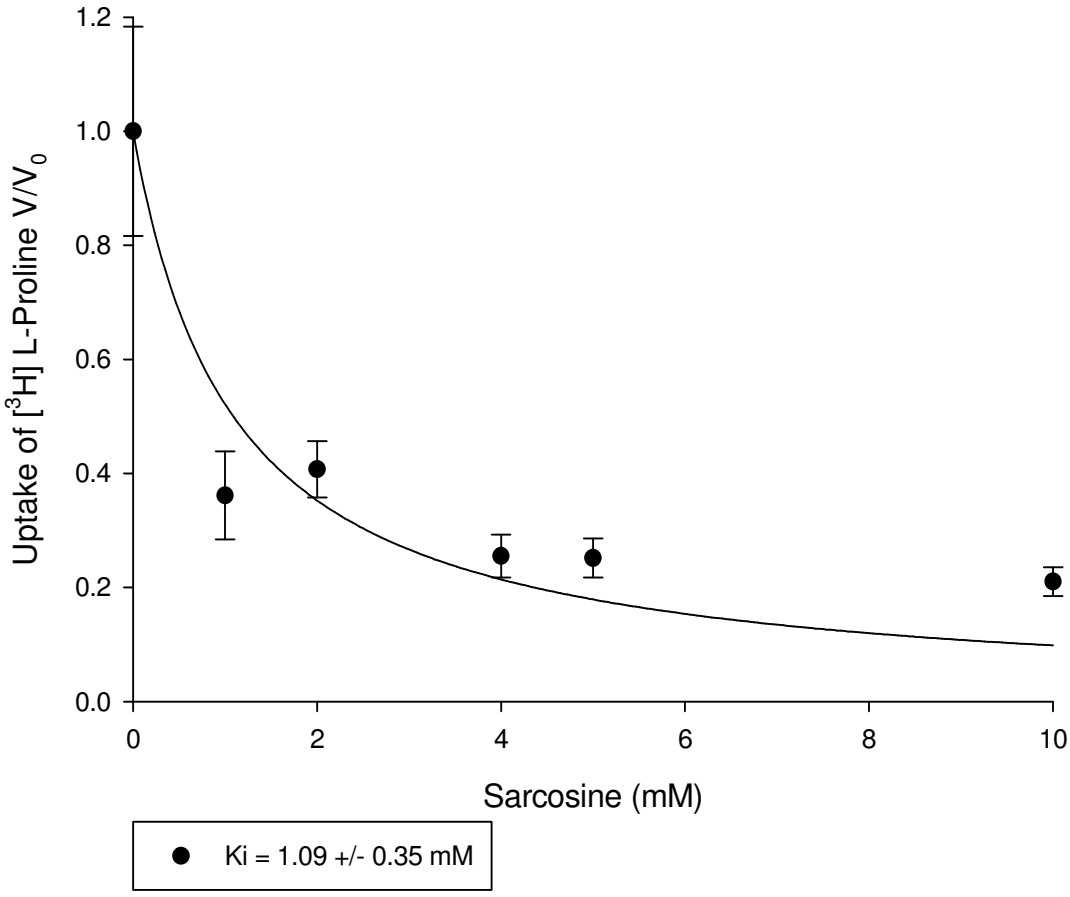

Figure 4.11: Concentration dependence of hPAT4-mediated sarcosine inhibition of [^{3}H] L-Proline uptake. Ki was 1.09 ± 0.35 mM, n=2 oocyte preparations. V/V_0 at 1 is 20.35 +/- 3.41 fmol/oocyte/hour. Concentration of L-Proline in the tracer 0.25 µM.

4.12 The mysterious case of L-Leucine and hPAT4

Neither hPAT1 nor hPAT2 have shown any evidence of inhibition of L-Proline transport L-Leucine or of transport of this amino acid.

As discussed in section 4.6, hPAT4 uptake of radiolabelled L-Proline is not inhibited in the presence of excess L-Leucine. However, when influx assays were set up and [^{3}H] L-Leucine was used as a tracer, there was significant uptake by hPAT4 injected oocytes as compared to non injected oocytes. This is of particular interest considering the importance of the amino acid in signaling.

Leucine is found to be important in nutrient sensing and mTOR regulation. Inhibition assays are performed with the notion that with one binding site for two amino acids that are transported by a particular transporter, each can cause inhibition of the other. For instance [^{3}H] L-Tryptophan uptake is inhibited by L-Proline and vice versa.

However [^{3}H] L-Leucine uptake was not inhibited by L- Proline but leucine was transported across the membrane. It is possible that there is a separate binding site for L-Leucine from the one where L-Proline binds. If correct, this could also explain the role of PAT4 in sensing mechanisms (mTOR regulation).

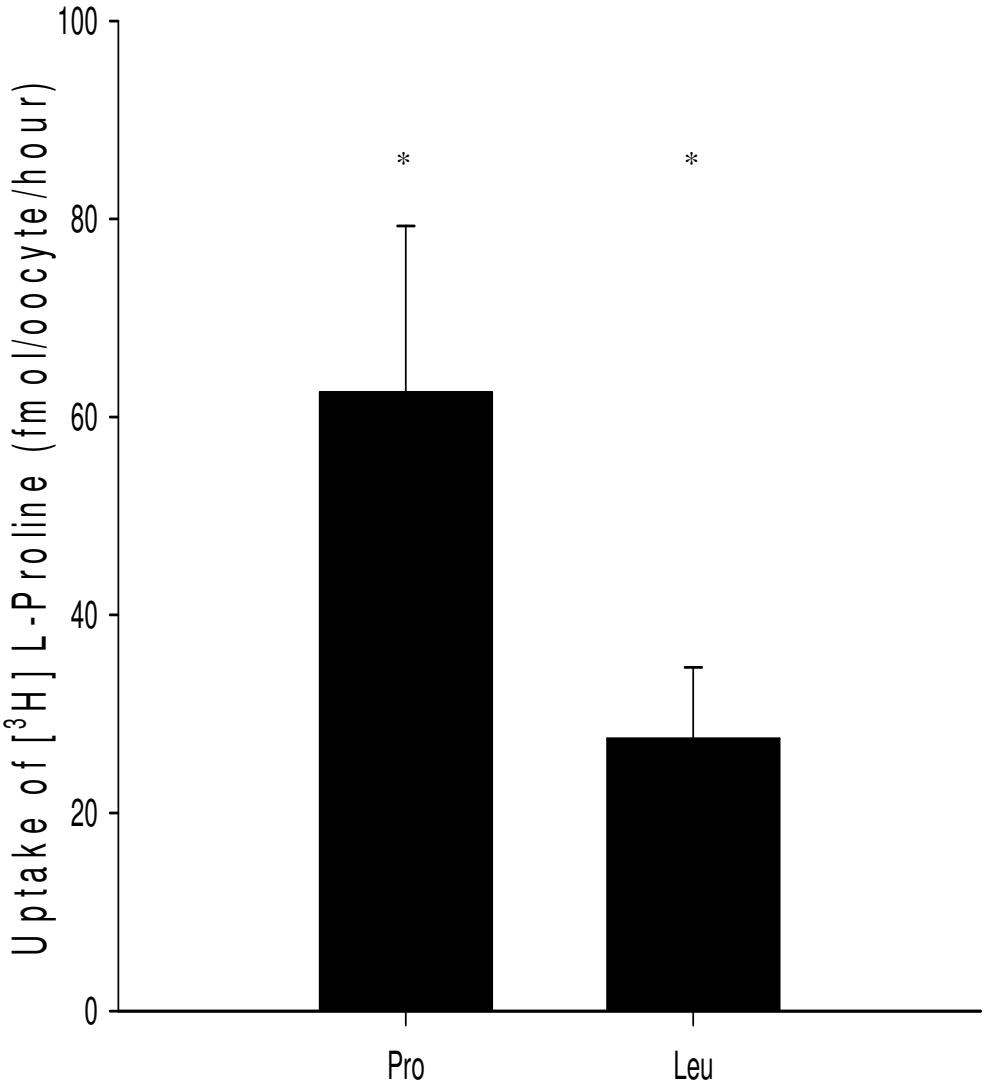

Figure 4.12: hPAT4 is shown to bring about a significant uptake of [^{3}H] L-Leucine. The data are corrected for non injected oocytes (* $p < 0.05$) . $n \geq 3$ oocyte preparation. Concentration of L-Proline and L-Leucine in the tracer 0.25 µM.

4.13 Chapter summary

- The human Proton coupled Aminoacid Transporter hPAT4 had always been categorised as an orphan transporter.
- For the first time this transporter was shown to bring about a pH dependent transport of amino acid across the membrane.
- The maximal transport activity was observed at a pH of 7.4
- Time course assays showed that the transporter still continued to show a liner uptake activity at 1 hour and was observed not to reach saturation.
- The transporter does not show a Na^+ dependence.
- The transporter is observed to have a very high affinity for L-Proline (Ki of 3 µM).
- Besides L-Proline, a variety of other substrates were also observed to inhibit [^{3}H] L-Proline uptake. These include, L-Alanine, L-Tryptophan, L-Glutamine.
- hPAT4 shows a high affinity for L-Tryptophan (range of 2 µM)
- hPAT4 shows a lower affinity for the other amino acids similar to that shown by the characterized hPATs 1 and 2, around 200 – 1000 µM.
- Of the radiolabelled amino acids that were tested, L-Tryptophan, L-Alanine were observed to be transported across the membrane, whereas amino acids like L-Serine, L-Cysterine and L-Tyrosine.
- Electrophysiological approaches indicated that the transporter is electroneutral. This was done by using voltage clamp experiments and by altering the membrane potential by changing the potassium concentration in the uptake medium.
- Among the non-proteogenic amino acids that were tested, sarcosine was found to inhibit [^{3}H] L-Proline uptake.
- L-Leucine could inhibit labeled L-Proline uptake and vice versa. However when labeled L-Leucine was used as a tracer, hPAT4 was shown to bring about transport across the membrane.

Chapter 5 :
The Membrane Topology of hPAT1 and its Implications

5.1 Transmembrane domain prediction for hPAT1

As discussed in section 1.3, membrane transporters are difficult to study using techniques such as X-ray crystallography which are commonly employed in definitive structural studies.

In an effort to get around this, various programs have been designed which utilize algorithms that have taken into consideration factors such as hydrophobicity, the amino acids present and can attempt to predict the topology of the membrane transporter. Different transmembrane prediction software use different algorithms to do the same and as a result, as shown in table 5.1, hPAT1 is predicted to have anywhere from 9 to 11 transmembrane domains.

These predictions can be used to design and insert epitope tags using site directed mutagenesis, and the predictions then confirmed or refuted experimentally.

The epitope tag used is the eight amino acid sequence FLAG (DYKDDDDK). This epitope tag has previously been used to characterize PepT1 (Panitsas, Boyd and Meredith, 2006). As shown in figure 5.1 different numbers and regions were predicted for the TMDs of hPAT1. It was decided to use the MEMSAT3 (Jones, Taylor and Thornton, 1994) predictions to design the epitope tags. This was due to the positive results the predictions had given with PepT1 (Meredith and Price, 2006), predictions later confirmed when PepT1 was modelled on the peptide transporter PepT(So) for which a crystal structure is now available (Newstead *et al.*, 2011).

Program	Transmembrane domains										
	1	2	3	4	5	6	7	8	9	10	11
MEMSAT3	50-73	76-100	139-163	192-211	218-241	259-278	291-315	330-352	377-400	403-422	434-458
TMHMM	53-72	77-99	141-163	194-211	218-240	255-277	290-312	341-363	376-393	403-425	438-460
TopPred	52-72	75-95	143-163	216-236	258-278	289-309	335-355	398-418	437-457		
SVMtm	78-98	145-162	198-214	216-237	259-273	291-311	337-359	376-400	402-418	438-453	
TMpred	75-99	145-163	196-212	216-236	259-278	291-309	337-355	376-394	398-415	434-453	

Table 5.1: Transmembrane domain prediction of hPAT1. Depending on the software used hPAT1 is predicted to have 9-11 TMDs, each TMD being atleast 18 amino acids in length (as indicated by the first and last amino acid residue number for each predicted TMD).

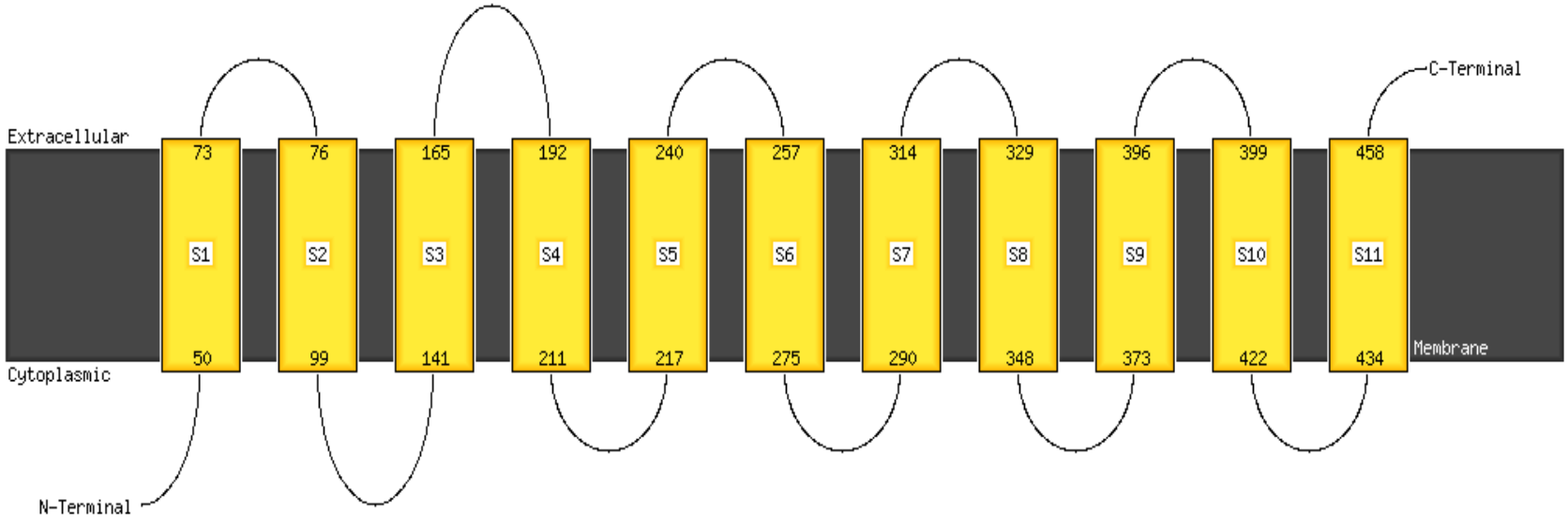

Figure 5.1: Diagram generated using MEMSAT3 transmembrane domain prediction software. The model consists of 11 transmembrane domains with an internal N-terminal and an external C-terminal.

MEMSAT3 was used in designing the epitope tags

5.2 Epitope tagging of hPAT1 – External FLAGs

5.2.1 Confirmation of epitope insertion

FLAG epitopes were inserted by site directed mutagenesis as described (materials and methods). The sequences were confirmed and the regions containing the inserts are shown in figure 5.2. The sequencing confirmed that there were no other mutations except the FLAG insert.

```
hPAT1           KGNIGTGLLGLPLAV-------KNAGIVMGPISLLIIGIVAVHCMGILVKCAHHFCRRLN 110
FLAG1           KGNIGTGLLGLPLAVDYKDDDDKNAGIVMGPISLLIIGIVAVHCMGILVKCAHHFCRRLN 117
                ***************       ************************************

hPAT1           VMYGLESSPCSWLRNHAHWGRRVVDFFLIVTQLGFCCVYFVFLADNF-------KQVIEA 172
FLAG2           VMYGLESSPCSWLRNHAHWGRRVVDFFLIVTQLGFCCVYFVFLADNFDYKDDDDKQVIEA 180
                ********************************************       ******

hPAT1           LVSLVMIYQFIVQRIPD-------PSHLPLVAPWKTYPLFFGTAIFSFEGIGMVLPLENK 281
FLAG3           LVSLVMIYQFIVQRIPDYKDDDDKPSHLPLVAPWKTYPLFFGTAIFSFEGIGMVLPLENK 288
                *****************       ************************************

hPAT1           ILYISLGCLGYLQFGANIQGS--------ITLNLPNCWLYQSVKLLYSIGIFFTYALQFY 352
Flag4           ILYISLGCLGYLQFGANIQGSDYKDDDDKITLNLPNCWLYQSVKLLYSIGIFFTYALQFY 360
                **********************        ********************************

hPAT1           VCLTCILAILIPRLDL--------VISLVGSVSSSALALIIPPLLEVTTFYSEGMSPLTI 412
FLAG5           VCLTCILAILIPRLDLDYKDDDDKVISLVGSVSSSALALIIPPLLEVTTFYSEGMSPLTI 420
                ****************        ************************************

hPAT1           LIQPSNAPIFINS--------TCAFI                                    476
FLAG6           LIQPSNAPIFINSDYKDDDDKTCAFI                                    484
                *************        *****
```

Figure 5.2: Sequence confirmation of external FLAGs F1, F2, F3, F4, F5 and F6.

5.2.2 Confirmation of functionality of the epitope tagged transporters (external FLAGs)

Mutations of genes with hPAT1 FLAG inserts F1, F2, F3, F4, F5 and F6 were checked for transport function. The uptake influx assays for transport were performed using oocytes injected with either hPAT1 (control) or the six external FLAG inserts or in non injected oocytes. Figure 5.3 shows the normalized uptake assays for these groups of oocytes.

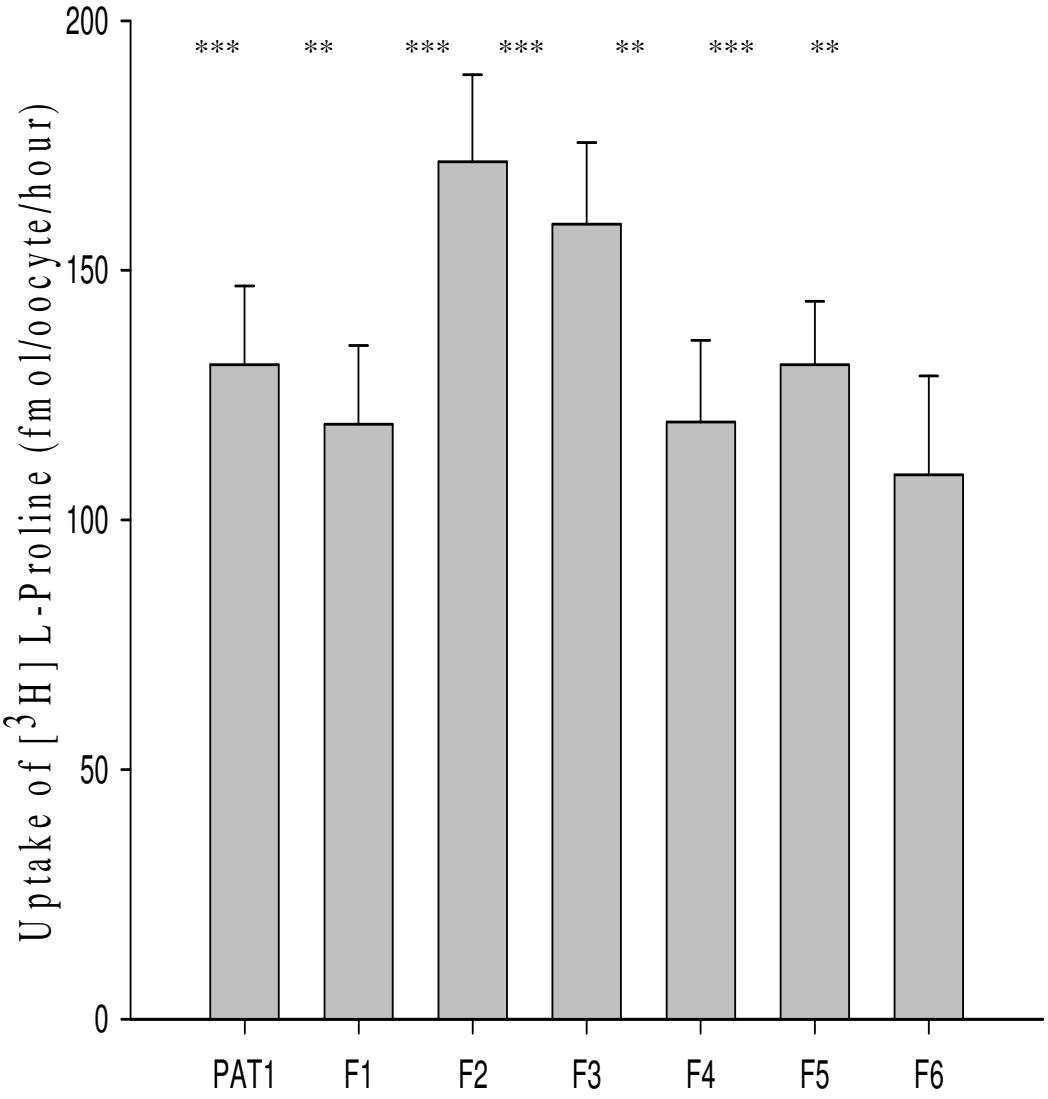

Figure 5.3: Transport of [^{3}H] L-Proline by external FLAGs. The figure shows the uptake of hPAT1, the putative external FLAGs F1, F2, F3, F4, F5 and F6 after correcting for non injected oocytes i.e the hPAT mediated uptake. The fmol/oocyte/hour reading for the non injected oocytes was 7.43. The significance levels are those when compared to non injected oocytes. (** $p < 0.01$, *** $p < 0.001$) n=7 oocyte preparations. Concentration of L-Proline in the tracer 0.25 µM.

5.2.3 Luminometry studies on the putative external FLAGs

Luminometry assays were performed on the same batch of oocytes as those studied in figure 5.3. The luminometry set up was performed as discussed (materials and methods). hPAT1 was used as a control along with non injected oocytes. This was done to ensure that hPAT1 did not show any reactivity with the anti-FLAG antibody.

The results showed that the luminometry data for the putative external FLAGs was significantly higher than those injected with wild type hPAT1. The luminometry readings obtained for the FLAG inserts were significantly higher than either PAT1 or non injected oocytes (Figure 5.4).If the region where the epitope tags were inserted lies outside the membrane, the HRP tagged antibody can readily access it and its presence can then be detected by luminescence.

The results obtained thus indicate that the regions which were tagged lay external region of the oocyte plasma membrane.

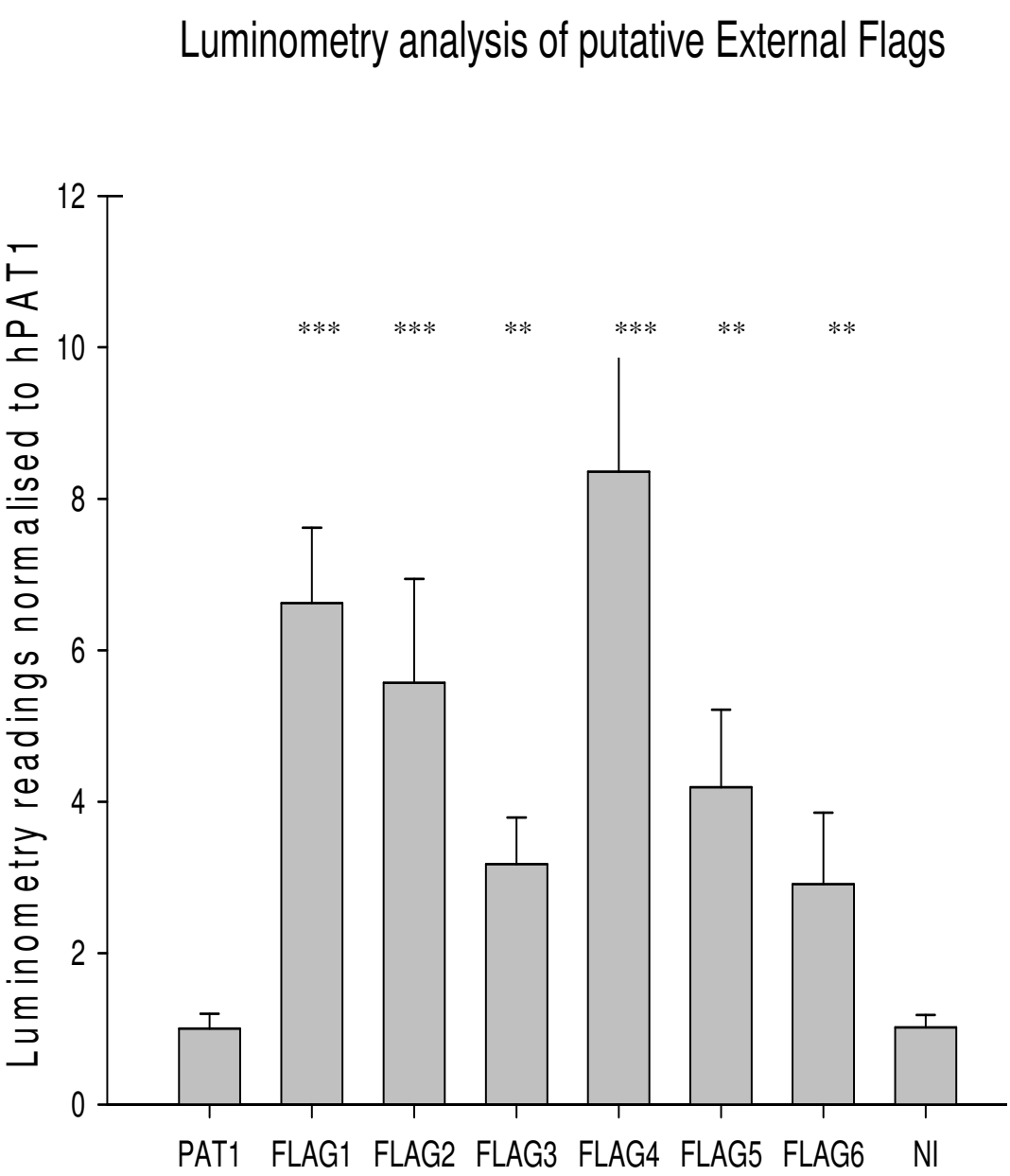

Figure 5.4: Normalised luminometry results of the putative external FLAGs. Luminometry experiments were set up on hPAT1, the external FLAGs F1, F2, F3, F4, F5, F6 and non injected oocytes. The oocytes injected with cRNA for the external FLAGs were compared to the hPAT1 injected oocytes to determine significance. (* $p < 0.05$, **$p < 0.01$, *** $p < 0.01$) n=7 oocyte preparations.

5.3 Epitope tagging of hPAT1 – Internal FLAGs

5.3.1 Confirmation of epitope insertion

Sequencing reactions were performed to confirm that FLAGs were inserted in the appropriate locations. No other mutations were observed in the sequence. Figure 5.5 shows the epitope insertions. As discussed in the materials and methods internal FLAGs 1, 2, 3, 5 and 6 were designed, but it was not possible to get a successful insertion of the internal epitope FLAG 4 on account of the region being extremely G+C rich.

```
hPAT1           MSTQRLRNEDYHDY--------SSTDVSPEESPSEGLNNLSSPGSYQRFGQSNSTTWFQT 52
iFlag1          MSTQRLRNEDYHDYKDDDDKDYSSTDVSPEESPSEGLNNLSSPGSYQRFGQSNSTTWFQT 60
                **************        ************************************

hPAT1           IGTGLLGLPLAVKNAGIVMGPISLLIIGIVAVHCMGILVKCAHHFCRRLN--------KS 112
iFlag2          IGTGLLGLPLAVKNAGIVMGPISLLIIGIVAVHCMGILVKCAHHFCRRLNKDYKDDDDKS 120
                ************************************************        **

hPAT1           HNNETVILTPTMDSRLYMLSFLPFLVLLVFIRN--------LRALSIFSLLANITMLVSL 232
iFlag3          HNNETVILTPTMDSRLYMLSFLPFLVLLVFIRNDYKDDDDKLRALSIFSLLANITMLVSL 240
                *********************************        ******************

hPAT1           FFVSRAPEHCE--------LVVDLFVRTVLVCLTCILAILIPRLDLVISLVGSVSSSALA 412
iFlag5          FFVSRAPEHCEDYKDDDDKLVVDLFVRTVLVCLTCILAILIPRLDLVISLVGSVSSSALA 420
                ***********         ****************************************

hPAT1           VTTFYSEGMSP--------LTIFKDALISILGFVGFVVGTYEALYELIQPSNAPIFINST 472
iFlag6          VTTFYSEGMSPDYKDDDDKLTIFKDALISILGFVGFVVGTYEALYELIQPSNAPIFINST 480
                ***********         ****************************************
```

Figure 5.5: Shows the sequence confirmation of internal FLAGs iF1, iF2, iF3, iF5 and iF6.

5.3.2 Confirmation of functionality of the internal epitope tagged transporters

The putative internal FLAGs iF1, iF2, iF3, iF5 and iF6 were used for cRNA synthesis. The cRNA for the internal FLAGs along with hPAT1 was injected into oocytes. Influx assays were performed using these injected oocytes and non injected ones. The internal FLAGs (figure 5.6) did not cause a change in transport activity compared to [^{3}H] L-Proline uptake by wild type hPAT1. This indicates that the insertion of the epitopes did not cause a loss of function.

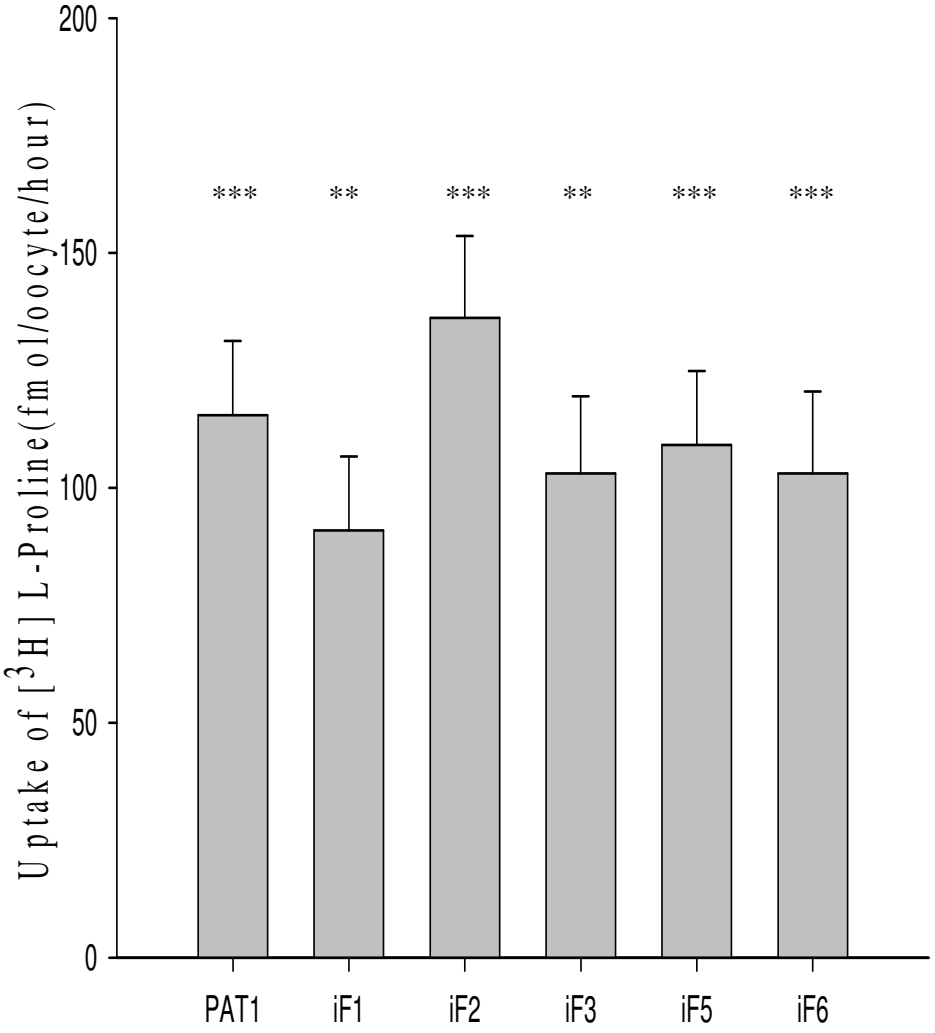

Figure 5.6: Transport of [^{3}H] L-Proline by the internal FLAGs. The figure shows the normalized uptake of the internal FLAGs when normalized to hPAT1. The uptake of the injected oocytes was corrected for the non injected oocytes (i.e. the mediated transport). The fmol/oocyte/hour reading for the non injected oocytes was 5.30. The significance levels are those observed on comparison with the non injected oocytes. (* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$) n=5 oocyte preparations. Concentration of L-Proline in the tracer 0.25 µM.

5.3.3 Luminometry studies on the internal FLAGs

Oocytes injected with cRNA for the internal FLAGs iF1, iF2, iF3, iF5, iF6, hPAT1 and non injected oocytes were used to set up luminometry experiments. As for the external FLAG experiments, hPAT1 expressing oocytes were used as a control to compare the internal FLAG injected oocytes against.

The results indicated that the predicted internal regions showed no significant increase when compared to wild type hPAT1 injected oocytes.

This indicates that the regions where these epitopes were inserted was not accessible from the extracellular medium, that is that the tags were present in the intracellular region or in the trans membrane domain (generally an insertion in the trans membrane region might be expected to cause a change in confirmation and a loss of function. Figure 5.7 shows the luminometry readings of the internal FLAGs, hPAT1 and non injected oocytes).

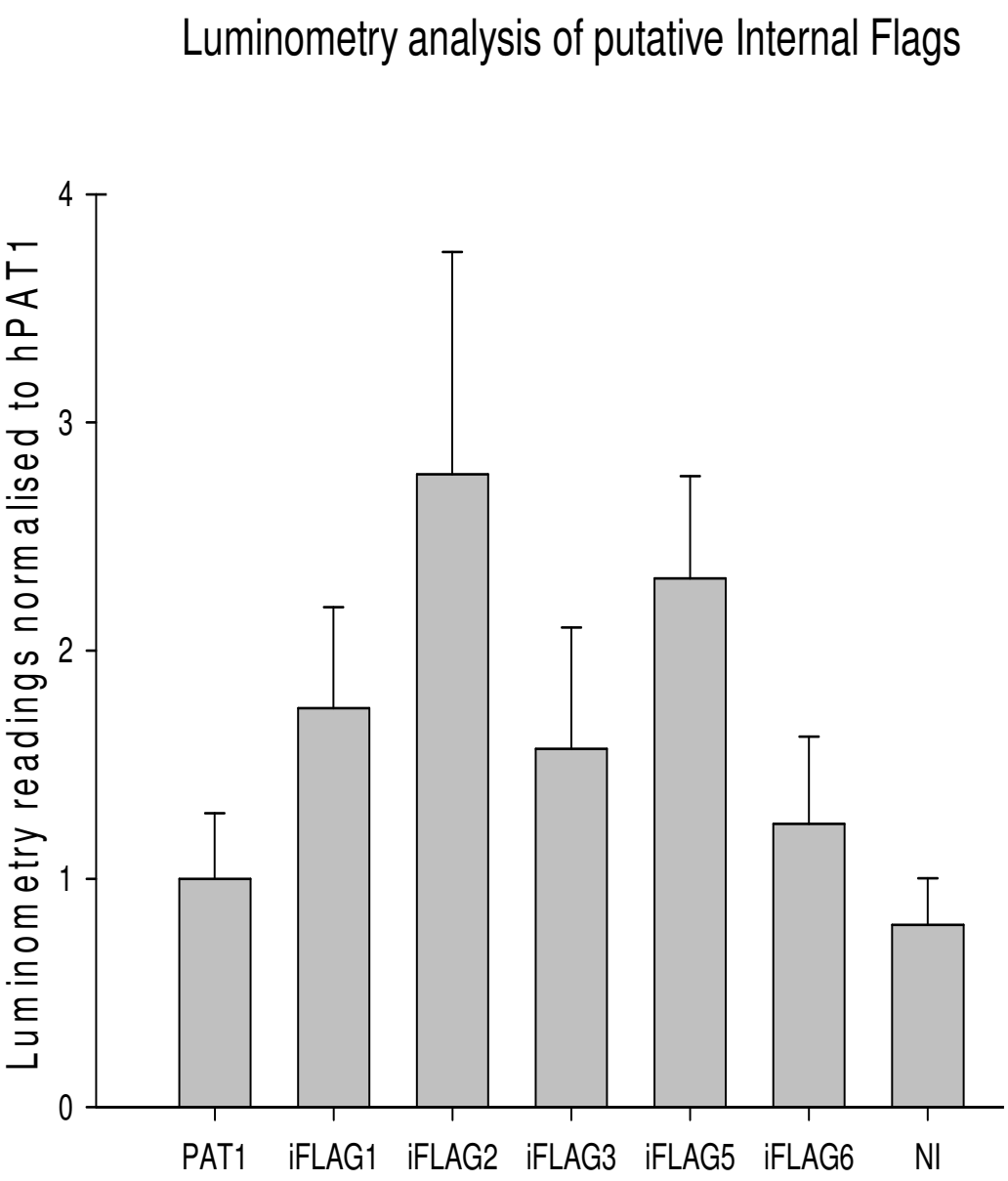

Figure 5.7: Normalised luminometry analysis of the predicted internal FLAGs. The internal FLAGs were compared to the hPAT1 injected oocytes. The readings of antibody binding for any of the individual FLAGs were nomalised to that of hPAT1. Compared to hPAT no significant difference was observed. n=5 oocyte preparations.

5.3.4 Permeabilisation of oocytes

The experiments on the internal FLAGs indicate that these regions are in the intracellular region (or possibly the trans membrane region) when performed in intact oocytes. To confirm this, oocytes which were injected with the cRNA for the internal FLAGs iF1, iF2, iF3, iF5, iF6 (with hPAT1 injected oocytes and non injected oocytes as control) were permeabilised. Permeabilisation was performed using TritonX (0.01%) through all the stages of luminometry from the initial blocking stages to the final wash just prior to addition of the ELISA substrate. Prior to couting the plates the oocytes were checked to make sure that they weren't damaged. In addition to the permeabilisation of the oocytes with the internal FLAG, the oocytes injected with the external FLAG were also performed as a control to check that the increase observed in both of them was similar. To ensure that none of the oocytes had been damages, they were inspected by eye and against the non injected and hPAT1 injected controls.

The results showed that permeabilised cells show an increase in all the luminometry readings compared to that in intact cells. However when compared to hPAT1 injected oocytes, the internal FLAGs showed a significant increase compared to hPAT1 and non injected. This is consistent with the hypothesis that in permeabilised cells the antibody is able to interact with the epitope tag indicating that the FLAGs are in the internal region (Figure 5.8a).

In case of the external FLAGs an increase is still observed since there will also be an increase since there will be transport proteins that have not reached the surface of the membrane yet and are being detected (Figure 5.8b). However perbeabilisation studies with both the external and the internal flags show very similar results indicating that the net amount of tagged transporter present within the oocyte is similar.

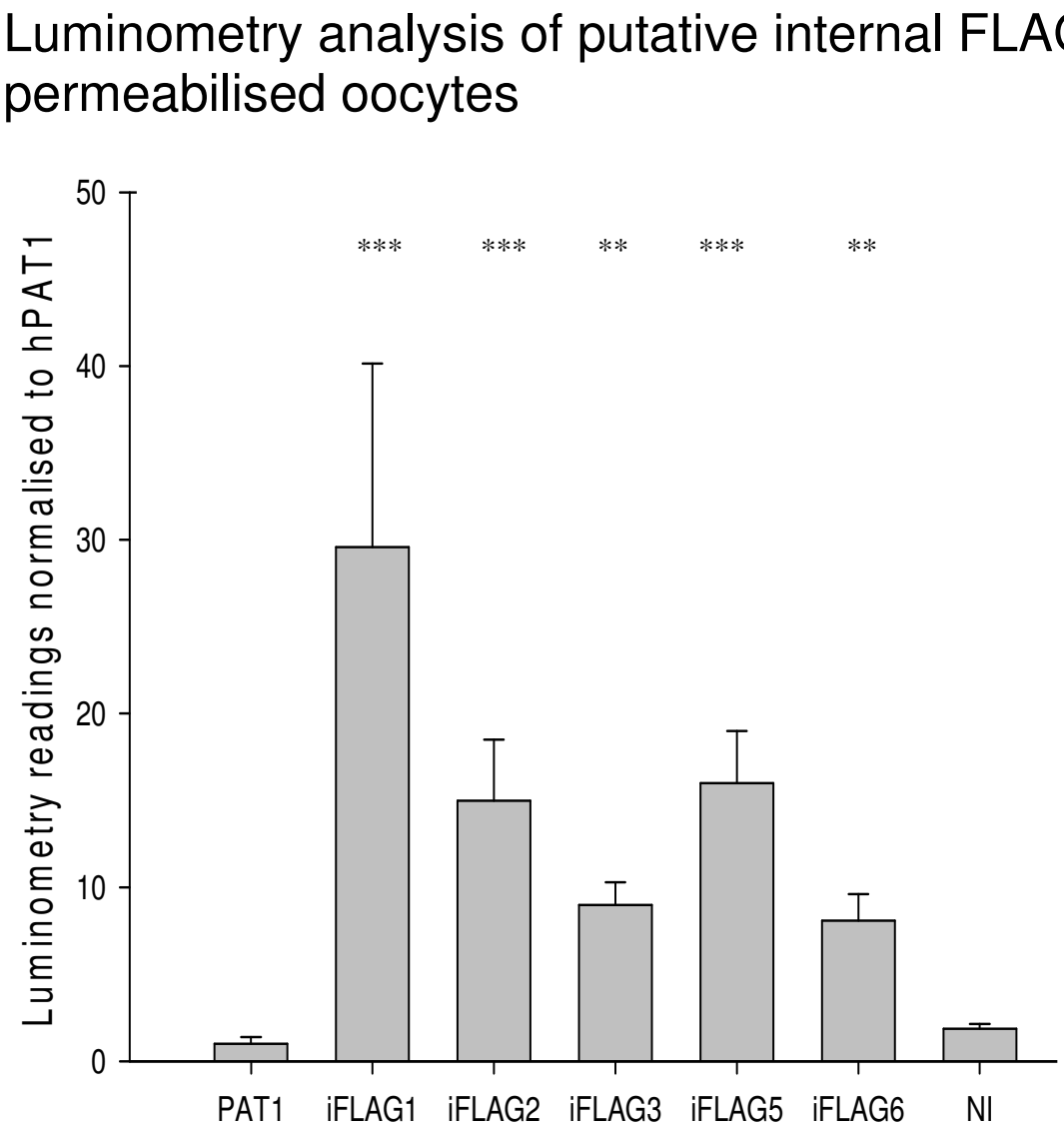

Figure 5.8a: Normalised luminometry readings performed on permeabilised oocytes. TritonX was used as the permeabilising reagent. The significance levels are those observed on comparison with the hPAT1 injected oocytes. (** $p < 0.01$, ***$p < 0.001$). n=4 oocyte preparations.

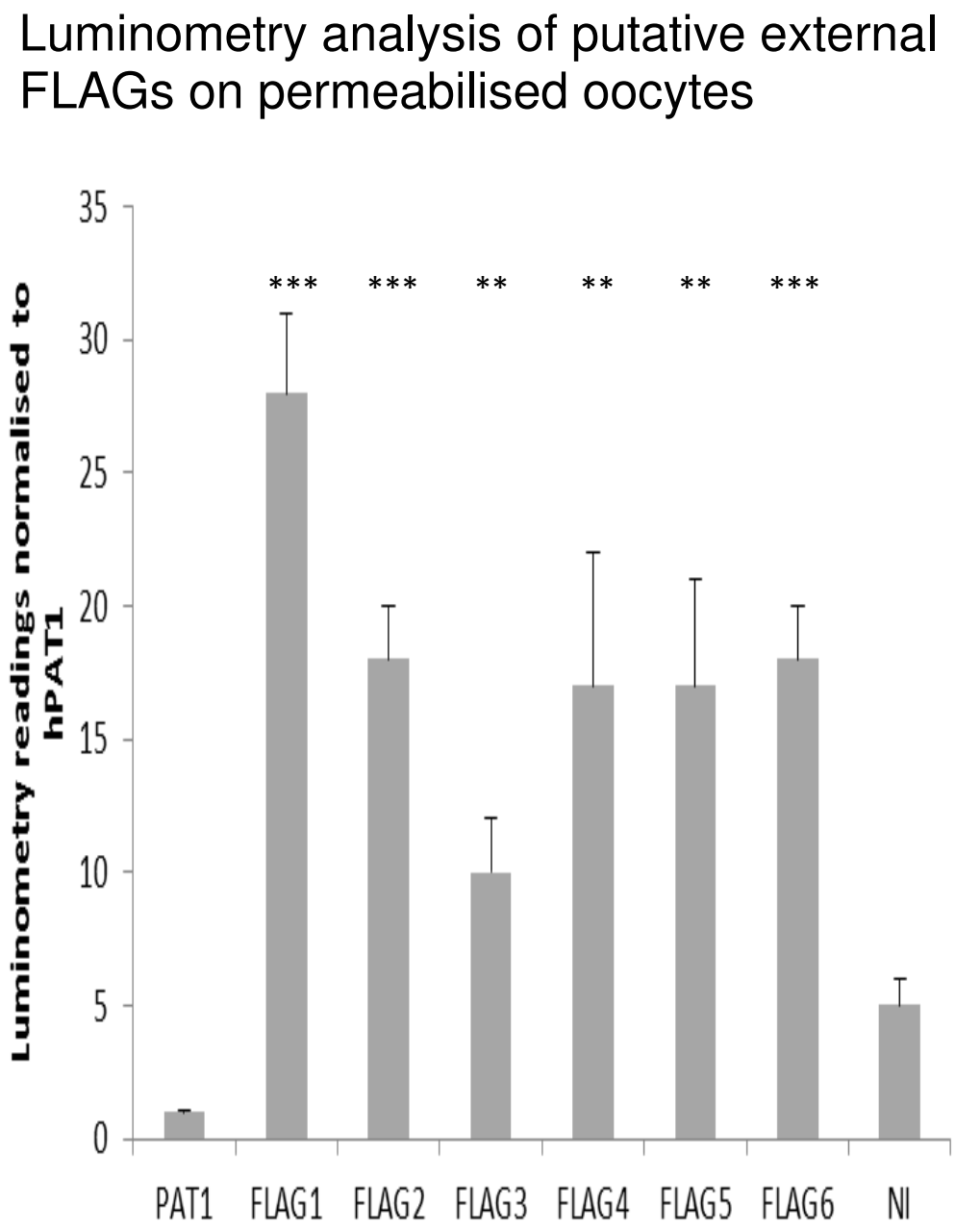

Figure 5.8b: Normalised luminometry readings performed on permeabilised oocytes (external FLAGs). TritonX was used as the permeabilising reagent. The significance levels are those observed on comparison with the hPAT1 injected oocytes. (** $p < 0.01$, ***$p < 0.001$). n=4 oocyte preparations.

5.4 Modelling studies on hPAT1

Due to the difficulties associated with crystallizing transporters and obtaining a tertiary structure, there is an increasing amount of focus on modelling studies. In recent years, a number of different software have been developed to study transporters using homology modeling. Different algorithms are used to analyse amino acid sequence of the protein and predict the where the helices lie (similar to TMD prediction software). This data is then used to model the transporter against a transporter for which the crystal structure is available.

One such modelling software is provided on the LOMETS server (Zhang lab, University of Michigan, Wu, S. and Zhang, Y., 2007). Based on homology modelling, the software modelled hPAT1 onto the *E.coli* transporter AdiC. According to this model hPAT1 has 11 TMDs. The model is produced based on the hydropathy plots obtained from PSI-PRED. The results obtained from this prediction are shown in figure 5.9 across the following two pages


Conf:
Pred:
Pred: CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
AA: MSTQRLRNEDYHDYSSTDVSPEESPSEGLNNLSSPGSYQR
10 20 30 40
Conf:
Pred:
Pred: CCCCCCCCHHHHHHHHHHHHHHHHHHHHHHHHHHCCHHHH
AA: FGQSNSTTWFQTLIHLLKGNIGTGLLGLPLAVKNAGIVMG
50 60 70 80
Conf:
Pred:
Pred: HHHHHHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCHHHHH
AA: PISLLIIGIVAVHCMGILVKCAHHFCRRLNKSFVDYGDTV
90 100 110 120
Conf:
Pred:
Pred: HHCCCCCCHHHHHHCCCCHHHHHHHHHHHHHHHHHHHHH
AA: MYGLESSPCSWLRNHAHWGRRVVDFFLIVTQLGFCCVYFV
130 140 150 160
Conf:
Pred:
Pred: HHHHHHHHHHHHHCCCCCCCCCCCCCCCCCCCCHHHHHHH
AA: FLADNFKQVIEAANGTTNNCHNNETVILTPTMDSRLYMLS
170 180 190 200
Conf:
Pred:
Pred: HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHEEEEC
AA: FLPFLVLLVFIRNLRALSIFSLLANITMLVSLVMIYQFIV
210 220 230 240
Conf:
Pred:
Pred: CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
AA: QRIPDPSHLPLVAPWKTYPLFFGTAIFSFEGIGMVLPLEN
250 260 270 280

Figure 5.9: Results used for prediction of structure by LOMETS. LOMETS generates hydropathy plots and then uses the information to generate the model.

Before generating a model, the LOMETS server analyses the scores for target to template alignment using 8 programs, FUGENE, HHsearch, MUSTER, PPA, PROSPECT2, SAMT02, SPARKS and SP3. Based on the data generated from these, the LOMETS server then provides a summary of the various transporter molecules that the test transporter molecule has been aligned to and the scores obtained.

The hPAT model obtained by this prediction is shown in figure 5.10. Highlighted in red are the first and last amino acid residues as predicted by MEMSAT3. The predictions according to the model and MEMSAT3 (epitope tagging experiments) are in agreement. Also indicated in the model (in dark blue) is the amino acid 63 which will be discussed more in detail in the next section.

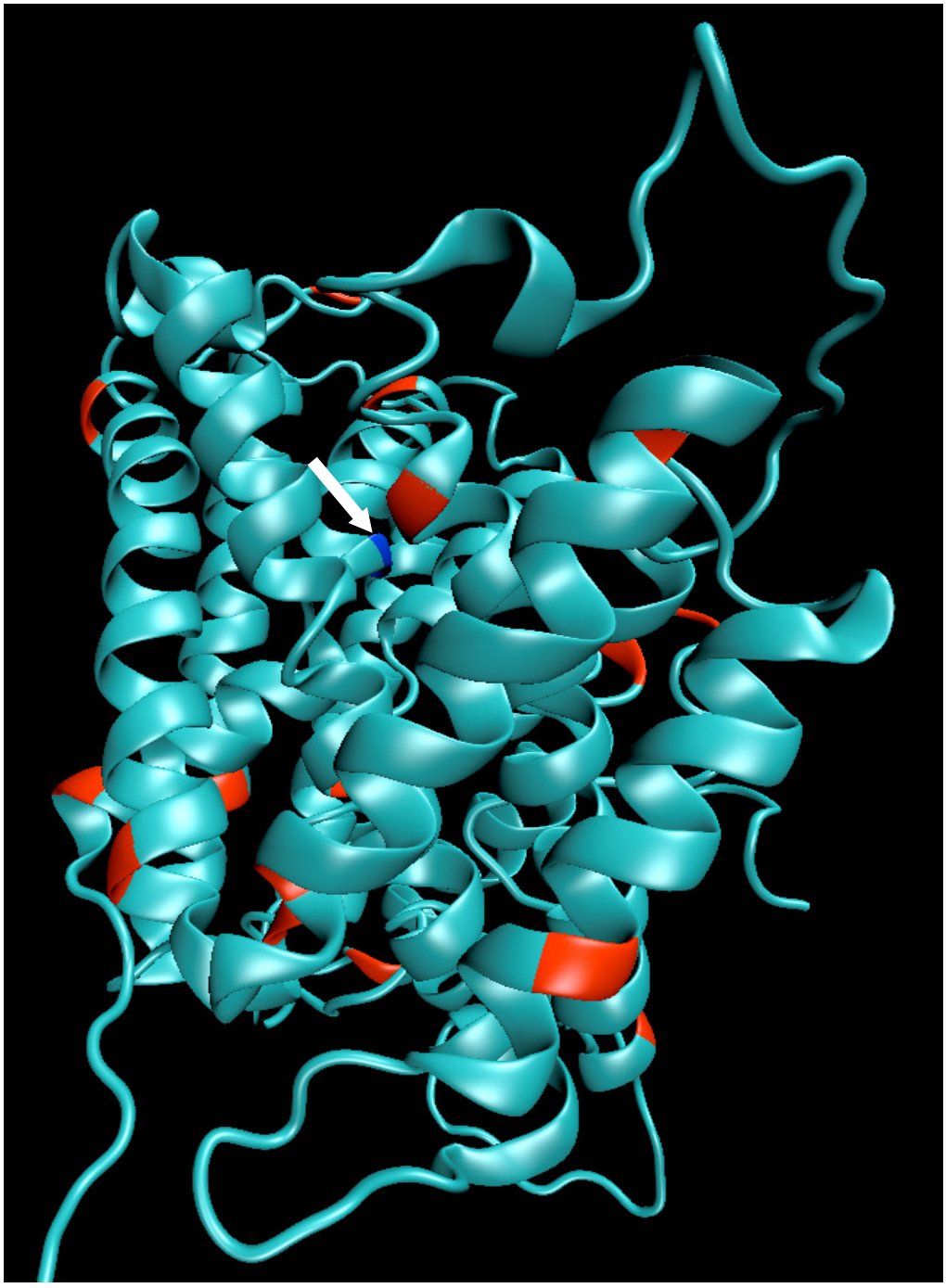

Figure.5.10: hPAT1 model as predicted by homolog modeling hPAT1 against Adic using LOMETS. There are 11 TMDs. The figure shows an inernal N-terminal and an external C-terminal. The first and last amino acids of each TMD as predicted by MEMSAT3 are shown in red. Amino acid 63 (which is discussed further in the next section is shown in dark blue and indicated with the white arrow).

There have also been predictions made using the crystal structures from LeuTAa. According to predictions made from these transporter families SLC6, SLC7, SLC36 and SLC38 show an inverted 5+5 repeat. SLC6 and dSLC7 show 12 TMDs i.e. 2 more TMDs after the 5+5 inverted repeat. SLC36 and SLC38 do not show the 12th TMD and have an 11 TMD structure (Broer and Palacin 2011).

Figure 5.11: The 5+5 inverted repeat structure which is shown by a few families of transporters including SLC6, SLC7, SLC36, and SLC38. SLC36 and 38 do not have the last TMD (Broer and Palacin 2011). Figure from Yamashita *et al.*, 2005.

5.5 The champagne mutation

The champagne mutation was first discovered in horses. The champagne coat colour is much sort after by horse breeders. Large scale genetic screens had been performed to check what the source of this coat colour was. A single point mutation was observed in exon 2 of the PAT1 gene (Cook *et al.*, 2008). The mutation is at nucleotide position 76 of exon 2 and causes a C to a G base. This in turn causes a change in the amino acid being formed at position 63 from a threonine to an arginine. The threonine residue is observed to be conserved across species as shown in Figure 5.12.

Figure 5.12: Sequence alignment of the PAT1 across species showing a conserved Threonine residue at amino acid position 63. From Cook *et al.*, 2008

Using the model obtained from LOMETS, amino acid 63 was marked onto it (figure. 5.9). According to the LOMETS prediction this site is also predicted to be one of the potential substrate binding sites. The location of this site (based on the model), in the middle of a TMD which is fairly close to the middle of the transporter protein.

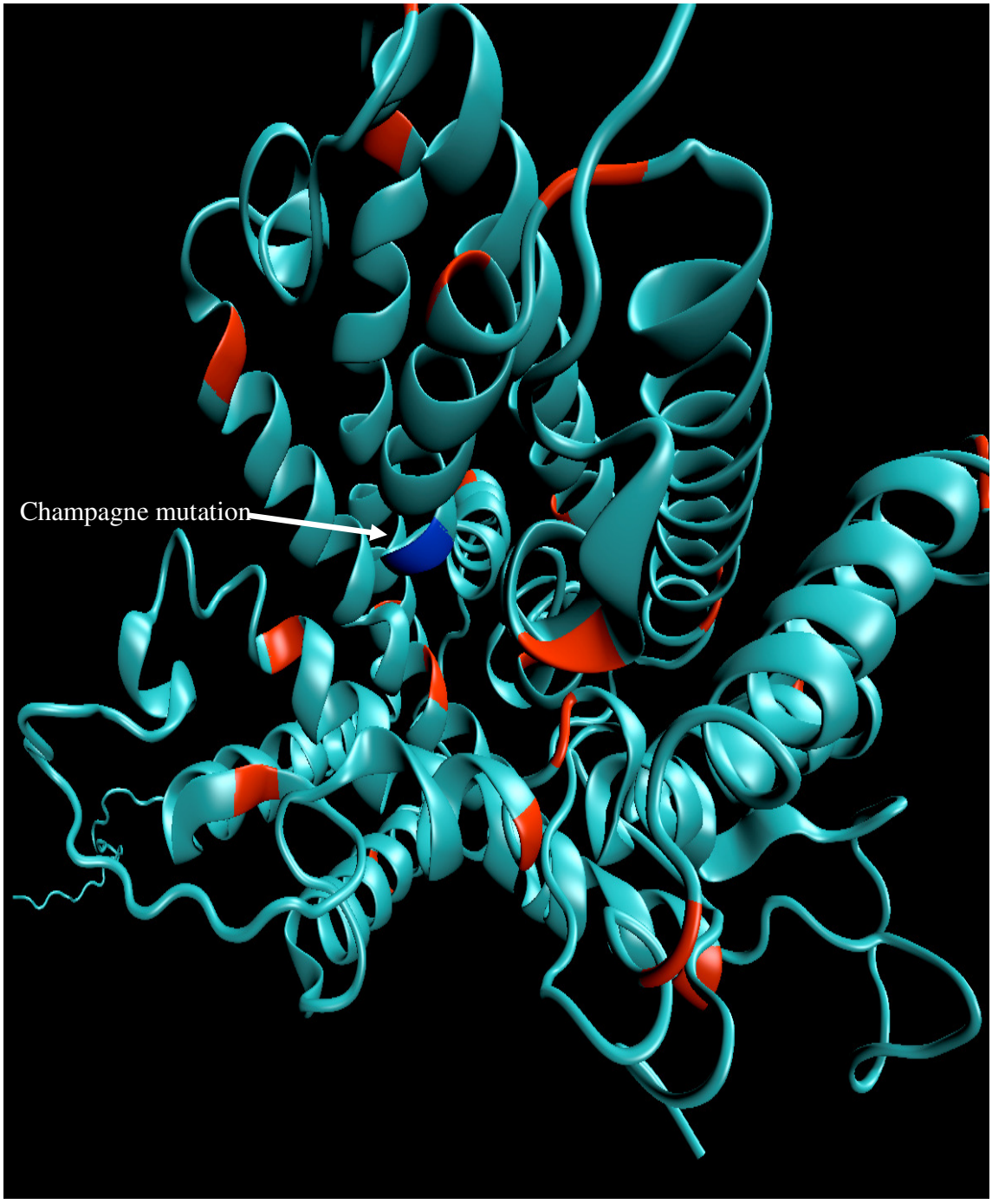

Figure 5.13: Modelling of hPAT1 on AdiC with the champagne mutation. The orientation is such that the transporter is viewed from the extracellular region into the intracellular region. The champagne mutation is marked in dark blue and is noticed to be in the first TMD both according to the model and MEMSAT3 prediction.

5.6 Chapter summary

- Various trans membrane domain (TMD) prediction software predict between 9 – 11 TMDs.
- MEMSAT3 prediction suggested 11 TMDs with an internal N-terminal and an external C-terminal.
- Six putative external FLAGs were designed. These were confirmed for transport function prior to performing luminometry assays. Each of these FLAGs was observed to show a significantly higher luminometry reading compared to the hPAT1 and non injected oocytes.
- Five putative interrnal FLAGs were designed. These were confirmed for transport function prior to performing luminometry assays. Each of the FLAGs was observed to show no difference in luminometry compared to hPAT1 and non injected oocytes. To confirm that the absence of signal was due to the region being internal rather than due to the fact that the antibody cannot access the epitope, the oocytes were permeabilised. On permeabilisation, the oocytes were observed to show a significantly higher uptake compared to the non injected oocytes. Similar data was also observed on permeabilisation of oocytes injected with the external epitope tags.
- Modeling studies using LOMETs also indicates an 11 TMD structure.
- The LOMETs prediction also shows that the champagne residue at amino acid 63 is found to lie within the first TMD and is one of the regions thought to play a role in substrate binding/recognition.

Chapter 6:
Characterization of hPAT3

hPAT3, like hPAT4 has always been characterized as an orphan transportrer as discussed in the introduction. Unlike hPAT1 and 2, which are expressed in a range of organs and hPAT4 which is expressed ubiquitously, hPAT3 expression has only been noted in mouse testis. 3.1 refers to the hPAT3 variant 1 which is 123 bp longer than 3.2 (variant 2).

6.1 Testing hPAT3 function in oocytes

Both the clones of hPAT3 (variant 1 and variant 2) were subcloned into the pXT7 Xenopus oocyte vector (as described in section materials and methods). Transport assays using [^{3}H] L-Proline were set up under different conditions. Proline was selected as a substrate since it was observed to be a good substrate for both the transporters hPAT1 and 2 and the orphan transporter hPAT4 which we characterized. Basic influx assays were set up across a range of pH. The pHs at which hPAT3 transport activity was tested were pH 5.5 (which is the optimal pH for hPAT1 and hPAT2), pH 7.4 (which is the pH at which hPAT4 showed optimal transport activity), pH 8.4 and pH9.4. The reason for selecting an alkaline pH higher than 7.4 was the localization of hPAT3 in the testis and that due to transporters including those such as HCO_3^- transporters it is possible that hPAT3 works best in an alkaline environment.
The experiments were performed along with hPAT4 as control.
Preliminary experiments indicated some pH dependence for hPAT3 transport with transport being more rapid at higher pH. However repeated experiments indicated that hPAT3 did not always transport proline across the membrane and the uptake wasn't always significant. It was therefore considered that hPAT3 perhaps has an ionic dependence (eg. Na^+ dependence).
Experiments were set up for influx assays at different pHs but with Na^+ containing medium. The pH dependence then appeared to shift towards 7.4 with maximum uptake at this pH. Despite this however the uptakes were not consistently significantly greater than non injected oocytes. It was therefore decided to use cell lines to study PAT3 function.

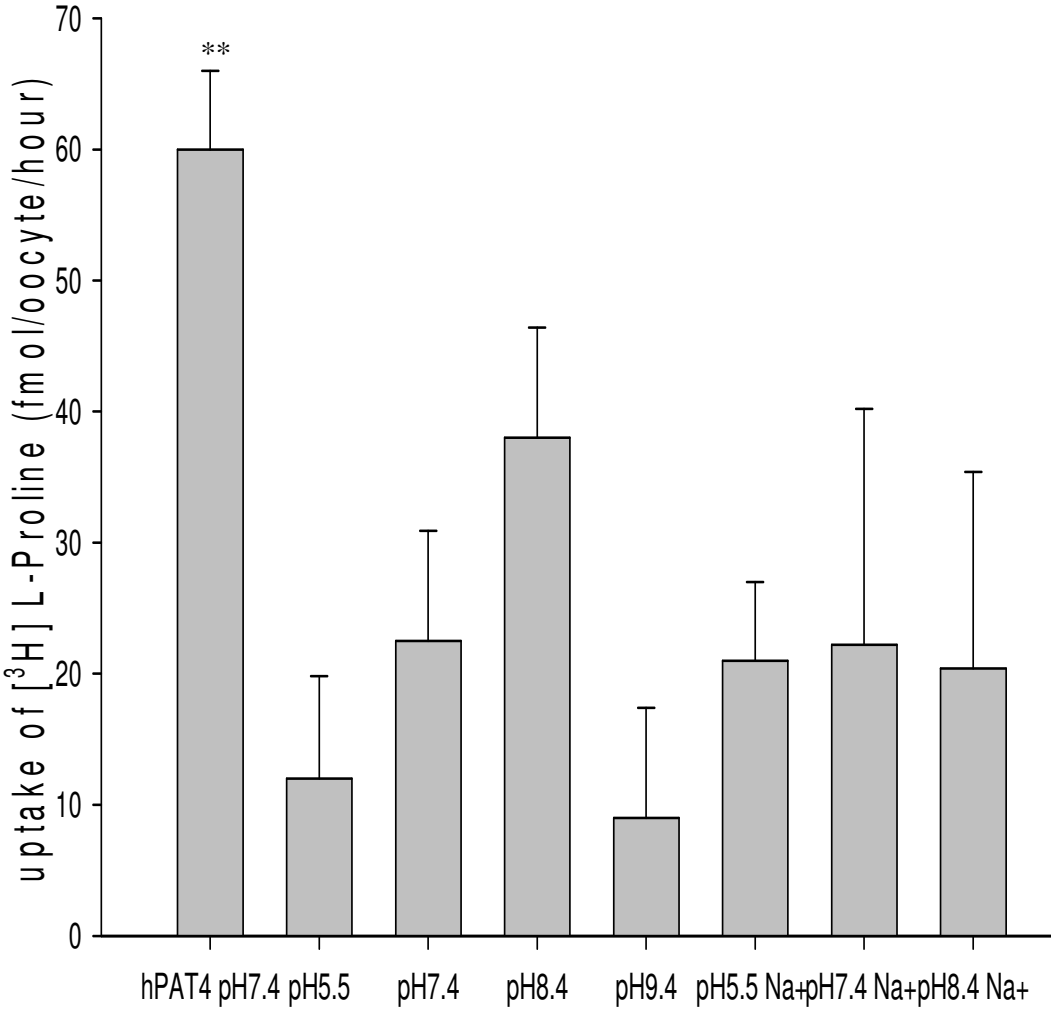

Figure 6.1: Mediated uptake of [^{3}H] L-Proline by hPAT3.2. Uptake was corrected for non injected oocytes. hPAT4 in Na^+ free medium at pH5.5 was used as a positive control. (** $p < 0.01$) n>5 oocyte preparations. Concentration of L-Proline in the tracer 0.25 µM.

6.2 Testing PAT endogenous expression in the TM4 cell line

6.2.1 Introduction

TM4 is a mouse cell line. Its origin is testicular cells (Sertoli cells) from a 11-13 day old mouse pup. This cell line is commonly used to study features which are common to the testicular cells. As discussed in the introduction PAT3 was found to be specific only to the testis.

Using the mouse testicular cell line presented two options regarding how to proceed with the experimental analysis

i) To check if mPAT3 (murine PAT3) was endogenously expressed in TM4 cells. If this was the case we could directly use this cell line to perform transport assays and characterize its function

ii) If the cell line did not express mPAT3, we could transfect this with hPAT3. Since the cell line is of testicular origin it would give us a suitable environment to study the transport function.

6.2.2 Checking for endogenous expression of mPAT3 in TM4 cells

To check for the presence of mPAT3 in TM4 cells, the principle of reverse transcription polymerase chain reaction (RT-PCR) was used. In this process RNA from cells was used to synthesize cDNA which was then used as a template for PCR reaction

RT-PCR reactions were set up using + RT and –RT template with primers specific to exon-exon boundaries. GAPDH was used as a positive control. The sample from the PCR was run on a gel and the results analyzed.

There were no fragments observed for the mPAT3 +RT samples as shown in figure 6.2. There was however a fragment for the positive control GAPDH. Since mPAT3 was absent in the TM4 cells, it was decided to transfect these cells with hPAT3.

Prior to transfection however two things needed to be done

1) To check if TM3 expressed any other PATs (i.e. mPATs)

2) To subclone the hPATs into a mammalian vector

Primers spanning exon-exon boundaries were designed for mPATs 1, 2 and 4. Repeated experiments were performed using different batches of cells and RNA but no fragments were observed. Different annealing temperatures and $MgCl_2$ concentrations were also used to ensure that the absence of fragments were to ensure that the absence of fragments was not due to the use of non optimal PCR conditions.
cDNA was synthesised as explained in the materials and methods and used as a template for PCR reactions. GAPDH was used as a positive control. The PCR products were then analysed on a 1% gel. The results obtained are shown in figure 6.3.

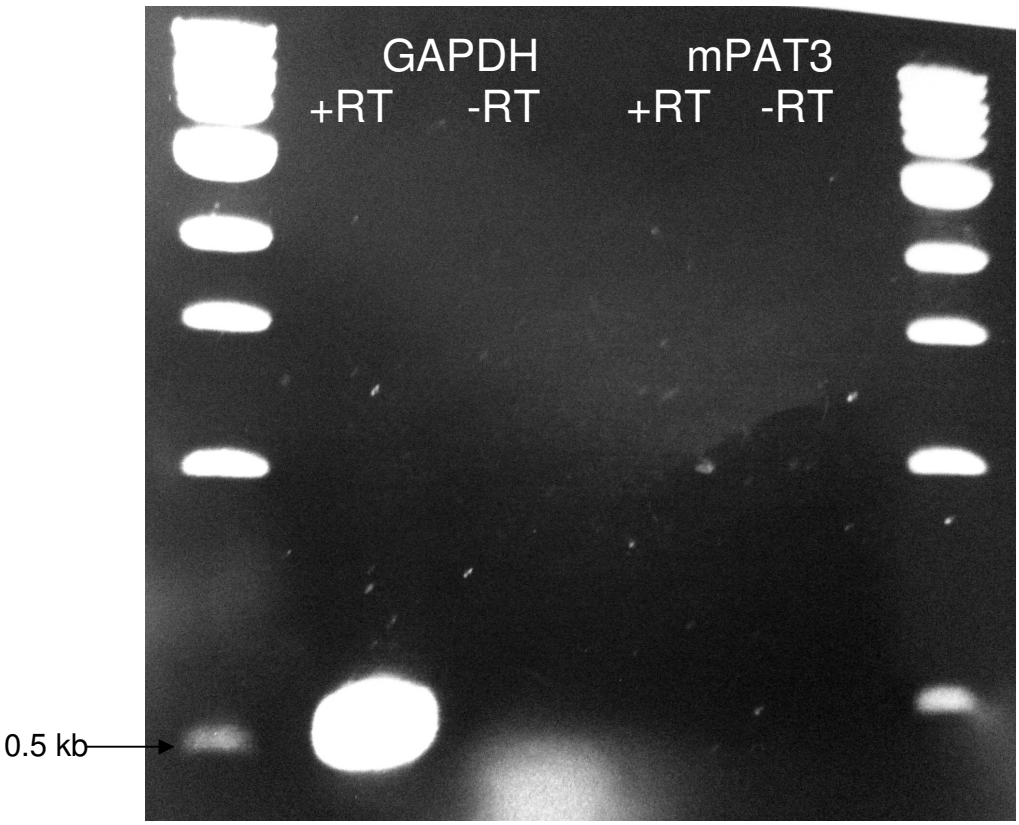

Figure 6.2: PCR products from reverse transcription PCR of mPAT3 and GAPDH. +RT and –RT indicate the presence and absence of reverse transcriptase in the cDNA synthesis process respectively.

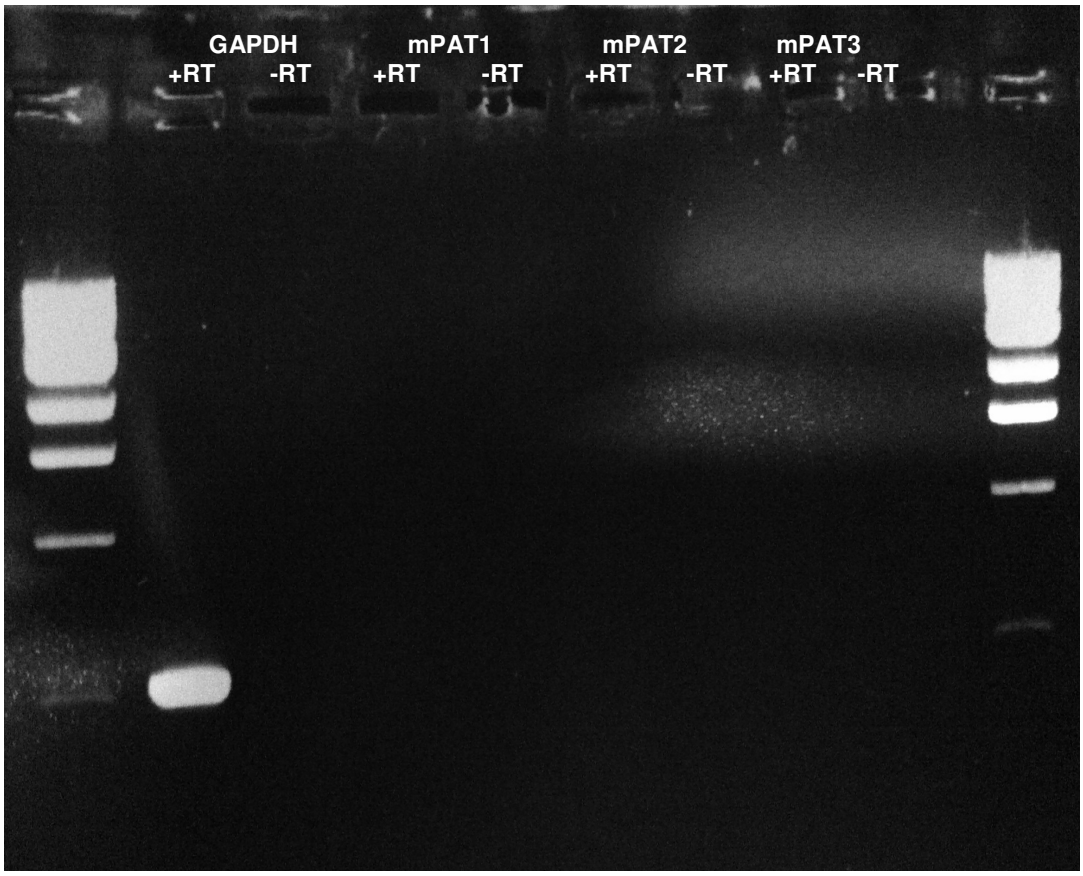

Figure 6.3: PCR products from reverse transcription PCR of mPAT1, PAT2, mPAT4 and GAPDH. +RT and –RT indicate the presence and absence of reverse transcriptase in the cDNA synthesis process respectively.

6.3 hPAT3 studies in TM4 and HEK293 cell lines

HEK293 was used as a control to check for transfection efficiencies and compare the results obtained.

6.3.1 Confocal microscopy

TM4 cells and HEK293 cells were analysed using confocal microscopy to study the transfection efficiencies in these cells. EGFP-C and EGFP-N were obtained from Dr. Ryan Pink (Oxford Brookes University) and both were observed to give similar efficiencies. Figure 6.4a and 6.4b show the confocal images obtained when co-transfection experiments were performed in HEK293 cells and TM4 cells respectively. These experiments were performed to check whether there was any difference in the transfections observed for GFP controls. The results from imaging analysis show that the efficiencies did not vary depending on the kind of GFP used. Therefore, it was decided to use EGFP-C in the cotransfection experiments.

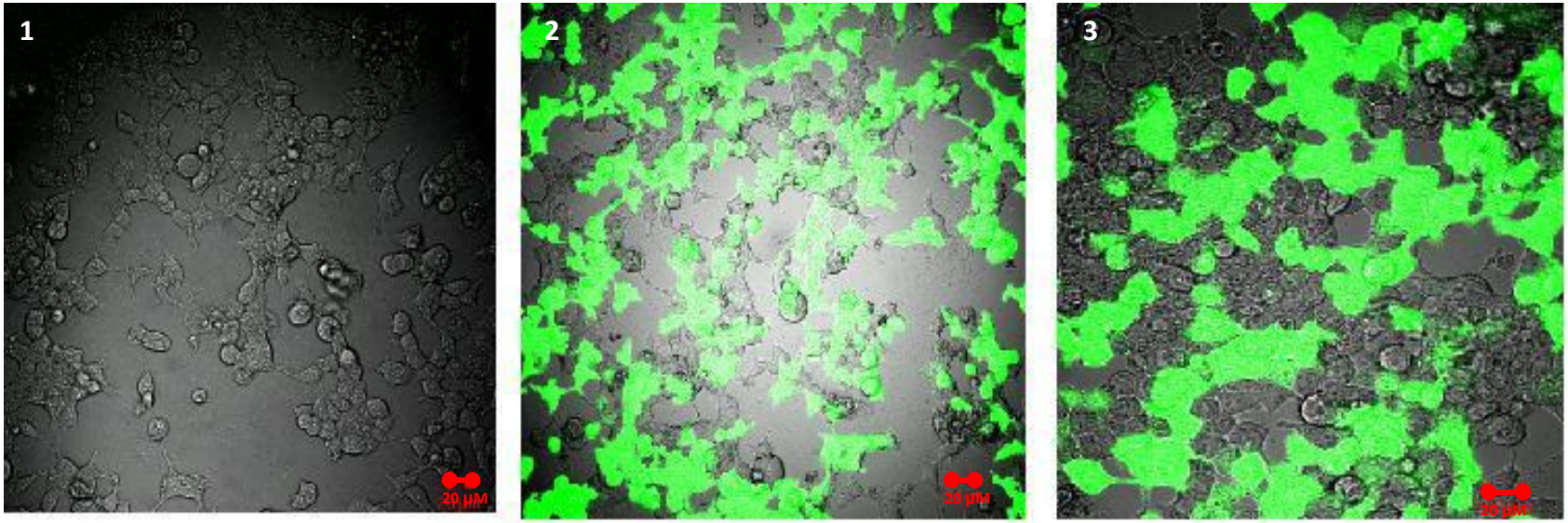

Figure 6.4a: Images obtained for HEK293 cells using confocal microscopy. The cells were grown in confocal compatible dishes and imaged after 48 hours. In both panels A and B, 1 is the cells transfected with pcDNA3.1(-) alone, 2 is the EGFP-C transfected cells and 3 is the EGFP-N transfected cells. The length between the two red dots in each images is 20 microns. In each case the amount of DNA used was constant and for the EGFP transfections, half the amount of pcDNA vector was used and the remaining was the EGFP construct.

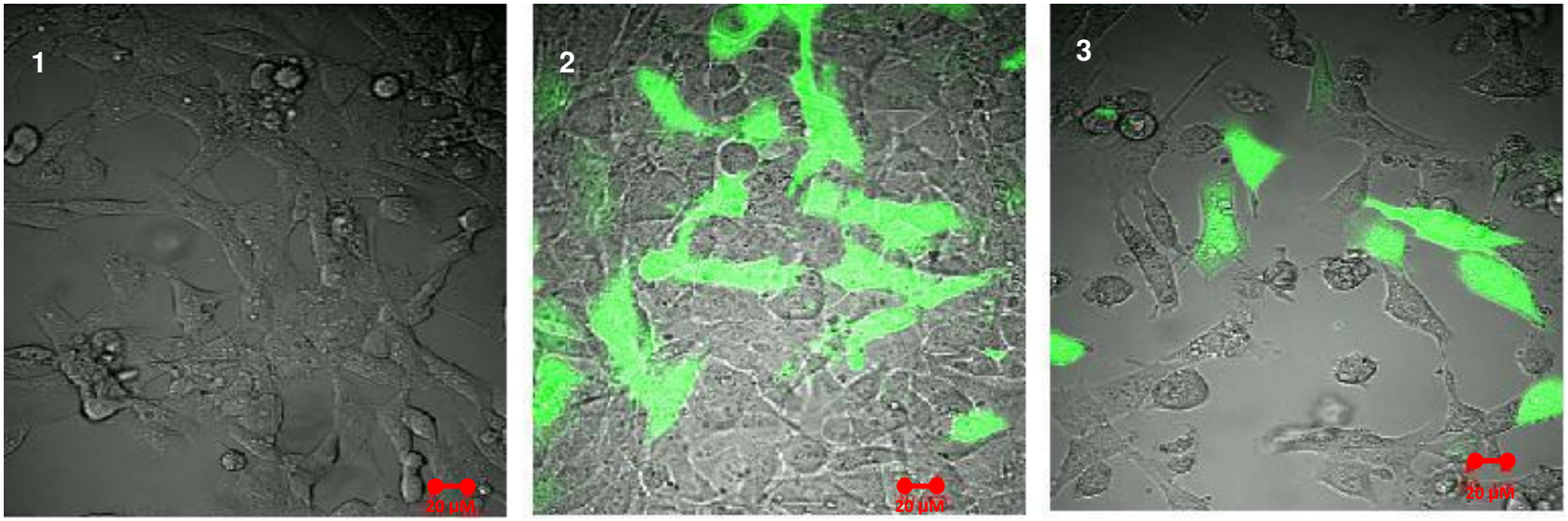

Figure 6.4b: Images obtained for TM4 cells using confocal microscopy. The cells were grown in confocal compatible dishes. 1 is the cells transfected with pcDNA3.1(-) alone, 2 is the EGFP-C transfected cells and 3 is the EGFP-N transfected cells. The length between the two red dots in each images is 20 microns. In each case the amount of DNA used was constant and for the EGFP transfections, half the amount of pcDNA vector was used and the remaining was the EGFP construct.

EGFP-C was purified to obtain very high quality DNA and used in co transfection experiments. This means that half the amount of DNA was EGFP-C control and remaining was the test plasmid (i.e. the empty vector or vector with hPAT).

HEK cells were observed to show a high efficiency in the range of >80%, 48 hours post transfection. TM4 cells on the other hand showed a lower efficiency, in the range of <50 %. As expected, the GFP was observed to localize to the entire cytoplasmic region. Results obtained from cotransfection experiments maintaining the total amount of DNA constant and using the same amount of EGFP-C in each case are shown in figure 6.5a (for HEK cells) and 6.5b (for HEK293 cells).

The images show that the efficiencies observed for the different co transfection was uniform and these cells could then be used to perform transport assays and check for the amount of protein.

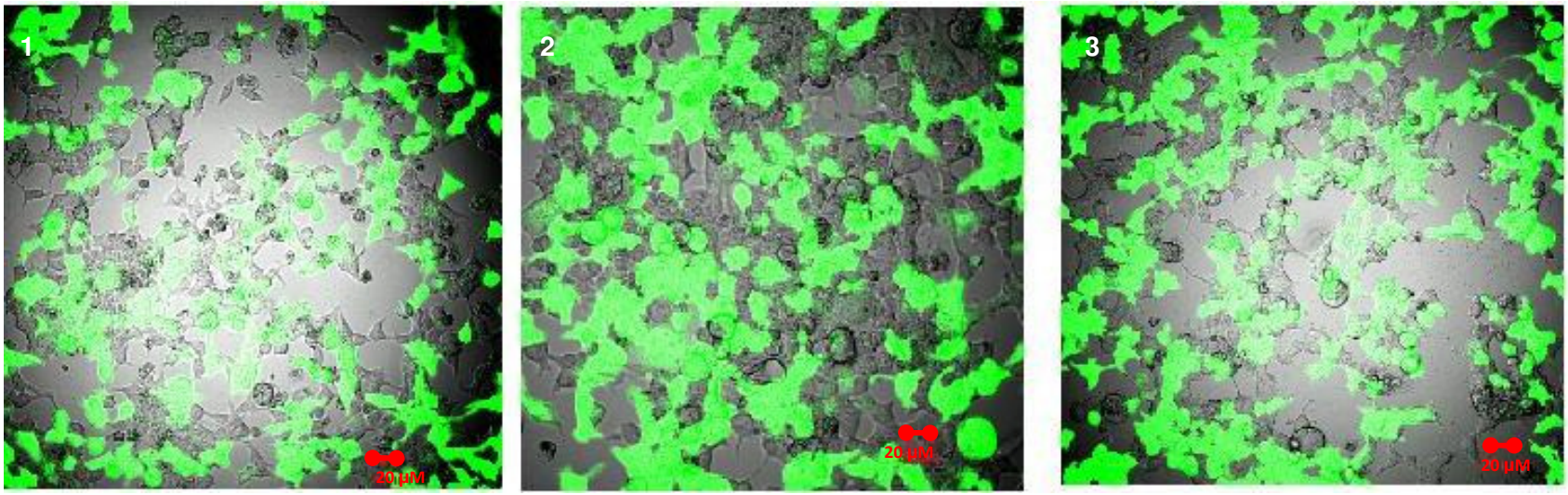

Figure 6.5a: Confocal microscopy of HEK293 cells showing results from cotransfection experiments. hPATs 2, 3.1 and 3.2 were cotransfected with EGFP-C and the results obtained are shown in panels 1, 2 and 3 respectively.

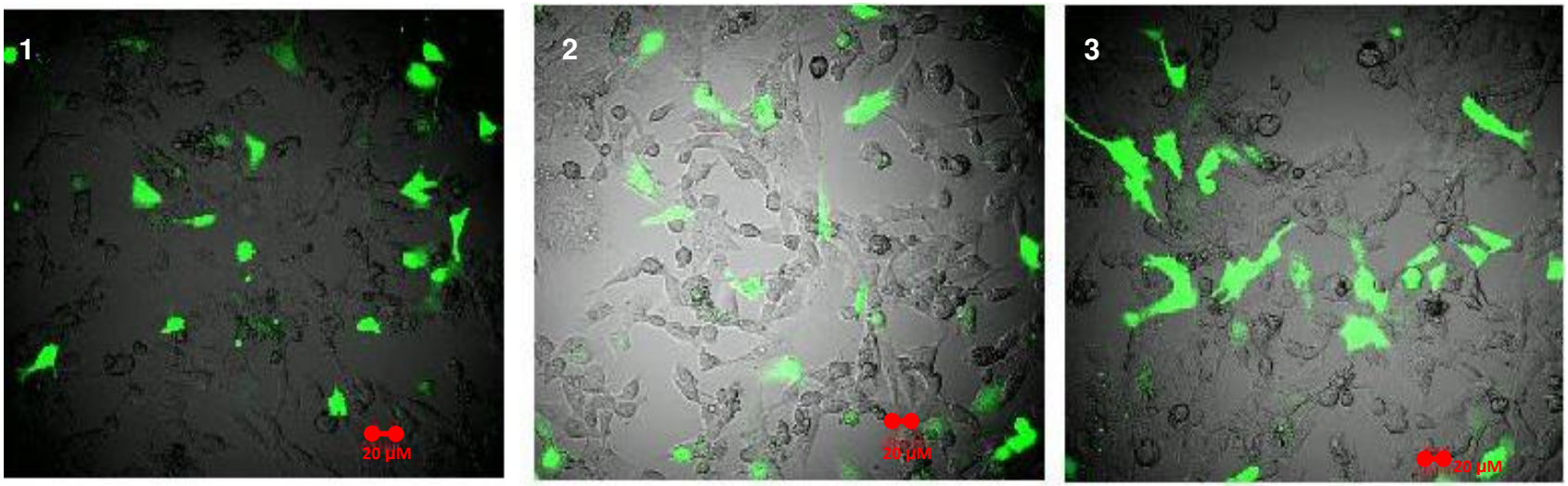

Figure 6.5b: Confocal microscopy of TM4 cells showing results from cotransfection experiments. hPATs 2, 3.1 and 3.2 were cotransfected with EGFP-C and the results obtained are shown in panels 1, 2 and 3 respectively.

6.3.2 hPAT3 transport studies in cell lines

Fresh Krebs Ringer solution was prepared and used (materials and methods) for the different stages of uptake assays in the TM4 cell line. The media was prepared at three different pH conditions 5.5, 7.4 and 8.4. Experiments were performed using [^{3}H] L-Proline as tracer. Uptake experiments were performed for 15 mins. After washing with Krebs Ringers solution at the same pH three times, the cells were washed, lysed and triplicates of the lysis products were analysed. A portion of the lysis product was also used to measure the protein content when compared to BSA standards. The average of the data obtained was then corrected for the amount of protein and represented as fmol/mg of protein. The values for the hPATs were then compared to the control (empty pcDNA vector).

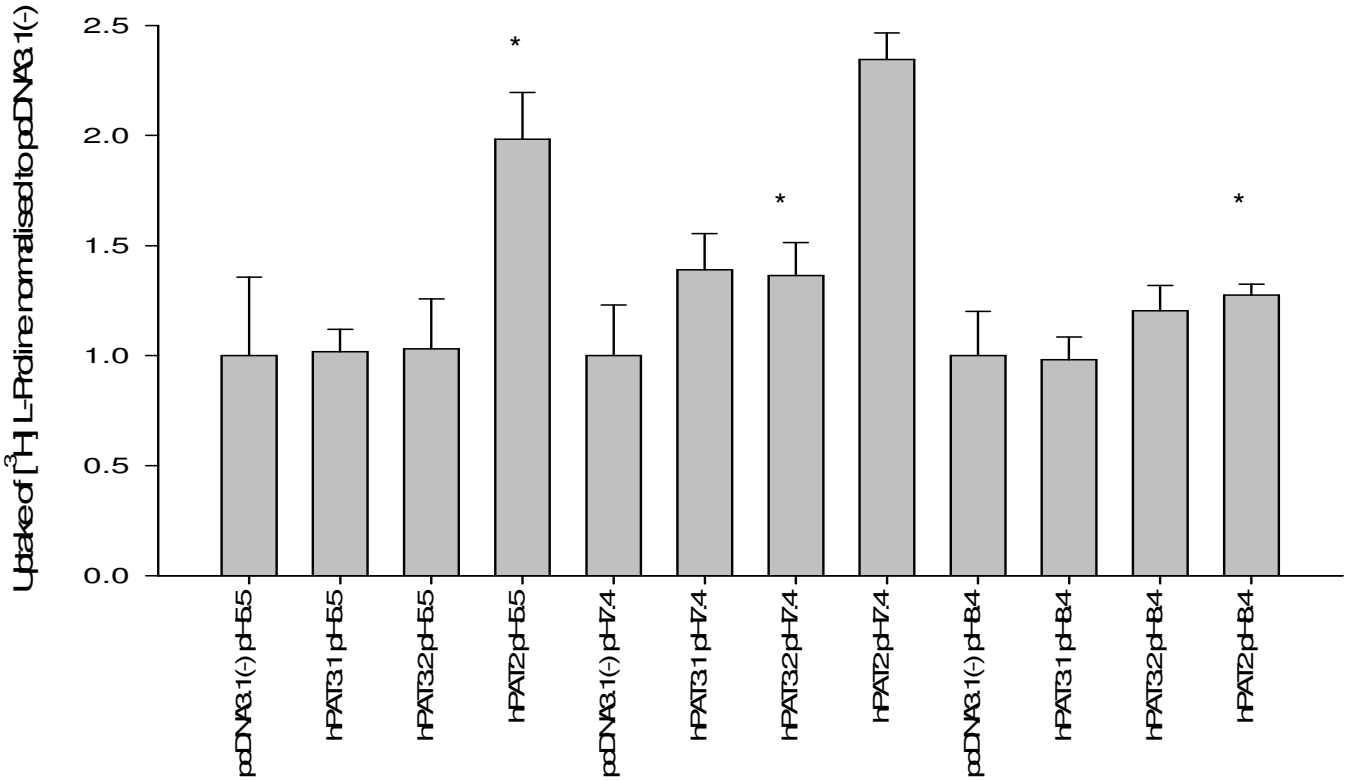

Figure 6.6: Uptake studies of [^{3}H] L-Proline in TM4 cells expressig either hPAT3.1 or hPAT3.2. The cells were co-transfected with EGFP-C and the hPAT. The data has been normalised to the uptake observed for pcDNA3.1(-) at the corresponding pH. The values for pcDNA3.1(-) at pH 5.5, 7.4 and 8.4 were 9.46 +/- 1.20 fmol/mg protein, 36.20 +/- 8.31 fmol/mg protein and 57.53+/- 11.6 fmol/mg protein respectively (* $p<0.05$). n=2 to 7 experiments.

Concentration of L-Proline in the tracer 0.25 µM.

6.4 Chapter summary

- An attempt to characterize hPAT3 (the two splice variants, here referred to as hPAT3.1 and hPAT3.2) was made in *Xenopus laevis* oocytes and in cell culture.
- Attempts to chatracterize the transporter included testing whether it could bring about transport of [^{3}H] L-Proline across the membrane depending on the pH and whether the transporter showed a Na^+ dependence.
- To test the expression of this transporter in cell lines, the mouse Sertoli cell line TM4 was selected alongside HEK293 (which is a very well characterized cell line).
- No endogenous expression of mPAT3 was detected in these cells and so it was decided to transfect this cell line with hPAT3.1 and hPAT3.2 in pcDNA3.1(-), while performing an EGFP co-transfection to check for transfection.
- Experiments were performed using co transfection of hPAT 2, 3.1 or 3.2 and EGFP in the Sertoli cell line. Preliminary experiments have showed that hPAT3.2 appears to transport at pH7.4. No transport function was observed for hPAT3.1.

Chapter 7:
Discussion

7.1 Introduction

In recent years transporters have received a lot of attention due to the important role they play in trafficking specific drugs across the membrane of target cells and organelles. There are a few transporter families that still rely on H^+ as its driving force, these include PepT1 and PATs.

The increasing attention they have drawn is driven by the broad range of substrates they can transport. For instance for PepT1, apart from peptides which are transported, there are a variety of other substrates including therapeutic drugs like β lactam antibiotics, ACE inhibitors, rennin inhibitors and bestatin which also bind to PepT1 and are successfully transported across the membrane (Han and Amidon 2000).

Similarly the PAT family of transporters (PAT1 and 2) which are primarily transporters for the small neutral amino acids glycine, alanine and proline have also been shown to transport therapeutic drugs such as δ-aminolevulinic acid (Frølund *et al*. 2010).

7.2 Functional studies on hPAT4

7.2.1 Summary of findings and its relevance

PAT4 had been placed into the PAT family based on sequence homology and until the studies reported here had been categorised as an orphan transporter. Based on the experiments performed using expression of cRNA in Xenopus laevis oocytes, we could show that hPAT4 is a pH dependent transporter which functions best at a pH of 7.4. Like hPATs 1 and 2, this transporter was Na^+ independent. But unlike PATs 1 and 2, hPAT4 was shown to be electroneutral and the transport did not appear to be proton coupled.

PATs 1 and 2 had the highest affinities for proline, alanine and glycine (with proline having the highest affinity and glycine the lowest). hPAT4 on the other hand could transport both proline and alanine but could neither bind nor transport glycine.

A variety of other substrates were tested including the 20 naturally occurring amino acids. Of these amino acids [^{3}H] L-Proline uptake was inhibited by proline, alanine,

tryptophan, cysteine, threonine, glutamine and methionine. A very high affinity was observed for proline and tryptophan (3.1+/-1.1 μM for proline and 1.7+/-1.1 μM for tryptophan) when compared to the others.

7.2.2 PATs and mTOR signaling

To understand the role of amino acid sensing and its significance in maintaining cellular homeostasis and nutrition levels, it is important to understand the mTOR signaling pathway. The mammalian Target of Rapamycin (mTOR) plays a critical role in nutrient sensing and growth.

Rapamycin was isolated from soil samples and was observed to exhibit immune suppressive properties (Heitman, Movva and Hall, 1991). In view of this Rapamycin analogs are being used in various clinical trials as a potential treatment for cancer.

TOR is a highly conserved Serine Threonine kinase which is highly conserved across species. It has been shown to play an important role in a variety of processes including cellular metabolism and cell survival. In more recent years however, TOR has been receiving a lot of attention on account of data which implicates it in a variety of diseases including cancer and neurodegenerative disorders. The role of mTOR is via two separate factors mammalian Target of Rapamycin Component 1 (mTORC1) and mTORC2. TOR binds Rapamycin and inhibits the Target of Rapamycin component 1(TORC1), though evidence also suggests that TORC2 might also be inhibited (Sarbassov *et al*. 2006). mTORC1 has been observed to play an important role in responding to changes in the growth factor and amino acid levels. The mechanism by which mTOR detects these changes remains largely unknown. In response to the stimulation by amino acids, mTOR is shuttled to the late endosomes and lysosomes where it can bind the Ragulator-Reg complex thereby being assembled into the active mTORC1 (Goberdhan, 2010).

In recent years, a variety of transporters have been observed in mTOR activation (mTOR stands for the mammalian target of rapamycin). mTOR regulation is governed by the local nutrient availability and is particularly sensitive to amino acids. These transporters have been observed to be very different from each other in terms

of the amino acids they transport and the affinities. However all of them have been observed to transport the essential amino acids into the cell, especially leucine.

PAT1 has been found to localize to the lysosomal membrane. The localization of hPAT4 on the other hand remains unknown. Taking into account the characteristics of hPAT4 (properties such as enzyme kinetics), it is likely that both the PAT transporters (hPAT4 and hPAT1) are expressed in the same region and interact to aid in the mTOR signaling pathway. This becomes more likely when taking into account the upregulation of mTOR, hPAT1 and hPAT4 in cancer cell lines.

7.2.3 The role of Leucine in signalling

Leucine has been shown to play a very important role in amino acid sensing by a number of groups (Hyde, Taylor and Hundal, 2003). In the model proposed we take into account the findings of this thesis and the recent work by Sabine et al. (ref). To summarise the results obtainined by performing the various transport assays, leucine was directy transported by hPAT4 as the data in this thesis shows. Surprisingly however leucine transport could not be inhibited by an excess of leucine and leucine transport could not be inhibited by proline. It is therefore suggested that leucine is transported by hPAT4 either by conjugating with another transport molecule i.e. a heterodimeric protein or by causing a change in conformation of a different transporter and bringing about an increase in transport of leucine.

The role of PATs in signaling

In the recent years a lot of attention has been drawn to the role of PATs in signaling. PAT1 and PAT4 have been found to be most important from this point of view. According to the studies performed in various cancer cell lines, PAT1 and PAT4 have been proposed to act in conjunction and aid in signaling.

PAT1 has been found to localize in the lysosomal membrane and in the brush border membrane of the intestinal epithelium (and the plasma membrane in some cell types). The sub cellular localization of PAT4 continues to remain unknown. Sabatini et al (year) have shown that PAT4 does not localize to the mitochondrial membrane. Taking into account the fact that PAT4 has been found to transport amino acids only at a pH of 7.4, this decreases the regions where PAT4 may be localized. It is however

possible that at a lower pH such as in the range of 6 which is observed in the late endosomes, PAT4 is found to bind substrates but not transport them and it is the binding which acts as a signal to sense the amino acid levels. Since between PAT1 and PAT4 they cover a wide range of amino acid substrates it is possible that they act in coordination to participate in mTOR signaling.

The amino acids are brought into the cytoplasm by transporters including PAT1 from the extracellular medium or via export from the lysosomes (the role of PAT4 at this stage depends on its localization). After

Due to the extremely high affinity of hPAT4 for amino acids like proline and tryptophan, it is likely that under normal physiological conditions the primary role of this protein is not to function as a transporter (since it will be saturated). However, along with PAT1, this transporter could be binding the substrates without necessarily transporting the substrates across the membrane, rather aiding in sensing the amino acid concentrations.

It is also likely that PAT4, when not saturated with amino acids could be causing an alteration in a different transporter that causes leucine transporter.

A change in the change in the amino acid concentration causes the activation of the mTOR pathway. Heublein *et al.* (2010) showed that hPAT1 and hPAT4 were necessary in cell lines such as HEK293 and the breast cancer cell line MCF7. In addition to this mTOR activation following amino acid stimulation also required PAT1 and PAT4. According to the model they predict, PATs regulate the activity of mTORC1 by controlling the intracellular response to amino acids and thus participate in mTOR regulation.

7.2.4 The PAT signalling story

The surprisingly high affinity of hPAT4 appears to indicate that hPAT4 may be important as a transceptor. Transceptors are molecules that along side bringing about transport of substrates across the membrane are also observed to act as receptors which participate in different processes including signalling pathways (Kriel *et al.* 2011). It is believed these transporter/receptors can sense the level of amino acid and act as a trigger for nutrient sensing (Hyde, Taylor and Hundal, 2003) Hundal and

Taylor, 2009). Further evidence for this was obtained by experiments performed in cell culture (Heublein *et al.*, 2010) which highlighted the impact of hPAT4 and its role in the signalling cascade (mTOR signalling). Following evidence from cell culture experiments, it is believed that the amino acid concentration is sensed by the hPATs (most likely hPAT4 or an interaction between hPAT1 and hPAT4).

One of the amino acids important in the mTOR signalling pathway is leucine. Our preliminary experiments involving inhibition of [^{3}H] L-Proline uptake had indicated that leucine could not inhibit the uptake. However when [^{3}H] L-Leucine was used a tracer, hPAT4 could bring about its transport across the membrane. Inhibition experiments indicated that L-proline could not inhibit the uptake of [^{3}H] L-Leucine

Yet another indication of the role of hPAT4 in nutrient sensing is based on experiments performed in *Drosophila*. As discussed in the introduction there are 12 homologs of the PATs in *Drosophila*. One of these homologs, *path* was observed to have properties similar to PAT4. These included factors such as its substrate affinity (which was in the range of 2 μM) (Goberdhan *et al*., 2005) compared to 3 μM found when hPAT4 was expressed in *Xenopus laevis* oocytes. This lead to the conclusion that *path* was in all likelihood the *Drosophila* homolog of PAT4.

The name *path* was given on the basis that flies that lacked this gene were observed to be a lot smaller than the wild type, hence the name pathetic or path. Apart from being smaller in size, the flies with path knock down were also observed to show much less development in the omatidia. Overexpression of this gene on the other hand, was found to result in flies which were nearly twice as large as the wild type with much more pronounced omatidia (numbers, size) in the eye (Goberdhan *et al.*, 2005).

Sequence alignment of PAT1 and PAT4 across species (Appendix 2) also shows a > 60% similarity. The suggested role of hPAT4 is in conjugation with hPAT1 where it forms a complex prior to facilitating in amino acid sensing and signalling in the mTOR pathway. Studies are also being undertaken to study the influence that the PATs have on regulating protein synthesis and influencing muscle growth in neonates (Suryawan and Davis, 2011).

7.3 Membrane topology studies on hPAT1

7.3.1 Studies using site directed mutagenesis (epitope tagging) and luminometry

Membrane transport structures are difficult to study on account of their dynamic nature. Obtaining a crystal structure for transport molecules is complicated by the fact that isolating sufficient amount of proteins that are in the same conformation is difficult. Currently there is no crystal structure available for any of the PAT family transporters despite the attention that they have received in recent years. Hydropathy plots of the AAAP family members (to which the PAT family belongs) indicated that these transporters are more likely to show the 11 transmembrane domain structures (Young, Jack *et al.*, 1998).

Following predictions obtained from TMD prediction software and epitope tagging studies, we could show that hPAT1 has 11 TMDs, an internal N-terminal and an external C-terminal. The results from the FLAG epitope tagging studies are summarised in table 7.1a and b and figure 7.1.

External FLAG	Transport	Luminometry
F1	✔	✔
F2	✔	✔
F3	✔	✔
F4	✔	✔
F5	✔	✔
F6	✔	✔

Table 7.1a: Summary of the results obtained from transport assays and luminometry experiments using the external epitope tags.

Internal FLAG	Transport	Luminometry
iF1	✔	✘
iF2	✔	✘
iF3	✔	✘
iF5	✔	✘
iF6	✔	✘

Table 7.1b: Summary of the results obtained from transport assays and luminometry experiments using the internal epitope tags.

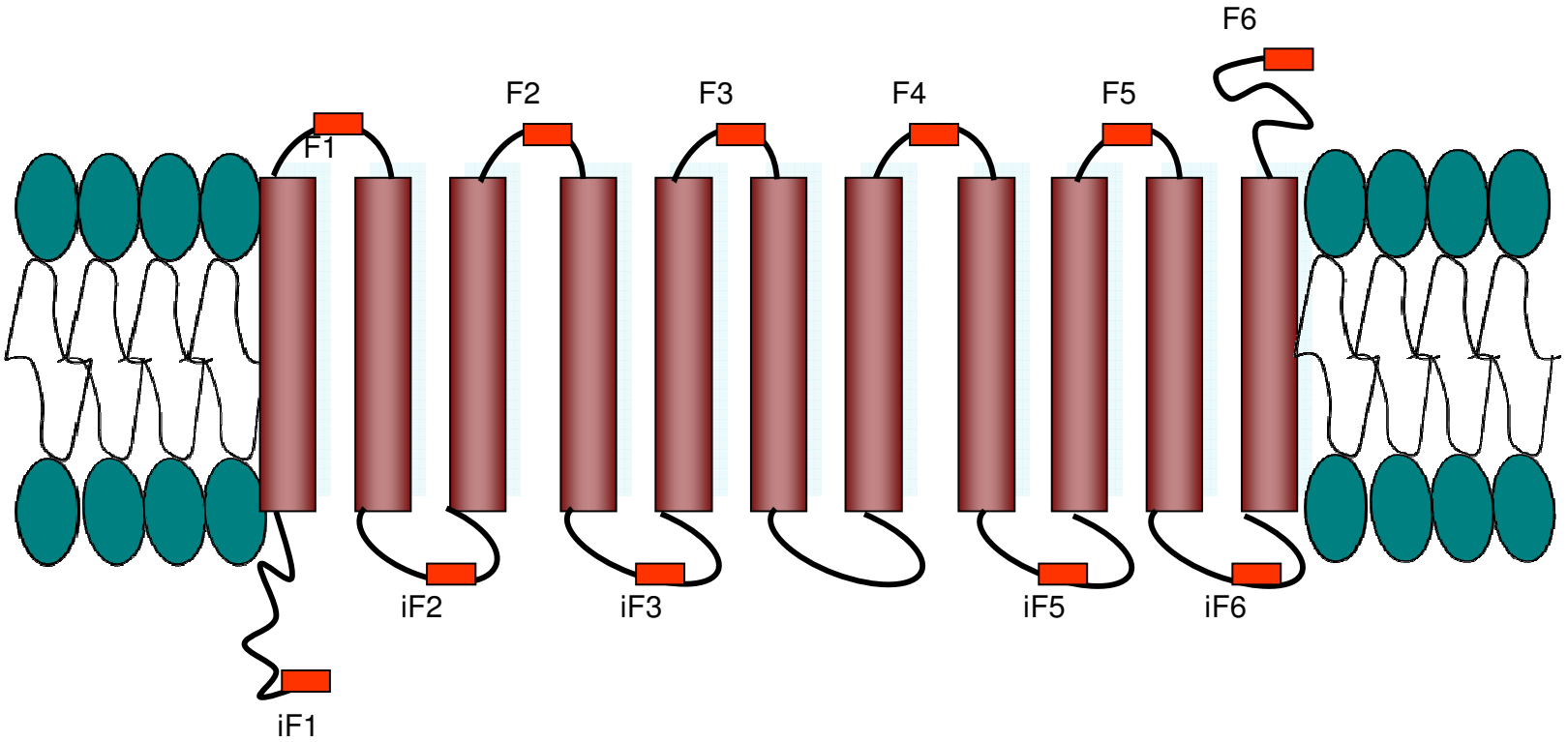

Figure 7.1: Diagrammatic representation of the epitope tagging and orientation of the hPAT1 molecule with 11 TMDs.

The predictions from modelling studies using LOMETS also agreed with our results and also largely agreed with the MEMSAT3 predictions.

The significance of the hPAT1 His-55 residue was shown by Metzner *et al*. (2008). According to the model we propose, this site lies in the first trans membrane domain and together with residue 63 it could play an important role in the substrate binding site.

Based on the positive results which were obtained from the MEMSAT predictions it was decided to try and used TMD prediction software to check for the number of TMDs for the remaining PATs as well. The data obtained from these are summarised in tables 7.3 a to d

hPAT2

Program	Amino acid										
	TMD1	TMD2	TMD3	TMD4	TMD5	TMD6	TMD7	TMD8	TMD9	TMD10	TMD11
MEMSAT3	56-80	83-107	147-171	199-218	225-246	264-285	297-321	336-356	380-403	406-429	441-465
TMHMM	84-106	148-170	201-218	225-247	262-284	297-319	344-366	379-401	406-428	441-463	
TopPred	59-79	82-102	149-169	223-243	265-285	297-317	342-362	405-425	444-464		
SVMtm	84-102	150-168	205-220	223-243	266-280	298-316	343-368	387-401	410-425	445-459	
TMpred	82-106	152-170	203-219	223-243	266-285	294-313	344-362	383-401	405-432	441-460	

Table 7.2a: Summary of TMD predictions for hPAT2

hPAT3.1

Program	Amino acid										
	TMD1	TMD2	TMD3	TMD4	TMD5	TMD6	TMD7	TMD8	TMD9	TMD10	TMD11
MEMSAT3	46-64	68-97	176-203	225-247	253-275	294-311	327-350	368-393	410-430	434-458	471-492
TMHMM	72-94	177-199	230-247	254-276	291-313	326-348	377-399	412-429	439-461	474-493	
TopPred	47-67	75-95	179-199	230-250	293-313	325-345	371-391	434-454	473-493		
SVMtm	74-94	178-197	228-250	252-274	291-308	327-346	373-388	419-436	438-459	474-488	
TMpred	72-94	177-198	232-250	252-276	288-308	323-342	373-391	414-430	434-461	474-492	

Table 7.2b: Summary of TMD predictions for hPAT3.1

hPAT3.2

Program	Amino acid										
	TMD1	TMD2	TMD3	TMD4	TMD5	TMD6	TMD7	TMD8	TMD9	TMD10	TMD11
MEMSAT3	46-62	66-95	131-161	184-206	212-234	253-270	286-309	327-352	369-389	393-417	430-451
TMHMM	72-94	136-158	189-206	213-235	250-272	285-307	336-358	371-388	398-420	433-452	
TopPred	47-67	75-95	138-158	189-209	252-272	284-304	330-350	393-413	432-452		
SVMtm	74-94	136-156	187-209	211-233	250-267	286-305	332-347	378-395	397-418	433-447	
TMpred	72-94	136-157	191-207	211-235	247-267	282-301	332-350	373-389	393-420	433-451	

Table 7.2c: Summary of TMD predictions for hPAT3.2

hPAT4

Program	Amino acid										
	TMD1	TMD2	TMD3	TMD4	TMD5	TMD6	TMD7	TMD8	TMD9	TMD10	TMD11
MEMSAT3	61-85	88-112	154-178	206-229	233-253	272-290	305-329	346-369	389-412	415-438	449-473
TMHMM	89-111	153-175	209-226	233-255	275-293	306-328	357-379	392-409	419-441	454-476	
TopPred	64-84	87-107	155-175	231-251	273-293	309-329	351-371	414-434	453-473		
SVMtm	89-106	155-173	212-226	232-251	275-289	310-324	353-368	397-428	451-470		
TMpred	87-104	157-175	211-227	232-251	274-293	306-324	350-371	393-410	414-432	454-472	

Table 7.2d: Summary of TMD predictions for hPAT4

According to the predictions of MEMSAT3, all for hPAT members are predicted to have 11 TMDs. This includes both variants of hPAT3.

7.3.2 Studies on the champagne mutation

The C to G point mutation in exon 2 of PAT1 has been found to be responsible for the champagne coat colour in horses. Based on membrane topology predictions this region in hPAT1 appears to lie within the first TMD and is thought to be one of the sites that is responsible for substrate binding.

No previous transport studies have been performed using this site mutation. Preliminary studies were performed to check whether there was a change in the transport mechanisms of PAT1. Since the champagne mutation causes a difference in the coat colour one of the substrates it could be affecting is one of the components in the melanin synthesis pathway. Figure 7.2 shows the various compounds that lead to the synthesis of melanin. Tyrosine and DOPA are the stable compounds of this pathway which are the likely candidates whose transport PAT1 could be altering.

Figure 7.2: Melanin synthesis pathway. The different products in the melanin synthesis pathway are shown (Ando, Kondoh, Ichibashi, and and Hearing 2007). The stable products in the synthesis pathway are tyrosine and DOPA.

Preliminary studies were performed in *Xenopus laevis* oocytes injected with hPAT1 wildtype (referred to as hPAT), hPAT1 champagne (referred to as champ) and non injected oocytes.

Inhibition assays were performed using an excess of L-Proline, L-Tyrosine and L-DOPA. Both hPAT1 and champ are inhibited by an excess of L-Proline. Neither L-Tyrosine nor L-DOPA are observed to be inhibited by hPAT1. However preliminary experiments show that L-Tyrosine and L-DOPA both inhibit the uptake of $^{[3H]}$ L-Proline. The results obtained are summarised in Figure 7.3.

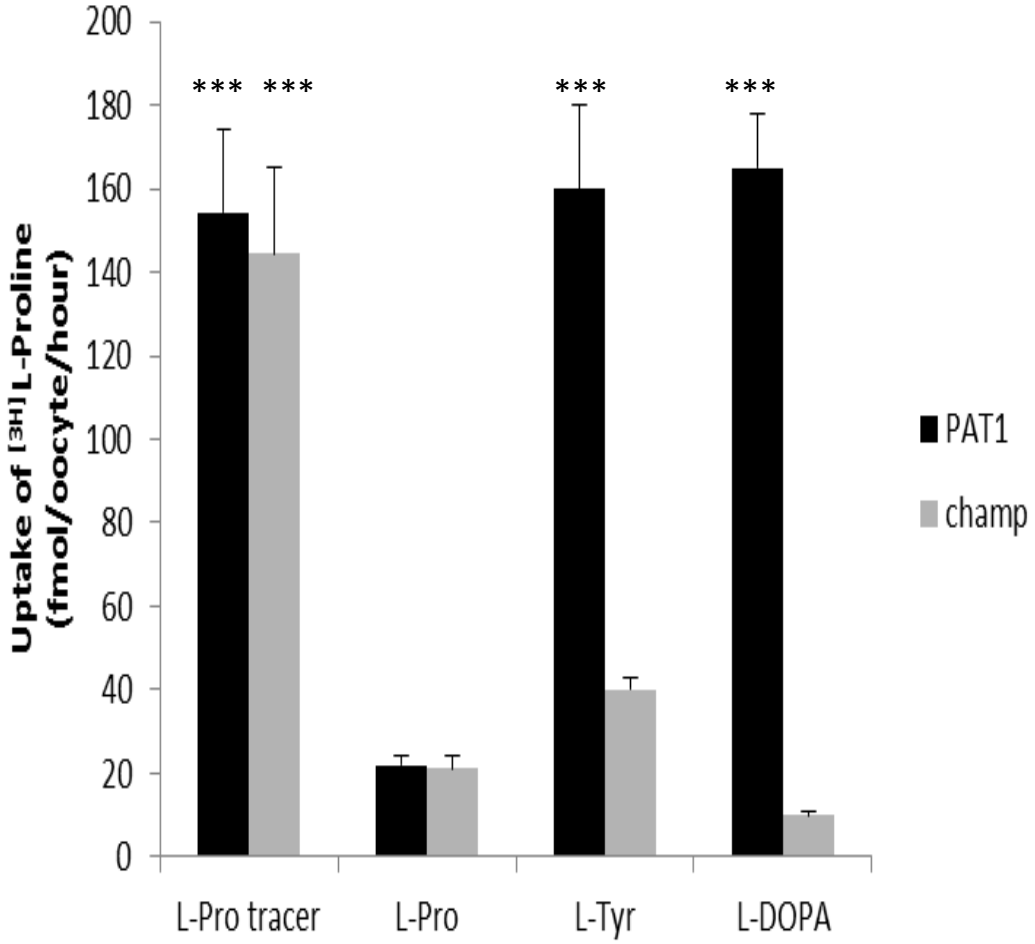

Figure 7.3: Mediated uptake of [3H] L-Proline by hPAT1 and champ. The significance levels are those obtained when compared to non injected oocytes. Both hPAT1 and champ bring about transport of tracer across the membrane. L-Proline inhibits tracer uptake by both hPAT1 and champ. Uptake of L-Proline is not inhibited in the presence of an excess of L-Tyr or L-DOPA; on the other hand both L-Tyr and L-DOPA inhibit uptake of L-Proline in oocytes injected with champ RNA. (*** $p < 0.001$). Concentration of L-Proline in the tracer 0.25 µM.

7.4 Future work

In this section, the future work that can be done on the hPATs in the context of the aims of this thesis will be discussed

- **To elucidate the function of hPAT4 (using the oocyte expression system)**
- **To characterize substrate affinities, specificity and other features of the transporter**

hPAT4 has been characterized functionally. Besides its affinities for the proteogenic amino acids and a few compounds like sarcosine, DOPA and β-Alanine, its substrate recognition still need to be studied in more detail.

In addition to these, the interesting results obtained from the studies using L-Leucine can be further investigated, especially in the context of the work done in mTOR regulation in cell lines (Heublein *et al.*, 2010).

- **To predict the Trans Membrane Domain structure using different membrane prediction software**
- **To confirm the predicted structure using the techniques of site directed mutagenesis, epitope tagging and luminometry**

Epitope tagging studies have confirmed the 11 TMD model as proposed based on the data from membrane prediction software (secondary structureof the protein). Using LOMETS, it was attempted to extend this to a tertiary structure prediction, which agreed with the 11 TMD model as well. The epitope tagging experiments could be further extended to the remaining PATs as well.

- **To study the champagne mutation using expression in *Xenopus* laevis oocytes and to study how the mutation might produce effects.**

According to this model, the champagne mutation appears to be in a region which could be playing a role in the substrate binding site. This information along with the significance of His-55 (Metzner *et al*., 2008) as the catalytic site can be further used to study the possible interactions between these two sites.

Preliminary studies using the champagne mutation in Xenopus laevis have shown that there appears to be an alteration in the subrate specificity. There is no change in the transport of L-Proline, however L-DOPA which is not a substrate for hPAT1 causes a change in current in the mutants (two electrode voltage clamp experiments). Similarly, initial inhibition experiments performed in *Xenopus laevis* oocytes also indicate that for the oocytes injected with champagne hPAT1, L-DOPA inhibits [^{3}H] L-Proline uptake as compared to the wild type hPAT1. L-DOPA is one of the products in the melanin synthesis pathway and this difference in the transport of DOPA might explain the champagne coat colour observed in horses.

While further investigation needs to be made with regards to investigating the role of the Threonine 63 residue in substrate binding, based on the preliminary experiments it seems to participate/alter the binding of amino acids. In addition to this residue, there is also a histidine at position 55. It is possible that this mutation is important for the binding of H^+ prior to the translocation of substrate across the membrane.

- **To use *Xenopus laevis* oocytes and cell culture techniques to study the functions of hPAT3**

Transport assays under different conditions were performed in both *Xenopus laevis* oocytes and TM4 cell culture. There appeared to be some transport, but no significant uptake was observed for hPAT3 in repeated experiments. The function of this transporter needs to be investigated further. It is the only PAT family member whose distribution is restricted to just one organ (the testis). The initial idea was that perhaps the transporter is coupled to a different ion. However, the absence of any significant transport even at different pHs makes this less likely. The alternative suggestion is to check for its role in signalling by studying at what stage during development it is expressed or if it is constantly expressed throughout the life of the organism.

There is a lot more information now available about the different transporter proteins due to the various advancements in the field of molecular biology; despite this many of them remain orphans. The relationship between transporters and signalling has gained a lot of attention in recent years and better understanding of the different

pathways involved will push forward the tranceptor studies. With more crystal structures being made available, the structural studies will become quicker and the data from membrane prediction studies a lot more accurate and this can be used for membrane prediction studies and for modelling a transporter whose structure is unknown against a range of different known transporters to study the best fit

APPENDIX 1: hPAT sequences and primers for subcloning and sequencing

Appendix 1

AMINO ACID SEQUENCES OF THE hPATs

hPAT1

>gi|41352721|ref|NP_510968.2| proton-coupled amino acid transporter 1 [Homo sapiens]

MSTQRLRNEDYHDYSSTDVSPEESPSEGLNNLSSPGSYQRFGQSNSTTWFQTLIHLLKGNIGTGLLGLPL
AVKNAGIVMGPISLLIIGIVAVHCMGILVKCAHHFCRRLNKSFVDYGDTVMYGLESSPCSWLRNHAHWGR
RVVDFFLIVTQLGFCCVYFVFLADNFKQVIEAANGTTNNCHNNETVILTPTMDSRLYMLSFLPFLVLLVF
IRNLRALSIFSLLANITMLVSLVMIYQFIVQRIPDPSHLPLVAPWKTYPLFFGTAIFSFEGIGMVLPLEN
KMKDPRKFPLILYLGMVIVTILYISLGCLGYLQFGANIQGSITLNLPNCWLYQSVKLLYSIGIFFTYALQ
FYVPAEIIIPFFVSRAPEHCELVVDLFVRTVLVCLTCILAILIPRLDLVISLVGSVSSSALALIIPPLLE
VTTFYSEGMSPLTIFKDALISILGFVGFVVGTYEALYELIQPSNAPIFINSTCAFI

hPAT2

>gi|119582082|gb|EAW61678.1| solute carrier family 36 (proton/amino acid symporter), member 2 [Homo sapiens]

MSVTKSTEGPQGAVAIKLDLMSPPESAKKLENKDSTFLDESPSESAGLKKTKGITVFQALIHLVKGNMGT
GILGLPLAVKNAGILMGPLSLLVMGFIACHCMHILVKCAQRFCKRLNKPFMDYGDTVMHGLEANPNAWLQ
NHAHWGRHIVSFFLIITQLGFCCVYIVFLADNLKQVVEAVNSTTNNCYSNETVILTPTMDSRLYMLSFLP
FLVLLVLIRNLRILTIFSMLANISMLVSLVIIIQYITQEIPDPSRLPLVASWKTYPLFFGTAIFSFESIG
VVLPLENKMKNARHFPAILSLGMSIVTSLYIGMAALGYLRFGDDIKASISLNLPNCWLYQSVKLLYIAGI
LCTYALQFYVPAEIIIPFAISRVSTRWALPLDLSIRLVMVCLTCLLAILIPRLDLVISLVGSVSGTALAL
IIPPLLEVTTFYSEGMSPLTIFKDALISILGFVGFVVGTYQALDELLKSEDSHPFSNSTTFVR

hPAT3 variant 1 i.e. 3.1

>gi|71681851|gb|AAI01094.1| SLC36A3 protein [Homo sapiens]

MSLLGRDYNSELNSLDNGPQSPSESSSSITSENVHPAGEAGLSMMQTLIHLLKCNIGTGLLGLPLAIKNA
GLLVGPVSLLAIGVLTVHCMVILLNCAQHLSQRLQKTFVNYGEATMYGLETCPNTWLRAHAVWGRWNLAL
SPRLECSGKISAHCNPHLQGSSNSPAQASRVAGIYRYTVSFLLVITQLGFCSVYFMFMADNLQQMVEKAH
VTSNICQPREILTLTPILDIRFYMLIILPFLILLVFIQNLKVLSVFSTLANITTLGSMALIFEYIMEGIP
YPSNLPLMANWKTFLLFFGTAIFTFEGVGMVLPLKNQMKHPQQFSFVLYLGMSIVIILYILLGTLGYMKF
GSDTQASITLNLPNCWLYQSVKLMYSIGIFFTYALQFHVPAEIIIPFAISQVSESWALFVDLSVRSALVC
LTCVSAILIPRLDLVISLVGSVSSSALALIIPALLEIVIFYSEDMSCVTIAKDIMISIVGLLGCIFGTYQ
ALYELPQPISHSMANSTGVHA

hPAT3 variant 2 i.e. 3.2

>gi|72533330|gb|AAI01096.1| Solute carrier family 36 (proton/amino acid symporter), member 3 [Homo sapiens]

MSLLGRDYNSELNSLDNGPQSPSESSSSITSENVHPAGEAGLSMMQTLIHLLKCNIGTGLLGLPLAIKNA

Appendix 1

GLLVGPVSLLAIGVLTVHCMVILLNCAQHLSQRLQKTFVNYGEATMYGLETCPNTWLRAHAVWGRYTVSF
LLVITQLGFCSVYFMFMADNLQQMVEKAHVTSNICQPREILTLTPILDIRFYMLIILPFLILLVFIQNLK
VLSVFSTLANITTLGSMALIFEYIMEGIPYPSNLPLMANWKTFLLFFGTAIFTFEGVGMVLPLKNQMKHP
QQFSFVLYLGMSIVIILYILLGTLGYMKFGSDTQASITLNLPNCWLYQSVKLMYSIGIFFTYALQFHVPA
EIIIPFAISQVSESWALFVDLSVRSALVCLTCVSAILIPRLDLVISLVGSVSSSALALIIPALLEIVIFY
SEDMSCVTIAKDIMISIVGLLGCIFGTYQALYELPQPISHSMANSTGVHA

hPAT4

>gi|28703727|gb|AAH47374.1| Solute carrier family 36 (proton/amino acid symporter), member 4 [Homo sapiens]
MEAAATPAAAGAARREELDMDVMRPLINEQNFDGTSDEEHEQELLPVQKHYQLDDQEGISFVQTLMHLLK
GNIGTGLLGLPLAIKNAGIVLGPISLVFIGIISVHCMHILVRCSHFLCLRFKKSTLGYSDTVSFAMEVSP
WSCLQKQAAWGRSVVDFFLVITQLGFCSVYIVFLAENVKQVHEGFLESKVFISNSTNSSNPCERRSVDIR
IYMLCFLPFIILLVFIRELKNLFVLSFLANVSMAVSLVIIYQYVVRNMPDPHNLPIVAGWKKYPLFFGTA
VFAFEGIGVVLPLENQMKESKRFPQALNIGMGIVTTLYVTLATLGYMCFHDEIKGSITLNLPQDVWLYQS
VKILYSFGIFVTYSIQFYVPAEIIIHGITSKFHTKWKQICEFGIRSFLVSITCAGAILIPRLDIVISFVG
AVSSSTLAIILPPLVEILTFSKEHYNIWMVLKNISIAFTGVVGFLLGTYITVEEIIYPTPKVVAGTPQSP
FLNLNSTCLTSGLK

Appendix 1

PRIMERS FOR SUB CLONING THE hPATs INTO pXT7 AND THEIR RELATIVE POSITIONS

Primer name	Sequence	Tm	%GC	Secondary structure	Primer/ Dimer
PAT1 Forward	GCTGGTACCCCATGTCCAC GCAGAGACTTCG	71.21	61.29	Weak	No
PAT1 Reverse	AGCCGGCCGTCCCTATATG AAGGCACAGGTGGAATTG	72.18	56.76	Moderate	No
PAT2 Forward	GCTGGTACCGCTCCTTGTTT CTTAAGCAGTCATGTCTGT G	72.17	50	Weak	No
PAT2 Reverse	AGCCGGCCGTCGGGTGCTG GTAGGCAAGGA	72.93	70	Weak	No
PAT3 Forward	GCTGGTACCGTTGAAGATG TCATTGCTTGGAAGGGACT AC	72.58	50	Weak	No
PAT3 Reverse	AGCCGGCCGGAATAAAAAC AGATAATTATGCATGGACA CCTGTGG	72.77	46.67	Moderate	No
PAT4 Forward	GCTGGTACCGGCGGCGTCC TGGAGACC	72.9	74.07	Weak	No
PAT4 Reverse	AGCCGGCCGGAAGACTCAT GATTCTGCTTTTACTATTTC AAACCAG	71.37	45.65	Weak	no

KEY: Ala Res site Forward: KpnI and reverse: EagI Untranslated region

The regions shaded in grey are the untranslated regions. The primer sequence present in the gene are indicated in bold

Appendix 1

hPAT1

GGGCTGTGCTGAAGCCAGAGCCGGAGCCGGAGCTGGGGCCAGAACCCGAGCAGTGAGTTCCTCCACTGGC
TGCCGGCTGGCGGCGCTCGCCGCCTTGGGCAGGACCCACCTCGCCTTCCTCCCGGCGTGGCAGATGCTCC
AGCTGCCATGTCCACGCAGAGACTTCGGAATGAAGACTACCACGACTACAGCTCCACGGACGTGAGCCCT
GAGGAGAGCCCGTCGGAAGGCCTCAACAACCTCTCCTCCCCGGGCTCCTACCAGCGCTTTGGTCAAAGCA
ATAGCACAACATGGTTCCAGACCTTGATCCACCTGTTAAAAGGCAACATTGGCACAGGACTCCTGGGACT
CCCTCTGGCGGTGAAAAATGCAGGCATCGTGATGGGTCCCATCAGCCTGCTGATCATAGGCATCGTGGCC
GTGCACTGCATGGGTATCCTGGTGAAATGTGCTCACCACTTCTGCCGCAGGCTGAATAAATCCTTTGTGG
ATTATGGTGATACTGTGATGTATGGACTAGAATCCAGCCCCTGCTCCTGGCTCCGGAACCACGCACACTG
GGGAAGACGTGTTGTGGACTTCTTCCTGATTGTCACCCAGCTGGGATTCTGCTGTGTCTATTTTGTGTTT
CTGGCTGACAACTTTAAACAGGTGATAGAAGCGGCCAATGGGACCACCAATAACTGCCACAACAATGAGA
CGGTGATTCTGACGCCTACCATGGACTCGCGACTCTACATGCTCTCCTTCCTGCCCTTCCTGGTGCTGCT
GGTTTTCATCAGGAACCTCCGAGCCCTGTCCATCTTCTCCCTGTTGGCCAACATCACCATGCTGGTCAGC
TTGGTCATGATCTACCAGTTCATTGTTCAGAGGATCCCAGACCCCAGCCACCTCCCCTTGGTGGCCCCTT
GGAAGACCTACCCTCTCTTCTTTGGCACAGCGATTTTTTCATTTGAAGGCATTGGAATGGTTCTGCCCCT
GGAAAACAAAATGAAGGATCCTCGGAAGTTCCCACTCATCCTGTACCTGGGCATGGTCATCGTCACCATC
CTCTACATCAGCCTGGGGTGTCTGGGGTACCTGCAATTTGGAGCTAATATCCAAGGCAGCATAACCCTCA
ACCTGCCCAACTGCTGGTTGTACCAGTCAGTTAAGCTGCTGTACTCCATCGGGATCTTTTTCACCTACGC
ACTCCAGTTCTACGTCCCGGCTGAGATCATCATCCCCTTCTTTGTGTCCCGAGCGCCCGAGCACTGTGAG
TTAGTGGTGGACCTGTTTGTGCGCACAGTGCTGGTCTGCCTGACATGCATCTTGGCCATCCTCATCCCCC
GCCTGGACCTGGTCATCTCCCTGGTGGGCTCCGTGAGCAGCAGCGCCCTGGCCCTCATCATCCCACCGCT
CCTGGAGGTCACCACCTTCTACTCAGAGGGCATGAGCCCCCTCACCATCTTTAAGGACGCCCTGATCAGC
ATCCTGGGCTTCGTGGGCTTTGTGGTGGGGACCTATGAGGCTCTCTATGAGCTGATCCAGCCAAGCAATG
CTCCCATCTTCATCAATTCCACCTGTGCCTTCATATAGGGATCTGGGTTCGTCTCTGCAGCTGCCTACCC
CTGCCCCATGTGTCCCCCGTTACCTGTCCTCAGAGCCTCAGGTATGGTCCAGGCTCTGAGGAAAGTCAGG
GTTGCTGTGTGGGAACCCCTCTGCCTGGCACCTGGATACCCTGGGCCAGGTAACCTGAGGGC

hPAT2

CTGTGCTGGGGTGTACATCTACACTAGACACCTTCCTGCTTCCCTCCTTCCAGAGCAGACCTCTTTGTCA
CCCCGAGCTCCTTGTTTCTTAAGCAGTCATGTCTGTGACAAAAAGTACTGAGGGTCCCCAGGGAGCCGTT
GCCATCAAATTGGACCTTATGTCGCCTCCTGAAAGTGCCAAGAAGTTGGAGAACAAGGACTCTACATTCT
TGGATGAAAGTCCTTCAGAGTCGGCAGGCTTGAAGAAGACCAAGGGCATAACAGTGTTCCAGGCCTTGAT
TCACCTGGTGAAAGGCAACATGGGCACAGGGATCCTGGGACTACCCCTCGCTGTGAAGAACGCGGGCATC
CTGATGGGCCCACTCAGTCTGCTGGTGATGGGCTTCATTGCCTGCCACTGTATGCACATCCTGGTCAAGT
GTGCCCAGCGCTTCTGTAAGAGGCTTAACAAGCCCTTTATGGACTATGGGGACACGGTGATGCATGGACT
AGAAGCCAACCCCAACGCCTGGCTCCAGAATCACGCTCACTGGGGAAGGCATATCGTGAGCTTCTTCCTT
ATTAACACCCAACTTGGCTTCTGCTGTGTGTACATTGTGTTTTTGGCTGATAATTTAAAACAGGTAGTGG
AAGCTGTTAATAGCACAACCAACAACTGCTATTCCAATGAGACGGTGATTCTGACCCCCACCATGGACTC
GCGACTCTACATGCTCTCCTTCCTGCCCTTCCTGGTGCTGCTGGTCCTCATCCGGAACCTCAGGATCTTG
ACCATCTTCTCCATGCTGGCCAACATCAGCATGCTGGTCAGCTTGGTCATCATCATACAGTACATTACCC
AGGAAATCCCAGACCCCAGCCGGTTGCCACTGGTAGCAAGCTGGAAGACCTACCCTCTCTTCTTCGGAAC

Appendix 1

AGCCATTTTTTCTTTTGAAAGCATTGGTGTGGTTCTGCCTCTGGAAAACAAGATGAAGAATGCCCGCCAC
TTCCCAGCCATCCTGTCTTTGGGAATGTCCATCGTCACTTCCCTATACATTGGCATGGCGGCTCTGGGCT
ACCTGCGGTTTGGAGATGACATCAAGGCCAGCATAAGCCTTAACCTGCCTAACTGCTGGCTGTACCAGTC
TGTCAAGCTTCTCTACATTGCCGGCATCCTGTGCACCTATGCCCTGCAGTTCTACGTCCCTGCAGAAATC
ATCATCCCCTTTGCCATCTCCCGGGTGTCAACACGCTGGGCACTGCCTCTGGATCTGTCCATTCGCCTCG
TCATGGTCTGCCTGACATGCCTCCTGGCCATCCTCATCCCTCGCCTGGACCTGGTCATCCCCCTGGTGGG
CTCCGTGAGTGGCACCGCCCTGGCCCTCATCATCCCACCGCTCCTGGAGGTCACCACGTTCTACTCAGAG
GGCATGAGCCCCCTCACCATCTTCAAGGACGCCCTGATCAGCATCCTGGGCTTCGTGGGCTTTGTGGTGG
GGACCTACCAGGCCCTGGACGAGCTGCTCAAGTCAGAAGACTCTCACCCCTTTTCCAACTCCACCACTTT
TGTTCGGTGAGCCTGGCACTGCTCCTTGCCTACCAGCACCCGACTTTTAATTATATGGATCTCTTTTTTT
TCTTTTTTTTTTTTTTGAGACGGAGTTTCTGTCTTGTTGCCCAGACTGGAGCACAATGATGCGATCTCAG

hPAT3.1

TTCAGCCAGCAGCACGCATCCTTGGGGTTAGGAGCCAGCATCTCAGGTCAGCAGCCATGGTTTCTCTGGA
GAAAGACCCATCAGAGGCTCATTCAGAGCCTTAACCCTGAGCCTCTGCCACCTACCCACCGTGTTGAAGA
TGTCATTGCTTGGAAGGGACTACAACAGTGAGCTGAACTCCTTGGACAACGGACCTCAGTCACCCTCAGA
GAGCAGCAGTAGCATTACTTCAGAGAATGTCCATCCTGCTGGAGAAGCTGGACTATCGATGATGCAAACT
TTGATCCACTTGTTGAAATGCAACATTGGCACAGGGCTCCTGGGGCTTCCCCTGGCCATAAAGAATGCCG
GCTTGTTGGTCGGTCCTGTCAGCCTTCTGGCCATCGGGGTCCTCACCGTGCACTGCATGGTCATCCTGTT
GAACTGTGCTCAACACCTCAGCCAGAGACTGCAGAAGACTTTTGTGAACTATGGAGAGGCCACGATGTAC
GGCCTTGAAACCTGCCCGAACACCTGGCTGAGGGCCCATGCAGTGTGGGGAAGATGGAATTTGGCTCTGT
CACCCAGGCTGGAGTGCAGTGGCAAGATCTCAGCTCACTGCAACCCCCACCTTCAGGGTTCAAGCAATTC
TCCTGCCCAAGCCTCCCGAGTAGCTGGGATTTACAGGTACACTGTCAGCTTCTTATTAGTCATCACCCAG
CTGGGCTTCTGCAGTGTTTATTTTATGTTTATGGCAGACAATTTACAACAGATGGTGGAAAAAGCCCACG
TGACCTCCAACATCTGCCAGCCCAGGGAGATTCTGACGCTGACCCCCATCCTGGACATTCGTTTCTACAT
GCTGATAATCCTGCCCTTCCTGATCCTGTTGGTGTTTATCCAGAACCTCAAGGTGCTGTCCGTCTTCTCG
ACATTGGCCAACATCACCACCCTTGGGAGCATGGCTCTGATCTTTGAGTATATCATGGAGGGGATTCCAT
ATCCCAGCAACCTACCCTTGATGGCAAACTGGAAGACCTTCTTGCTGTTCTTTGGTACAGCCATCTTCAC
ATTTGAAGGCGTCGGTATGGTTCTGCCTCTCAAAAACCAGATGAAGCATCCACAGCAGTTTTCTTTTGTT
CTGTACTTGGGGATGTCCATTGTCATCATCCTCTATATCTTACTGGGGACACTGGGCTACATGAAGTTTG
GGTCAGACACCCAGGCCAGCATCACCCTCAACTTGCCCAATTGCTGGTTGTACCAGTCAGTCAAGCTGAT
GTACTCTATCGGCATCTTCTTCACCTATGCCCTCCAGTTCCACGTCCCAGCTGAGATCATCATCCCGTTT
GCCATCTCCCAAGTGTCAGAGAGCTGGGCACTGTTTGTAGACCTGTCTGTCCGCTCAGCCTTGGTCTGTC
TAACCTGTGTCTCAGCCATCCTCATCCCCCGCCTGGACTTGGTCATCTCCCTGGTAGGCTCCGTGAGCAG
CAGCGCCCTGGCTCTCATCATCCCAGCCCTCCTGGAGATCGTCATCTTTTACTCTGAGGACATGAGCTGT
GTCACCATTGCCAAGGACATCATGATTAGCATCGTGGGCCTTTTAGGGTGTATATTTGGGACATACCAAG
CCCTCTATGAGTTGCCCCAACCCATCAGCCATTCCATGGCCAACTCCACAGGTGTCCATGCATAATTATC
TGTTTTTATTCTAATAGCTCTCCCTTCCTCCCATCCCCAGTTTGACTTCCATGTGGATGTTATATACCTT
CATCAAATCCCAACATCTCTATATTAATTAGTGGCGTCTTTATCTTTCCAAGAGAAATGCAGATGAGAAA

Appendix 1

hPAT3.2

AGCAGCACGCATCCTTGGGGTTAGGAGCCAGCATCTCAGGTCGGCAGCCATGGTTTCTCTGGAGAAAGAC
CCGTCAGAGGCTCATTCAGAGCCTTAACCCTGAGCCTCTGCCACCTACCCACCGTGTTGAAGATGTCATT
GCTTGGAAGGGACTACAACAGTGAGCTGAACTCCTTGGACAACGGACCTCAGTCACCCTCAGAGAGCAGC
AGTAGCATTACTTCAGAGAATGTCCATCCTGCTGGAGAAGCTGGACTATCGATGATGCAAACTTTGATCC
ACTTGTTGAAATGCAACATTGGCACAGGGCTCCTGGGGCTTCCCCTGGCCATAAAGAATGCCGGCTTGTT
GGTCGGTCCTGTCAGCCTTCTGGCCATCGGGGTCCTCACCGTGCACTGCATGGTCATCCTGTTGAACTGT
GCTCAACACCTCAGCCAGAGACTGCAGAAGACTTTTGTGAACTATGGAGAGGCCACGATGTACGGCCTTG
AAACCTGCCCGAACACCTGGCTGAGGGCCCATGCAGTGTGGGGAAGGTACACTGTCAGCTTCTTATTAGT
CATCACCCAGCTGGGCTTCTGCAGTGTTTATTTTATGTTTATGGCAGACAATTTACAACAGATGGTGGAA
GAAGCCCACGTGACCTCCAACATCTGCCAGCCCAGGGAGATTCTGACGCTGACCCCCATCCTGGACATTC
GTTTCTACATGCTGATAATCCTGCCCTTCCTGATCCTGTTGGTGTTTATCCAGAACCTCAAGGTGCTGTC
CGTCTTCTCGACATTGGCCAACATCACCACCCTTGGGAGCATGGCTCTGATCTTTGAGTATATCATGGAG
GGGATTCCATATCCCAGCAACCTACCCTTGATGGCAAACTGGAAGACCTTCTTGCTGTTCTTTGGTACAG
CCATCTTCACATTTGAAGGCGTCGGTATGGTTCTGCCTCTCAAAAACCAGATGAAGCATCCACAGCAGTT
TTCTTTTGTTCTGTACTTGGGGATGTCCATTGTCATCATCCTCTATATCTTACTGGGGACACTGGGCTAC
ATGAAGTTTGGGTCAGACACCCAGGCCAGCATCACCCTCAACTTGCCCAATTGCTGGTTGTACCAGTCAG
TCAAGCTGATGTACTCTATCGGCATCTTCTTCACCTATGCCCTCCAGTTCCACGTCCCAGCTGAGATCAT
CATCCCGTTTGCCATCTCCCAAGTGTCAGAGAGCTGGGCACTGTTTGTAGACCTGTCTGTCCGCTCAGCC
TTGGTCTGTCTAACCTGTGTCTCAGCCATCCTCATCCCCCGCCTGGACTTGGTCATCTCCCTGGTAGGCT
CCGTGAGCAGCAGCGCCCTGGCTCTCATCATCCCAGCCCTCCTGGAGATCGTCATCTTTTACTCTGAGGA
CATGAGCTGTGTCACCATTGCCAAGGACATCATGATTAGCATCGTGGGCCTTTTAGGGTGTATATTTGGG
ACATACCAAGCCCTCTATGAGTTGCCCCAACCCATCAGCCATTCCATGGCCAACTCCACAGGTGTCCATG
CATAATTATCTGTTTTTATTCTAATAGCTCTCCCTTCCTCCCATCCCCAGTTTGACTTCCATGTGGATGT
TATATACCTTCATCAAATCCCAACATCTCTATATTAATTAGTGGCGTCTTTATCTTTCCAAGAGAAATGC
AGATGAGAAAAGTTAGCACTGATGTCTCTCAGGCTACACCTCTTTTGGTTTTATATTTTTTGG

hPAT4

CCCTCCGGGCAGGGGAGGTAGGCCTGGGCCTGACGCCGGCCACGCAGCGGCGGGAGAGTGAGCACTCGGG
CGGCGGCGTCCTGGAGACCCGCGAGAGATGGAAGCGGCGGCGACGCCGGCGGCTGCCGGGGCGGCGAGGC
GCGAGGAGCTAGATATGGATGTAATGAGGCCCTTGATAAATGAGCAGAATTTTGATGGGACATCAGATGA
AGAACATGAGCAAGAGCTTCTGCCTGTTCAGAAGCATTACCAACTTGATGATCAAGAGGGCATTTCATTT
GTACAAACTCTTATGCACCTTCTTAAAGGAAATATTGGAACTGGCCTTTTAGGACTTCCATTGGCAATAA
AAAATGCAGGCATAGTGCTTGGACCAATCAGCCTTGTGTTTATAGGAATTATTTCTGTTCACTGTATGCA
CATATTGGTACGTTGCAGTCACTTTCTATGTCTGAGGTTTAAAAAGTCAACATTAGGTTATAGTGACACT
GTGAGCTTTGCTATGGAAGTGAGTCCTTGGAGTTGTCTTCAGAAGCAAGCAGCATGGGGGCGGAGTGTGG
TTGACTTTTTTCTGGTGATAACACAGCTGGGATTCTGTAGTGTTTATATTGTCTTCTTAGCTGAAAATGT
GAAACAAGTTCATGAAGGATTCCTGGAGAGTAAAGTGTTTATTTCAAATAGTACCAATTCATCAAACCCT
TGTGAGAGAAGAAGTGTTGACCTAAGGATATATATGCTTTGCTTTCTTCCATTTATAATTCTTTTGGTCT
TCATTCGTGAACTAAAGAATCTATTTGTACTTTCATTCCTTGCCAACGTTTCCATGGCTGTCAGTCTTGT
GATAATTTACCAGTATGTTGTCAGGAACATGCCAGATCCCCACAACCTTCCAATAGTGGCTGGTTGGAAG

Appendix 1

AAATACCCACTCTTTTTTGGTACTGCTGTATTTGCTTTTGAAGGCATAGGAGTGGTCCTTCCACTGGAAA
ACCAAATGAAAGAATCAAAGCGTTTCCCTCAAGCGTTGAATATTGGCATGGGGATTGTTACAACTTTGTA
TGTAACATTAGCTACTTTAGGATATATGTGTTTCCATGATGAAATCAAAGGCAGCATAACTTTAAATCTT
CCCCAAGATGTATGGTTATATCAATCAGTGAAAATTCTATATTCCTTTGGCATTTTTGTGACATATTCAA
TTCAGTTCTATGTTCCAGCAGAGATCATTATCCCTGGGATCACATCCAAATTTCATACTAAATGGAAGCA
AATCTGTGAATTTGGGATAAGATCCTTCTTGGTTAGTATTACTTGTGCCGGAGCAATTCTTATTCCTCGT
TTAGACATTGTGATTTCCTTCGTTGGAGCTGTGAGCAGCAGCACATTGGCCCTAATCCTGCCACCTTTGG
TTGAAATTCTTACATTTTCGAAGGAACATTATAATATATGGATGGTCCTGAAAAATATTTCTATAGCATT
CACTGGAGTTGTTGGCTTCTTATTAGGTACATATATAACTGTTGAAGAAATTATTTATCCTACTCCCAAA
GTTGTAGCTGGCACTCCACAGAGTCCTTTTCTAAATTTGAATTCAACATGCTTAACATCTGGTTTGAAAT
AGTAAAAGCAGAATCATGAGTCTTCTATTTTTGTCCCATTTCTGAAAATTATCAAGATAACTAGTAAAAT
ACATTGCTATATACATAAAAATGGTAACAAACTCTGTTTTCTTTGGCACGATATTAATATTTTGGAAGTA
ATCATAACTCTTTACCAGTAGTGGTAAACCTATGGAAAATCCTTGCTTTTAAGTGTTAGCAATAGTTCAA

Appendix 1

PRIMERS FOR SUB CLONING THE hPATs INTO pcDNA3.1(-) AND THEIR RELATIVE POSITIONS

Primer name	Sequence	Tm	%GC	Secondary structure	Primer/ Dimer
PAT2 Forward	GCTGGTACCGCTCCTTGTTTCTTAAGCAGTCATGTCTGTG	72.17	50	Weak	No
PAT2 Reverse	AGCGGTACCTCGGGTGCTGGTAGGCAAGGA	72.93	70	Weak	No
PAT3 Forward	GCTGGTACCGTTGAAGATGTCATTGCTTGGAAGGGACTAC	72.58	50	Weak	No
PAT3 Reverse	AGCGGTACCGAATAAAAACAGATAATTATGCATGGACACCTGTGG	72.77	46.67	Moderate	No

KEY

Ala Res site KpnI site Untranslated region

Appendix 1

hPAT2

CTGTGCTGGGGTGTACATCTACACTAGACACCTTCCTGCTTCCCTCCTTCCAGAGCAGACCTCTTTGTCA
CCCCGAGCTCCTTGTTTCTTAAGCAGTCATGTCTGTGACAAAAAGTACTGAGGGTCCCCAGGGAGCCGTT
GCCATCAAATTGGACCTTATGTCGCCTCCTGAAAGTGCCAAGAAGTTGGAGAACAAGGACTCTACATTCT
TGGATGAAAGTCCTTCAGAGTCGGCAGGCTTGAAGAAGACCAAGGGCATAACAGTGTTCCAGGCCTTGAT
TCACCTGGTGAAAGGCAACATGGGCACAGGGATCCTGGGACTACCCCTCGCTGTGAAGAACGCGGGCATC
CTGATGGGCCCACTCAGTCTGCTGGTGATGGGCTTCATTGCCTGCCACTGTATGCACATCCTGGTCAAGT
GTGCCCAGCGCTTCTGTAAGAGGCTTAACAAGCCCTTTATGGACTATGGGGACACGGTGATGCATGGACT
AGAAGCCAACCCCAACGCCTGGCTCCAGAATCACGCTCACTGGGGAAGGCATATCGTGAGCTTCTTCCTT
ATTAACACCCAACTTGGCTTCTGCTGTGTGTACATTGTGTTTTTGGCTGATAATTTAAAACAGGTAGTGG
AAGCTGTTAATAGCACAACCAACAACTGCTATTCCAATGAGACGGTGATTCTGACCCCCACCATGGACTC
GCGACTCTACATGCTCTCCTTCCTGCCCTTCCTGGTGCTGCTGGTCCTCATCCGGAACCTCAGGATCTTG
ACCATCTTCTCCATGCTGGCCAACATCAGCATGCTGGTCAGCTTGGTCATCATCATACAGTACATTACCC
AGGAAATCCCAGACCCCAGCCGGTTGCCACTGGTAGCAAGCTGGAAGACCTACCCTCTCTTCTTCGGAAC
AGCCATTTTTTCTTTTGAAAGCATTGGTGTGGTTCTGCCTCTGGAAAACAAGATGAAGAATGCCCGCCAC
TTCCCAGCCATCCTGTCTTTGGGAATGTCCATCGTCACTTCCCTATACATTGGCATGGCGGCTCTGGGCT
ACCTGCGGTTTGGAGATGACATCAAGGCCAGCATAAGCCTTAACCTGCCTAACTGCTGGCTGTACCAGTC
TGTCAAGCTTCTCTACATTGCCGGCATCCTGTGCACCTATGCCCTGCAGTTCTACGTCCCTGCAGAAATC
ATCATCCCCTTTGCCATCTCCCGGGTGTCAACACGCTGGGCACTGCCTCTGGATCTGTCCATTCGCCTCG
TCATGGTCTGCCTGACATGCCTCCTGGCCATCCTCATCCCTCGCCTGGACCTGGTCATCCCCCTGGTGGG
CTCCGTGAGTGGCACCGCCCTGGCCCTCATCATCCCACCGCTCCTGGAGGTCACCACGTTCTACTCAGAG
GGCATGAGCCCCCTCACCATCTTCAAGGACGCCCTGATCAGCATCCTGGGCTTCGTGGGCTTTGTGGTGG
GGACCTACCAGGCCCTGGACGAGCTGCTCAAGTCAGAAGACTCTCACCCCTTTTCCAACTCCACCACTTT
TGTTCGGTGAGCCTGGCACTGCTCCTTGCCTACCAGCACCCGACTTTTAATTATATGGATCTCTTTTTTT
TCTTTTTTTTTTTTTTGAGACGGAGTTTCTGTCTTGTTGCCCAGACTGGAGCACAATGATGCGATCTCAG

hPAT3.1

TTCAGCCAGCAGCACGCATCCTTGGGGTTAGGAGCCAGCATCTCAGGTCAGCAGCCATGGTTTCTCTGGA
GAAAGACCCATCAGAGGCTCATTCAGAGCCTTAACCCTGAGCCTCTGCCACCTACCCACCGTGTTGAAGA
TGTCATTGCTTGGAAGGGACTACAACAGTGAGCTGAACTCCTTGGACAACGGACCTCAGTCACCCTCAGA
GAGCAGCAGTAGCATTACTTCAGAGAATGTCCATCCTGCTGGAGAAGCTGGACTATCGATGATGCAAACT
TTGATCCACTTGTTGAAATGCAACATTGGCACAGGGCTCCTGGGGCTTCCCCTGGCCATAAAGAATGCCG
GCTTGTTGGTCGGTCCTGTCAGCCTTCTGGCCATCGGGGTCCTCACCGTGCACTGCATGGTCATCCTGTT
GAACTGTGCTCAACACCTCAGCCAGAGACTGCAGAAGACTTTTGTGAACTATGGAGAGGCCACGATGTAC
GGCCTTGAAACCTGCCCGAACACCTGGCTGAGGGCCCATGCAGTGTGGGGAAGATGGAATTTGGCTCTGT
CACCCAGGCTGGAGTGCAGTGGCAAGATCTCAGCTCACTGCAACCCCCACCTTCAGGGTTCAAGCAATTC
TCCTGCCCAAGCCTCCCGAGTAGCTGGGATTTACAGGTACACTGTCAGCTTCTTATTAGTCATCACCCAG
CTGGGCTTCTGCAGTGTTTATTTTATGTTTATGGCAGACAATTTACAACAGATGGTGGAAAAAGCCCACG
TGACCTCCAACATCTGCCAGCCCAGGGAGATTCTGACGCTGACCCCCATCCTGGACATTCGTTTCTACAT
GCTGATAATCCTGCCCTTCCTGATCCTGTTGGTGTTTATCCAGAACCTCAAGGTGCTGTCCGTCTTCTCG
ACATTGGCCAACATCACCACCCTTGGGAGCATGGCTCTGATCTTTGAGTATATCATGGAGGGGATTCCAT

Appendix 1

ATCCCAGCAACCTACCCTTGATGGCAAACTGGAAGACCTTCTTGCTGTTCTTTGGTACAGCCATCTTCAC
ATTTGAAGGCGTCGGTATGGTTCTGCCTCTCAAAAACCAGATGAAGCATCCACAGCAGTTTTCTTTTGTT
CTGTACTTGGGGATGTCCATTGTCATCATCCTCTATATCTTACTGGGGACACTGGGCTACATGAAGTTTG
GGTCAGACACCCAGGCCAGCATCACCCTCAACTTGCCCAATTGCTGGTTGTACCAGTCAGTCAAGCTGAT
GTACTCTATCGGCATCTTCTTCACCTATGCCCTCCAGTTCCACGTCCCAGCTGAGATCATCATCCCGTTT
GCCATCTCCCAAGTGTCAGAGAGCTGGGCACTGTTTGTAGACCTGTCTGTCCGCTCAGCCTTGGTCTGTC
TAACCTGTGTCTCAGCCATCCTCATCCCCCGCCTGGACTTGGTCATCTCCCTGGTAGGCTCCGTGAGCAG
CAGCGCCCTGGCTCTCATCATCCCAGCCCTCCTGGAGATCGTCATCTTTTACTCTGAGGACATGAGCTGT
GTCACCATTGCCAAGGACATCATGATTAGCATCGTGGGCCTTTTAGGGTGTATATTTGGGACATACCAAG
CCCTCTATGAGTTGCCCCAACCCATCAGCCATTCCATGGCCAACTCCACAGGTGTCCATGCATAATTATC
TGTTTTTATTCTAATAGCTCTCCCTTCCTCCCATCCCCAGTTTGACTTCCATGTGGATGTTATATACCTT
CATCAAATCCCAACATCTCTATATTAATTAGTGGCGTCTTTATCTTTCCAAGAGAAATGCAGATGAGAAA

hPAT3.2

AGCAGCACGCATCCTTGGGGTTAGGAGCCAGCATCTCAGGTCGGCAGCCATGGTTTCTCTGGAGAAAGAC
CCGTCAGAGGCTCATTCAGAGCCTTAACCCTGAGCCTCTGCCACCTACCCACCGTGTTGAAGATGTCATT
GCTTGGAAGGGACTACAACAGTGAGCTGAACTCCTTGGACAACGGACCTCAGTCACCCTCAGAGAGCAGC
AGTAGCATTACTTCAGAGAATGTCCATCCTGCTGGAGAAGCTGGACTATCGATGATGCAAACTTTGATCC
ACTTGTTGAAATGCAACATTGGCACAGGGCTCCTGGGGCTTCCCCTGGCCATAAAGAATGCCGGCTTGTT
GGTCGGTCCTGTCAGCCTTCTGGCCATCGGGGTCCTCACCGTGCACTGCATGGTCATCCTGTTGAACTGT
GCTCAACACCTCAGCCAGAGACTGCAGAAGACTTTTGTGAACTATGGAGAGGCCACGATGTACGGCCTTG
AAACCTGCCCGAACACCTGGCTGAGGGCCCATGCAGTGTGGGGAAGGTACACTGTCAGCTTCTTATTAGT
CATCACCCAGCTGGGCTTCTGCAGTGTTTATTTTATGTTTATGGCAGACAATTTACAACAGATGGTGGAA
GAAGCCCACGTGACCTCCAACATCTGCCAGCCCAGGGAGATTCTGACGCTGACCCCCATCCTGGACATTC
GTTTCTACATGCTGATAATCCTGCCCTTCCTGATCCTGTTGGTGTTTATCCAGAACCTCAAGGTGCTGTC
CGTCTTCTCGACATTGGCCAACATCACCACCCTTGGGAGCATGGCTCTGATCTTTGAGTATATCATGGAG
GGGATTCCATATCCCAGCAACCTACCCTTGATGGCAAACTGGAAGACCTTCTTGCTGTTCTTTGGTACAG
CCATCTTCACATTTGAAGGCGTCGGTATGGTTCTGCCTCTCAAAAACCAGATGAAGCATCCACAGCAGTT
TTCTTTTGTTCTGTACTTGGGGATGTCCATTGTCATCATCCTCTATATCTTACTGGGGACACTGGGCTAC
ATGAAGTTTGGGTCAGACACCCAGGCCAGCATCACCCTCAACTTGCCCAATTGCTGGTTGTACCAGTCAG
TCAAGCTGATGTACTCTATCGGCATCTTCTTCACCTATGCCCTCCAGTTCCACGTCCCAGCTGAGATCAT
CATCCCGTTTGCCATCTCCCAAGTGTCAGAGAGCTGGGCACTGTTTGTAGACCTGTCTGTCCGCTCAGCC
TTGGTCTGTCTAACCTGTGTCTCAGCCATCCTCATCCCCCGCCTGGACTTGGTCATCTCCCTGGTAGGCT
CCGTGAGCAGCAGCGCCCTGGCTCTCATCATCCCAGCCCTCCTGGAGATCGTCATCTTTTACTCTGAGGA
CATGAGCTGTGTCACCATTGCCAAGGACATCATGATTAGCATCGTGGGCCTTTTAGGGTGTATATTTGGG
ACATACCAAGCCCTCTATGAGTTGCCCCAACCCATCAGCCATTCCATGGCCAACTCCACAGGTGTCCATG
CATAATTATCTGTTTTTATTCTAATAGCTCTCCCTTCCTCCCATCCCCAGTTTGACTTCCATGTGGATGT
TATATACCTTCATCAAATCCCAACATCTCTATATTAATTAGTGGCGTCTTTATCTTTCCAAGAGAAATGC
AGATGAGAAAAGTTAGCACTGATGTCTCTCAGGCTACACCTCTTTTGGTTTTATATTTTTTGG

Appendix 1

PRIMERS (apart from T7F) WHICH WERE USED FOR CONFIRMATION OF FLAG EPITOPE INSERTION

Primer name	Sequence
PAT1.1 for sequencing	AGCTGCCATGTCCACGCA
PAT1.2 for sequencing	GTGAAAAATGCAGGCATCGT
PAT1.3 for sequencing	GACGCCTACCATGGACTC
PAT1.4 for sequencing	TCTGGGGTACCTGCAATTTG
PAT1.5 for sequencing	CTTGGCCATCCTCATCCC

APPENDIX 2: Primers used to check for the endogenous expression of mPATs in TM4 cells

Appendix 2

PRIMERS USED TO CHECK FOR THE ENDOGENOUS EXPRESSION OF mPATs AND THEIR RELATIVE POSITIONS

mPAT1

>gb|BC138556.1|:119-1546 Mus musculus solute carrier family 36 (proton/amino acid symporter), member 1, mRNA (cDNA clone MGC:170181 IMAGE:8861576), complete cds

ATGTCCACACAGAGGCTTCGGAACGAAGACTATCATGACTACAGTTCCACAGACGTGAGCCCCGAGGAGA
GCCCATCTGAAGGCCTTGGCAGCTTCTCCCCCGGCTCCTACCAACGCTTAGGAGAGAACAGTAGCATGAC
ATGGTTCCAGACCCTGATCCACCTGCTGAAAGGCAACATTGGCACCGGACTGCTGGGGCTGCCTCTGGCA
GTGAAGAACGCAGGCCTCCTGTTGGGTCCTCTCAGCCTGCTGGTGATTGGTATCGTGGCCGTGCACTGCA
TGGGTATCCTGGTGAAGTGTGCTCATCACTTATGTCGTAGACTGAACAAACCCTTTTTGGACTATGGGGA
CACGGTGATGTATGGACTAGAATGCAGCCCCAGCACCTGGGTCCGGAACCACTCCCACTGGGGAAGGCGC
ATCGTGGACTTCTTCCTCATCGTCACTCAGCTGGGATTCTGCTGTGTCTACTTCGTGTTTCTGGCGGACA
ACTTTAAGCAGGTGATAGAGGCAGCCAATGGGACCACCACCAACTGCAACAACAATGTGACCGTGATCCC
GACGCCCACCATGGACTCTCGACTCTACATGCTCTCCTTCCTGCCCTTCCTGGTGTTGCTGTCTTTCATC
AGGAACCTGCGTGTGTTGTCCATCTTCTCCCTGCTGGCCAACATCAGCATGTTCGTCAGCCTGATCATGA
TATACCAGTTCATTGTCCAGAGGATCCCAGACCCCAGCCACCTCCCCTTGGTGGCTCCATGGAAGACCTA
CCCTCTGTTCTTTGGCACAGCGATTTTTGCTTTTGAAGGCATTGGAGTGGTTCTACCCCTTGAGAACAAA
ATGAAGGACTCACAGAAATTTCCATTGATTCTGTATTTGGGGATGGCTATCATCACTGTACTCTACATCA
GCCTGGGGAGCCTGGGGTACCTGCAATTTGGAGCTAATATCAAGGGCAGCATCACCCTCAACCTGCCCAA
CTGCTGGTTGTACCAGTCGGTGAAGCTGCTGTACTCCATAGGCATCTTCTTCACCTATGCCCTCCAGTTC
TATGTCGCAGCTGAGATCATCATCCCGGCCATTGTGTCCCGAGTGCCTGAGCATTTCGAGCTGATGGTGG
ACCTTTGTGTGCGCACCGCCATGGTCTGTGTGACATGTGTGCTGGCCATCCTCATCCCACGCCTGGACCT
GGTCATCTCCCTGGTGGGCTCTGTGAGCAGCAGCGCCCTGGCCCTCATCATCCCGCCCCTGCTGGAGGTG
GTCACCTACTATGGAGAGGGCATTAGCCCCCTGACCGTCACCAAGGATGCCCTCATCAGCATCCTGGGCT
TCGTGGGCTTCGTGGTGGGGACCTACGAGTCTCTGTGTGAGCTGATCCAGCCAAGCCATAGCGACTCCTC
TACCAATTCCACCAGTGCCTTCATATAA

mPAT2

>gi|238624177:119-1555 Mus musculus solute carrier family 36 (proton/amino acid symporter), member 2 (Slc36a2), mRNA

ATGTCTGTGACCAAGAGTGCCAGGAGTCCGCAGGTAGCCACCCCTCTCAATCTGGACCTTCCTGAGAGTG
CCAAGAAGCTGCAGAGCCAGGATCCCAGTCCAGCGAATGGGAGCTCTTCAGAGTCATCAAAGAAGACCAA
GGGCATAACCGGGTTCCAGACATTGGTTCATCTGGTCAAAGGCAACATGGGCACAGGGATCCTCGGACTT
CCCCTGGCTGTGAAGAATGCGGGCATCTTGATGGGCCCACTCAGCTTGCTGGTAATGGGCCTCATCGCCT
GCCACTGTATGCACATCCTAGTCAGATGTGCCCAGCGCTTCTGTCACAGACTGAACAAGCCTTTCATGGA
CTATGGGGACACAGTGATGCACGGACTGGCTTTCAGTCCCAATGCCTGGCTGCAAAACCACGCCCACTGG
GGGAGGCGTGTCGTGAGCTTCTTCCTCATCGTCACCCAGCTGGGTTTCTGCTGTGTGTACATTGTGTTTC
TGGCGGACAATTTAAAACAGGTAGTAGAAGCTGTTAACAGCACAACCATCAGCTGCCACAAGAACGAGAC

Appendix 2

GGTAGTTCTGACGCCCACCATGGACTCTCGACTCTACATGCTCTCCTTCCTGCCCGTTCTGGGGCTTCTG
GTGTTCGTCAGGAACCTGCGTGTCCTCACCATCTTCTCCCTGCTGGCCAACATCAGCATGCTGGTCAGCC
TGGTCATCATTGCCCAGTACATCATCCAGGAAATTCCAGATGCCAGCCAGTTGCCACTGGTAGCAAGCTG
GAAGACCTACCCGCTCTTCTTTGGAACAGCCATTTTTTCATTTGAAAGCATCGGTGTGGTCCTGCCTCTG
GAAAACAAGATGAAGGATGCCCGCGGCTTCCCAACCATTCTGTCTTTGGGGATGTCCATCATCACCACCC
TGTACATTGCTATCGGGGCTCTGGGCTACCTGCGGTTTGGAGATGACATCAAAGCCAGCATCACTCTTAA
CCTGCCCAACTGCTGGCTGTACCAATCAGTCAAGCTCCTGTATGTCGTCGGTATCCTGTGTACCTATGCC
CTGCAGTTCTACGTCCCTGCGGAAATCATCATCCCCCTTGCTGTCTCTCAGGTGTCAAAACGCTGGGCAC
TGCCCGTGGACCTGTCTATCCGGCTTGCCCTGGTCTGCCTCACATGCATGCTGGCCATCCTCATCCCACG
CCTGGACCTGGTCCTCTCCCTGGTGGGCTCTGTGAGCAGCAGCGCCCTGGCCCTCATCATCCCGCCCCTG
CTGGAGGTGGTCACCTACTATGGAGAGGGCATTAGCCCCCTGACCGTCACCAAGGATGCCCTCATCAGCA
TCCTGGGCTTCATGGGCTTTGTGGTGGGGACCTACCAGGCCCTGGATGAGCTGATCAAGTCCGGGAACTC
TCCCGCCCTGTCCAACTCCACCATGTTTATTCAGTGA

mPAT3

>gi|262231847:255-1688 Mus musculus solute carrier family 36 (proton/amino acid symporter), member 3 (Slc36a3), mRNA

ATGGGGAATGTGCCGCTGCTTAGAGAAGTTGGCAAGTGTCAGCGGAATATGTTCGGTCGGTCTACAGCCT
CCTCAAAAGGCAGCAGCAACAGCAGAAGCAGCAGCAGTACCTCCCCAAAGAAGGGCCCTCGCAGGGAAGC
TGATGCGCTAATGTTTATACAGATCTTTATCCACCTGTTGAAAAGCAACATTGGCACAGGCTTCCTGGGA
CTGCCCCTGGCTGTGAAAAATGCTGGATTGTTGGTGGGGCCTGTCAGCCTCTTGGCCATCGGGGCGCTCA
CAGTGCACTGTATGGATATCCTGCTGAACTGTGCCTGCCATCTCACTCAGAGACTGCAGAGGAGTTTTGT
GAACTATGAAGAGACCACAATGTACAGCCTGGAAACGTGTCCAAGTCCTTGGCTGAGGACGCACTCAGTG
TGGGGCAGGTACGTTGTGAGCTTCCTGCTGATCGTCACTCAGCTGGGCTTCTGCAGCGTGTACTTCATGT
TTCTGGCGGACAACTTACAACAGATCATGGAAGAAGCCCACTTCACCTCCAACGTCTGCCAGCCGAGGCA
GAGCCTGGTGATGACCTCCATCCTGGACACTCGCTTCTATATGCTCACCATCCTGCCCTTCCTCATCCTG
CTGGTGCTCATCCAGAACCCACAGGTGTTGTCCATCTTCTCTACCTTGGCCACCATCACCACCCTGAGCA
GCCTGGCTCTGATCTTCGAGTATCTCATCCAGACCCCACACCACAGCAACCTGCCCCTGGTTGCAAACTG
GAAGACTTTCTTGCTGTTCTTTGGCACGGCTATCTTCACCTTTGAAGGCGTGGGGATGGTTCTGCCTCTC
AAAAGCCAGATGAAGAGCCCGCAGCAGTTCCCCGCTGTGCTGTATCTGGGGATGTCCTTTGTCATCTTCC
TCTACATCTGCCTGGGGACTCTGGGCTACATGAAGTTTGGGACAGACACTCAGGCCAGCATCACCCTCAA
CTTGCCCATTTGCTGGCTGTACCAGTCAGTCAAGCTGATGTACTCTGTGGGCATCTTCTTCACCTATGCC
CTCCAGTTCCACGTCCCAGCTGAGATCATCGTCCCCTATGTCGTCTCAAGAGTGTCTGAGAACTGGGCTC
TGTTCGTAGACCTGACTGTCCGCACAGCCTTGGTCTGCCTGACCTGTTTCTCAGCCGTCCTCATCCCCCG
CCTGGACCTGGTCATCTCCCTGGTGGGCTCTGTGAGCAGTAGCGCCCTGGCCATCATCATCCCGCCGCTG
CTGGAGATTGCCACATTTTACTCTGAGAACATAAGCTGTGCCACCATTGTCAAGGACATCATGATCAGCA
TCCTGGGCCTCTTAGGGTGTGTACTTGGGACATACCAAGCCCTGTACGAGATGACCCAGCAGACCCACTT
TTACATGGCCAACTCTACAAGGGTCCACATATAA

Appendix 2

mPAT4

>gb|BC115964.1|:25-1527 Mus musculus solute carrier family 36 (proton/amino acid symporter), member 4, mRNA (cDNA clone MGC:141261 IMAGE:40057255), complete cds

ATGGAAGCGCCGGCGCCGGCGGAGACGGCGGGATGCGAGGAGCTCGATATGGACGTGATGAGGCCCTTAA
TAAACGAGCAGAATTTCGATGGGTCGTCCGACGAGGAGCAGGAACAGACGCTTGTGCCCATACAGAAGCA
CTACCAGCTCGATGGGCAGCACGGGATTTCGTTCCTGCAGACCTTGGTGCATCTTCTCAAGGGGAACATC
GGGACTGGCCTTTTAGGGCTCCCCTTGGCTATAAAGAACGCGGGCATCGTGCTTGGACCAATCAGCCTTG
TGTTTATAGGAATTATTTCCGTCCACTGTATGCACATATTGGTACGTTGCAGTCACTTTCTATGTCAGAG
GTTTAAGAAGTCAACATTGGGGTACAGTGACACCGTGAGTTTTGCCATGGAGGCTAGTCCGTGGAGTTGC
CTTCAGCGACAGGCAGCATGGGGGCGGAGCGTGGTCGACTTCTTTCTGGTGATAACACAGCTGGGATTCT
GCAGCGTCTACATTGTCTTCTTAGCTGAGAATGTGAAACAGGTTCATGAAGGATTCCTGGGGAGCACGCC
GATTGTTTCAAATGGCTCGGACCTGTCTCATGCCTGTGAGAGAAGAAGTGTGGACCTCAGGGTCTATATG
CTCTGCTTTCTCCCACTTATAATTCTCTTGGTCTTCATTCGCGAGCTGAAGAATCTCTTTGTACTTTCAT
TCCTTGCCAACATTTCCATGGCTGCCAGTCTTGTGATAATTTACCAGTATGTTGTTAGGAACATGCCAGA
TCCCCACAACCTTCCAATAGTGGCCGGCTGGAAGAAATACCCACTGTTTTTTGGCACTGCTGTGTTTGCT
TTTGAAGGCATAGGAGTGGTGCTACCACTGGAAAACCAAATGAGAGAGTCCAAACGCTTCCCTCAGGCAT
TGAACATCGGCATGGCCATCGTCACCGTGCTGTACATCAGCCTAGCCACTTTAGGCTACATGTGTTTCCG
TGACGAGATCAAAGGCAGCATTACACTGAATCTTCCCCAGGACATGTGGTTGTATCAGTCAGTGAAAATT
CTGTATTCCTTTGGCATTTTTGTGACCTATTCAATTCAGTTCTATGTCCCAGCAGAGATCATTATCCCTG
GAGTCACTGCTAGACTTCATGCCAAATGGAAGCGCATTTGTGAATTTGGGATACGGTCCCTCTTGGTTAG
TATCACCTGTGCTGGGGCGATTCTCATTCCTCGCCTAGACATTGTGATCTCCTTCGTGGGGGCTGTGAGC
AGCAGTACACTAGCCCTGATCCTGCCCCCACTTGTGGAAATCCTTACATTTTCTAAGGACCACTACAACA
TATGGATGATCCTGAAAAACATTTCCATAGCGTTCACTGGAGTCGTGGGCTTCTTGTTGGGCACCTATGT
CACTGTTGAAGAAATTATTTATCCGACTACGGCAGTTGTTGCTGGCACCTCCCAGAGTCCCTTTCTGAAT
GTGAACTCTACATGCATAACAAGTGGTTTGTAA

mGAPDH

>gb|GU214026.1|:72-1073 Mus musculus glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, complete cds

ATGGTGAAGGTCGGTGTGAACGGATTTGGCCGTATTGGGCGCCTGGTCACCAGGGCTGCCATTTGCAGTG
GCAAAGTGGAGATTGTTGCCATCAACGACCCCTTCATTGACCTCAACTACATGGTCTACATGTTCCAGTA
TGACTCCACTCACGGCAAATTCAACGGCACAGTCAAGGCCGAGAATGGGAAGCTTGTCATCAACGGGAAG
CCCATCACCATCTTCCAGGAGCGAGACCCCACTAACATCAAATGGGGTGAGGCCGGTGCTGAGTATGTCG
TGGAGTCTACTGGTGTCTTCACCACCATGGAGAAGGCCGGGGCCCACTTGAAGGGTGGAGCCAAAAGGGT
CATCATCTCCGCCCCTTCTGCCGATGCCCCCATGTTTGTGATGGGTGTGAACCACGAGAAATATGACAAC
TCACTCAAGATTGTCAGCAATGCATCCTGCACCACCAACTGCTTAGCCCCCCTGGCCAAGGTCATCCATG
ACAACTTTGGCATTGTGGAAGGGCTCATGACCACAGTCCATGCCATCACTGCCACCCAGAAGACTGTGGA
TGGCCCCTCTGGAAAGCTGTGGCGTGATGGCCGTGGGGCTGCCCAGAACATCATCCCTGCATCCACTGGT
GCTGCCAAGGCTGTGGGCAAGGTCATCCCAGAGCTGAACGGGAAGCTCACTGGCATGGCCTTCCGTGTTC
CTACCCCCAATGTGTCCGTCGTGGATCTGACGTGCCGCCTGGAGAAACCTGCCAAGTATGATGACATCAA

Appendix 2

GAAGGTGGTGAAGCAGGCATCTGAGGGCCCACTGAAGGGCATCTTGGGCTACACTGAGGACCAGGTTGTC
TCCTGCGACTTCAACAGCAACTCCCACTCTTCCACCTTCGATGCCGGGGCTGGCATTGCTCTCAATGACA
ACTTTGTCAAGCTCATTTCCTGGTATGACAATGAATACGGCTACAGCAACAGGGTGGTGGACCTCATGGC
CTACATGGCCTCCAAGGAGTAA

APPENDIX 3: PAT Sequence Alignments

Appendix 3

Alignments of hPATs

```
hPAT1              --MSTQRLRN--EDYHDYSSTDVSPEESPSEGLNNLS--------SPGSYQRFGQSNSTT 48
hPAT2              --MSVTKSTEGPQGAVAIKLDLMSPPESAKKLENKDST---FLDESPSESAGLKKTKGIT 55
hPAT3_longer_      --MSLLGRDYN-SELNSLDNGPQSPSESSSS--------------ITSENVHPAGEAGLS 43
hPAT3              --MSLLGRDYN-SELNSLDNGPQSPSESSSS--------------ITSENVHPAGEAGLS 43
hPAT4              MEAAATPAAAGAARREELDMDVMRPLINEQNFDGTSDEEHEQELLPVQKHYQLDDQEGIS 60
                      :             .    *  . ..               .       . :

hPAT1              WFQTLIHLLKGNIGTGLLGLPLAVKNAGIVMGPISLLIIGIVAVHCMGILVKCAHHFCRR 108
hPAT2              VFQALIHLVKGNMGTGILGLPLAVKNAGILMGPLSLLVMGFIACHCMHILVKCAQRFCKR 115
hPAT3_longer_      MMQTLIHLLKCNIGTGLLGLPLAIKNAGLLVGPVSLLAIGVLTVHCMVILLNCAQHLSQR 103
hPAT3              MMQTLIHLLKCNIGTGLLGLPLAIKNAGLLVGPVSLLAIGVLTVHCMVILLNCAQHLSQR 103
hPAT4              FVQTLMHLLKGNIGTGLLGLPLAIKNAGIVLGPISLVFIGIISVHCMHILVRCSHFLCLR 120
                    .*:*:**:* *:***:******:****:::**:**: :*.:: *** **:.*:: :. *

hPAT1              LNKSFVDYGDTVMYGLESSPCSWLRNHAHWG----------------------------- 139
hPAT2              LNKPFMDYGDTVMHGLEANPNAWLQNHAHWG----------------------------- 146
hPAT3_longer_      LQKTFVNYGEATMYGLETCPNTWLRAHAVWGRWNLALSPRLECSGKISAHCNPHLQGSSN 163
hPAT3              LQKTFVNYGEATMYGLETCPNTWLRAHAVWG----------------------------- 134
hPAT4              FKKSTLGYSDTVSFAMEVSPWSCLQKQAAWG----------------------------- 151
                   ::*. :.*.::. ..:*  * : *: :* **

hPAT1              ------------RRVVDFFLIVTQLGFCCVYFVFLADNFKQVIE---AANGTTNNCHNNE 184
hPAT2              ------------RHIVSFFLIITQLGFCCVYIVFLADNLKQVVE---AVNSTTNNCYSNE 191
hPAT3_longer_      SPAQASRVAGIYRYTVSFLLVITQLGFCSVYFMFMADNLQQMVE---KAHVTSNICQPRE 220
hPAT3              ------------RYTVSFLLVITQLGFCSVYFMFMADNLQQMVE---KAHVTSNICQPRE 179
hPAT4              ------------RSVVDFFLVITQLGFCSVYIVFLAENVKQVHEGFLESKVFISNSTNSS 199
                               *  *.*:*::******.**::*:*:*.:*: *     :   . .   .

hPAT1              TVILTPTMDSRLYMLSFLPFLVLLVFIRNLRALSIFSLLANITMLVSLVMIYQFIVQRIP 244
hPAT2              TVILTPTMDSRLYMLSFLPFLVLLVLIRNLRILTIFSMLANISMLVSLVIIIQYITQEIP 251
hPAT3_longer_      ILTLTPILDIRFYMLIILPFLILLVFIQNLKVLSVFSTLANITTLGSMALIFEYIMEGIP 280
hPAT3              ILTLTPILDIRFYMLIILPFLILLVFIQNLKVLSVFSTLANITTLGSMALIFEYIMEGIP 239
hPAT4              NPCERRSVDIRIYMLCFLPFIILLVFIRELKNLFVLSFLANVSMAVSLVIIYQYVVRNMP 259
                          :* *:*** :***::***:*::*: * ::* ***::   *:.:* ::: . :*

hPAT1              DPSHLPLVAPWKTYPLFFGTAIFSFEGIGMVLPLENKMKDPRKFPLILYLGMVIVTILYI 304
hPAT2              DPSRLPLVASWKTYPLFFGTAIFSFESIGVVLPLENKMKNARHFPAILSLGMSIVTSLYI 311
hPAT3_longer_      YPSNLPLMANWKTFLLFFGTAIFTFEGVGMVLPLKNQMKHPQQFSFVLYLGMSIVIILYI 340
hPAT3              YPSNLPLMANWKTFLLFFGTAIFTFEGVGMVLPLKNQMKHPQQFSFVLYLGMSIVIILYI 299
hPAT4              DPHNLPIVAGWKKYPLFFGTAVFAFEGIGVVLPLENQMKESKRFPQALNIGMGIVTTLYV 319
                    * .**::* **.: ******:*:**.:*:****:*:**..::*.  * :** **  **:

hPAT1              SLGCLGYLQFGANIQGSITLNLPN-CWLYQSVKLLYSIGIFFTYALQFYVPAEIIIPFFV 363
hPAT2              GMAALGYLRFGDDIKASISLNLPN-CWLYQSVKLLYIAGILCTYALQFYVPAEIIIPFAI 370
hPAT3_longer_      LLGTLGYMKFGSDTQASITLNLPN-CWLYQSVKLMYSIGIFFTYALQFHVPAEIIIPFAI 399
hPAT3              LLGTLGYMKFGSDTQASITLNLPN-CWLYQSVKLMYSIGIFFTYALQFHVPAEIIIPFAI 358
hPAT4              TLATLGYMCFHDEIKGSITLNLPQDVWLYQSVKILYSFGIFVTYSIQFYVPAEIIIHGIT 379
                    :. ***: *  : :.**:****:  *******::*  **: **::**:*******

hPAT1              SRAPEHCELVVDLFVRTVLVCLTCILAILIPRLDLVISLVGSVSSSALALIIPPLLEVTT 423
hPAT2              SRVSTRWALPLDLSIRLVMVCLTCLLAILIPRLDLVISLVGSVSGTALALIIPPLLEVTT 430
hPAT3_longer_      SQVSESWALFVDLSVRSALVCLTCVSAILIPRLDLVISLVGSVSSSALALIIPALLEIVI 459
hPAT3              SQVSESWALFVDLSVRSALVCLTCVSAILIPRLDLVISLVGSVSSSALALIIPALLEIVI 418
hPAT4              SKFHTKWKQICEFGIRSFLVSITCAGAILIPRLDIVISFVGAVSSSTLAIILPPLVEILT 439
                   *:         :: :*  :*.:**  ********:***:**:**.::**:*:*.*:*:

hPAT1              FYSEGMSPLTIFKDALISILGFVGFVVGTYEALYELIQPS---------NAPIFINSTCA 474
hPAT2              FYSEGMSPLTIFKDALISILGFVGFVVGTYQALDELLKSE---------DSHPFSNSTTF 481
hPAT3_longer_      FYSEDMSCVTIAKDIMISIVGLLGCIFGTYQALYELPQP----------ISHSMANSTGV 509
hPAT3              FYSEDMSCVTIAKDIMISIVGLLGCIFGTYQA---------------------------- 450
hPAT4              FSKEHYNIWMVLKNISIAFTGVVGFLLGTYITVEEIIYPTPKVVAGTPQSPFLNLNSTCL 499
                   * .*  .   : *:  *:: *.:* :.*** :

hPAT1              FI--- 476
hPAT2              VR--- 483
hPAT3_longer_      HA--- 511
hPAT3              -----
hPAT4              TSGLK 504
```

Appendix 3

Alignments of PAT1 across species

```
Homo_sapiens          MSTQRLRNEDYHDYSSTDVSP--EESPSEGLNNLSSPGSYQRFGQSNSTTWFQTLIHLLK 58
Bos_taurus            MSTQRLRDEDYRDYSSTDASP--EESPSEGLNNFSSSGSYMRFGESNSTTWFQTLIHLLK 58
Mus_musculus          MSTQRLRNEDYHDYSSTDVSP--EESPSEGLGSFS-PGSYQRLGENSSMTWFQTLIHLLK 57
Rattus_norvegicus     MSTQRLRNEDYHDYSSTDVSP--EESPSEGLGSFS-PGSYQRLGENSSMTWFQTLIHLLK 57
Xenopus_laevis        MDTSRLTSDRYNDYSSTEVSPSEENSPGTIGNNVSRPRQYERLGEDSSTTWYQTLIHLLK 60
                      *.*.** .: *.*****:.**  *:**.   ...* . .* *:*:..* **:********

Homo_sapiens          GNIGTGLLGLPLAVKNAGIVMGPISLLIIGIVAVHCMGILVKCAHHFCRRLNKSFVDYGD 118
Bos_taurus            SNIGTGLLGLPLAVKNAGILMGPLSLLVIGLVAVHCMRILVKCAHHFCYRLNKPFVDYGD 118
Mus_musculus          GNIGTGLLGLPLAVKNAGLLLGPLSLLVIGIVAVHCMGILVKCAHHLCRRLNKPFLDYGD 117
Rattus_norvegicus     GNIGTGLLGLPLAVKNAGLLLGPLSLLVIGIVAVHCMGILVKCAHHLCRRLNKPFLDYGD 117
Xenopus_laevis        GNIGTGLLSLPLAVKNAGIVLGPLSLVFMGIIAVHCMDLLVKCAHHLCQREQRPFVDYGD 120
                      .*******.*********:::**:**:.:*::***** :*******:* * ::.*:****

Homo_sapiens          TVMYGLESSPCSWLRNHAHWGRRVVDFFLIVTQLGFCCVYFVFLADNFKQVIEAANGTTN 178
Bos_taurus            TVMYSLEASPISWLRNHAHWGRRMVDFFLIVTQLGFCCIYFVFLADNFKQVIEMANGTTN 178
Mus_musculus          TVMYGLECSPSTWVRNHSHWGRRIVDFFLIVTQLGFCCVYFVFLADNFKQVIEAANGTTT 177
Rattus_norvegicus     TVMYGLECSPSTWIRNHSHWGRRIVDFFLVVTQLGFCCVYFVFLADNFKQVIEAANGTTT 177
Xenopus_laevis        ALMYGMQGCPSQWLQRNSVWGRWIVGFFLILTQLGFCCVYFVFLADNIKQVVEAANGTTN 180
                      ::**.:: .*  *::.:: *** :*.***::*******:********:***:* *****.

Homo_sapiens          NCHNNETVILTPTMDSRLYMLSFLPFLVLLVFIRNLRALSIFSLLANITMLVSLVMIYQF 238
Bos_taurus            NCHNNETVILTPTMDSRLYMLTFLPFMVLLVFIRNLRALSIFSLLANITMAVSLVMIYQF 238
Mus_musculus          NCNNNVTVIPTPTMDSRLYMLSFLPFLVLLSFIRNLRVLSIFSLLANISMFVSLIMIYQF 237
Rattus_norvegicus     NCNNNETVILTPTMDSRLYMLTFLPFLVLLSFIRNLRILSIFSLLANISMFVSLIMIYQF 237
Xenopus_laevis        DCSANETVVLVESMDSRLYILSFLPFLILLVFITNLRYLSIFSLLANLSMLGSVIMIYQY 240
                      :*  * **: . :******:*:****::** ** *** *********::*  *::****:

Homo_sapiens          IVQRIPDPSHLPLVAPWKTYPLFFGTAIFSFEGIGMVLPLENKMKDPRKFPLILYLGMVI 298
Bos_taurus            TVQNIPDPSHLPLVASWKTYPLFFGTAIFAFEGIGMVLPLENKMKDPKKFSLILYVGMAI 298
Mus_musculus          IVQRIPDPSHLPLVAPWKTYPLFFGTAIFAFEGIGVVLPLENKMKDSQKFPLILYLGMAI 297
Rattus_norvegicus     IVQRIPDPSHLPLVAPWKTYPLFFGTAIFAFEGIGVVLPLENKMKDSQKFPLILYLGMAI 297
Xenopus_laevis        IGRDIPDPTHLSYVSSWRSFALFFGTAIFAFEGIGVVLPLENKMKIPHQFPVVLYVGMGI 300
                        : ****:**. *:.*:::.********:*****:********* .::*.::**:** *

Homo_sapiens          VTILYISLGCLGYLQFGANIQGSITLNLPNCWLYQSVKLLYSIGIFFTYALQFYVPAEII 358
Bos_taurus            VTALYVSLGILGYLHFGANIQGSITLNLPNCWLYQSVKLLYSVGIFFTYALQFYVPAEII 358
Mus_musculus          ITVLYISLGSLGYLQFGANIKGSITLNLPNCWLYQSVKLLYSIGIFFTYALQFYVAAEII 357
Rattus_norvegicus     ITVLYISLGSLGYLQFGADIKGSITLNLPNCWLYQSVKLLYSIGIFFTYALQFYVAAEII 357
Xenopus_laevis        VTILYISMGTLGFLRFGSSIQASITLNLPNCWFYQSVKLLYSFGIFITFALQFYVAAEII 360
                      :* **:*:* **:*:**:.*:.**********:*********.***:*:******.****

Homo_sapiens          IPFFVSRAPEHCELVVDLFVRTVLVCLTCILAILIPRLDLVISLVGSVSSSALALIIPPL 418
Bos_taurus            IPFFVARGPEHCELVIDLSVRTVLVCLTCILAILIPRLDLVISLVGSVSSSALALIIPPL 418
Mus_musculus          IPAIVSRVPEHFELMVDLCVRTAMVCVTCVLAILIPRLDLVISLVGSVSSSALALIIPPL 417
Rattus_norvegicus     IPAIVSRVPERFELVVDLSARTAMVCVTCVLAVLIPRLDLVISLVGSVSSSALALIIPPL 417
Xenopus_laevis        VPTVTLHVHDRWVRCMDLTVRAALVCLTCVLAILIPHLGLVISLVGSVSSSALALIIPPL 420
                      :* .. :  ::    :** .*:.:**:**:**:***:*.********************

Homo_sapiens          LEVTTFYSEGMSPLTIFKDALISILGFVGFVVGTYEALYELIQPSNAPIFINSTCAFI-- 476
Bos_taurus            LEITTYYSEGMSPITIVKDALISILGFVGFVVGTCLTLYELIQPSSAPIFINSTSAFV-- 476
Mus_musculus          LEVVTYYGEGISPLTVTKDALISILGFVGFVVGTYESLCELIQPSHSDSSTNSTSAFI-- 475
Rattus_norvegicus     LEVTTYYGEGISPLTITKDALISILGFVGFVVGTYESLWELIQPSHSDSSTNSTSAFI-- 475
Xenopus_laevis        LEILTYYTEGLSRWVIAKDIFISLVGFLGFVLGTYVALWELIVPEVSP-ALNETALFVVQ 479
                      **: *:* **:*  .: ** :**::**:***:**  :* *** *. :    *.*. *:
```

Appendix 3

Alignments of PAT4 across species

```
Homo_sapiens          MEAAATPAAAGAARREELDMDVMRPLIN-EQNFDGT------------SDEEHEQELLP- 46
Bos_taurus            MEAAA-PAAAEAAGREELDMDVMRPLIN-EQNFDGT------------SDEEQEQELLP- 45
Mus_musculus          MEAPA---PAETAGCEELDMDVMRPLIN-EQNFDGS------------SDEEQEQTLVP- 43
Rattus_norvegicus     MEAPA---PAEAAGCEELDMDVMRPLIN-EQNFDGS------------SDEEQEQTLLP- 43
Xenopus_laevis        MDATE---LGVSGEGEEIDMEVMRPLIESEDRFEGTYGEKKHLQRYLNSDNKKDEEVMKP 57
                      *:*.     . :.  **:**:******: *:.*:*:            **::::: ::

Homo_sapiens          -----------------VQKHYQLDDQEGISFVQTLMHLLKGNIGTGLLGLPLAIKNAGI 89
Bos_taurus            -----------------VQKHHQLDDQEGISFVQTLIHLLKGNIGTGLLGLPLAIKNAGI 88
Mus_musculus          -----------------IQKHYQLDGQHGISFLQTLVHLLKGNIGTGLLGLPLAIKNAGI 86
Rattus_norvegicus     -----------------MQKHYQLDGQHGISFVQTLMHLLKGNIGTGLLGLPLAIKNAGI 86
Xenopus_laevis        LIENEDDSDGTCDEHQYLQRHPDLDNKDGLTFFQTLIHLLKGNIGTGLLGLPLAMKNAGV 117
                                      :*:* :**.:.*::*.***:*****************:****:

Homo_sapiens          VLGPISLVFIGIISVHCMHILVRCSHFLCLRFKKSTLGYSDTVSFAMEVSPWSCLQKQAA 149
Bos_taurus            VLGPISLVFIGIISVHCMHILVRCSHFLCQRFKKSTLGYSDTVSFAMEVSPWSCLQKQTA 148
Mus_musculus          VLGPISLVFIGIISVHCMHILVRCSHFLCQRFKKSTLGYSDTVSFAMEASPWSCLQRQAA 146
Rattus_norvegicus     VLGPISLVFIGIISVHCMHILVRCSHFLCQRFKKSTLGYSDTVSFAMEASPWSCLQRQAA 146
Xenopus_laevis        LLGPISLLFFGIISIHCMNILVRCSHFLCQRYKKANLGYSDTVGLALEVGPG-VLQRHAS 176
                      :******:*:****:***:********** *:**:.*******.:*:*..*   **::::

Homo_sapiens          WGRSVVDFFLVITQLGFCSVYIVFLAENVKQVHEGFLESKVFISNSTNSSNPCERRSVDI 209
Bos_taurus            WGRNVVDFFLVITQLGFCSVYIVFLAENVKQVHEGFLESKVFVLNSTNSSSPCERRSIDL 208
Mus_musculus          WGRSVVDFFLVITQLGFCSVYIVFLAENVKQVHEGFLGSTPIVSNGSDLSHACERRSVDL 206
Rattus_norvegicus     WGRSVVDFFLVITQLGFCSVYIVFLAENVKQVHEGLLETTVVVSNSSDLSQVCERRSVDL 206
Xenopus_laevis        FGRNLVDWFLVVTQLGFCSVYFVFLAENIKQVFEVFLETKLQQSEIG-------IWSLDL 229
                      :**.:**:***:*********:******:***.* :* :.    :           *:*:

Homo_sapiens          RIYMLCFLPFIILLVFIRELKNLFVLSFLANVSMAVSLVIIYQYVVRNMPDPHNLPIVAG 269
Bos_taurus            RIYMLCFLPFLILLVFIRELKNLFVLSFLANISMAVSLVIIYQYVVRNMPDLHNLPIVAG 268
Mus_musculus          RVYMLCFLPLIILLVFIRELKNLFVLSFLANISMAASLVIIYQYVVRNMPDPHNLPIVAG 266
Rattus_norvegicus     RVYMLCFLPLLILLVFIRELKSLFVLSFLANISMAASLVIIYQYVVRSMPDPHNLPIVAG 266
Xenopus_laevis        RIYMFSFLPLIIPLVFIRDLKNLSLLSFFANVSMAISLLIVYQYVIRNLSDPRTLPLGTS 289
                      *:**:.***::* *****:**.* :***:**:*** **:*:****:*.:.* :.**: :.

Homo_sapiens          WKKYPLFFGTAVFAFEGIGVVLPLENQMKESKRFPQALNIGMGIVTTLYVTLATLGYMCF 329
Bos_taurus            WKKYPLFFGTAVFAFEGIGVVLPLENQMKESKRFPQALNIGMGIVTALYVSLATLGYMCF 328
Mus_musculus          WKKYPLFFGTAVFAFEGIGVVLPLENQMRESKRFPQALNIGMAIVTVLYISLATLGYMCF 326
Rattus_norvegicus     WKKYPLFFGTAVFAFEGIGVVLPLENQMRESKRFPQALNIGMAIVTVLYISLATLGYMCF 326
Xenopus_laevis        WKTYPLFFGTAIFAFEGIGVVLPLENRMRDKKDFSKALNIGMAIVTTLYISLATLGYFCF 349
                      **.********:**************:*::.* *.:******.***.**::******:**

Homo_sapiens          HDEIKGSITLNLPQDVWLYQSVKILYSFGIFVTYSIQFYVPAEIIIHGITSKFHTKWKQI 389
Bos_taurus            HDDIKGSITLNLPQDVWLYQSVKILYSFGIFVTYSIQFYVPAEIIIPVITSRFHAKWKQI 388
Mus_musculus          RDEIKGSITLNLPQDMWLYQSVKILYSFGIFVTYSIQFYVPAEIIIPGVTARLHAKWKRI 386
Rattus_norvegicus     RDEIKGSITLNLPQDMWLYQSVKILYSFGIFVTYSIQFYVPAEIIIPAVTARLHAKWKCI 386
Xenopus_laevis        GDQIKGSITLNLPQDSWLYQLVKILYSFGIYVTYAIQYYVPAEIILPAVTSRVQKTRKLL 409
                       *:************ **** *********:***:**:*******:  :*::.: . * :

Homo_sapiens          CEFGIRSFLVSITCAGAILIPRLDIVISFVGAVSSSTLAIILPPLVEILTFSKEHYNIWM 449
Bos_taurus            YEFAIRSFLVTITCAGAILIPRLDIVIAFVGAVSSSTLALILPPLVEILTFSKEHYSIWM 448
Mus_musculus          CEFGIRSLLVSITCAGAILIPRLDIVISFVGAVSSSTLALILPPLVEILTFSKDHYNIWM 446
Rattus_norvegicus     CDFGIRSLLVSITCAGAVLIPRLDIVISFVGAVSSSTLALILPPLVEILTFSKDHYNVWM 446
Xenopus_laevis        CEFTMRFFLVCLTCAVAVLIPRLDLVISFVGAVSSSTLALILPPLVEIITYHKENLSPWV 469
                       :* :* :** :*** *:******:**:***********:********:*: *:: . *:

Homo_sapiens          VLKNISIAFTGVVGFLLGTYITVEEIIYPTPKVVAGTPQSPFLNLNSTCLTSGLK 504
Bos_taurus            VLKNVSIVFTGVVGFLLGTYVTVEEIIYPTPRAITSTPHGPSLNLNSTCFTSGLK 503
Mus_musculus          ILKNISIAFTGVVGFLLGTYVTVEEIIYPTTAVVAGTSQSPFLNVNSTCITSGL- 500
Rattus_norvegicus     VLKNISIAFTGFVGFLLGTYVTVEEIIYPTTAVADGASQSLSLNVNSTCVSSGL- 500
Xenopus_laevis        IMKDVGIAVIGFVGFIAGTYVTIEEMIYPISYVPPNVSHSDFGVLNNTVLEGH-- 522
                      ::*::.*.. *.***: ***:*:**:*** . .  ...:.    :*.* . .
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