Distribution and Relative Abundance of Nutrient Transporter mRNA in the Gastrointestinal Tract of Black Bears

by

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(ABSTRACT)

Black bears are omnivorous, and tend to be opportunistic feeders, in that they will eat what is readily abundant or available. The end-products of intestinal digestion are absorbed by the body through the action of transporter proteins expressed on the brushborder membrane of small intestinal epithelial cells. The goal of this study was to increase the understanding of the physiological processes associated with nutrient assimilation by black bears. Distribution and relative abundance of mRNA of a peptide transporter (PepT1), a glucose transporter (SGLT1), two AA transporters (NBAT, $b^{o,+}AT$), and a digestive enzyme, aminopeptidase N (APN), in the intestinal tract of black bears were investigated. Ten bears were used for this study. For tissue collection, the intestine was removed from the animal and divided into five sections. Each collected section was opened longitudinally, rinsed in ice-cold PBS, and the mucosal scrapings were stored at -80°C. Total RNA was extracted and quantified by spectrophotometry. Abundance of PepT1, SGLT1, NBAT, $b^{o,+}AT$, and APN mRNA was determined by performing Northern blots, using bear cDNA probes. Northern blot data were quantified by densitometric analysis, with the abundance of each gene expressed relative to GAPDH. Abundance of PepT1 ($P < 0.05$), APN ($P < 0.05$), and SGLT1 ($P < 0.0001$) changed quadratically from the proximal to the distal intestine with abundance being greatest in the midregion. Abundance of $b^{o,+}AT$ mRNA increased linearly ($P < 0.05$) from the proximal to distal intestine. Abundance of NBAT mRNA did not change among intestinal segments.The absolute number of molecules of mRNA/ng of total RNA for each gene was determined using Real-Time PCR. Similar to the Northern results, abundance of PepT1 ($P < 0.0003$), SGLT1 ($P < 0.0003$), and APN ($P < 0.02$) changed quadratically from the proximal to distal intestine with abundance being greatest in the mid-region, and $b^{o,+}AT$ mRNA increased linearly ($P < 0.0001$) from the proximal to distal intestine. NBAT mRNA abundance also increased linearly ($P < 0.0001$) from proximal to distal intestine. PepT1 mRNA was present at tenfold or greater levels than AA transporter mRNA in all segments of the intestine, suggesting that di- and tripeptides constitute the major form in which AAs are absorbed. NBAT and $b^{o,+}AT$ mRNA abundance was greater towards the distal portion of the intestine, suggesting their importance in salvaging remaining unabsorbed AAs.These results indicate that the mRNA of nutrient transporters examined and APN are differentially expressed throughout the gastrointestinal tract of black bears, suggesting their involvement in nutrient assimilation.

Key words: Nutrient Transporter, Black Bear, PepT1, Gastrointestinal Tract, Northern Blot, Real-Time PCR

Dedication

I dedicate this thesis to the memory of my friend and mentor, Dr. Patrick Scanlon (September 16, 1941 – March 5, 2003).

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Chapter I

Introduction

The end-products of stomach digestion enter the small intestine where pancreatic and intestinal epithelial cell membrane-bound enzyme digestion results in absorbable end-products. Throughout the length of the small intestine, small finger-like projections called villi serve to maximize the absorptive surface area. Lining the intestinal villi are polarized, epithelial cells called enterocytes, which have a brushborder membrane (microvilli) facing the intestinal lumen that is in contact with nutrients, and contain the transport proteins responsible for nutrient absorption. Enterocytes originate in the crypts and migrate up the villi, during which time they mature and gain absorptive function.

In recent years, molecular cloning has allowed for extensive characterization of nutrient transporter genes expressed on the brushborder membrane of intestinal epithelial cells. Real-Time PCR has emerged as a sensitive, but accurate method for quantifying low-abundance transcripts in a tissue, and currently a peptide transporter (PepT1), 5 AA transporter proteins (NBAT, $b^{o,+}$AT, B^{o}AT1, ASCT2, and EAAC1), two monosaccharide transporters (SGLT1 and GLUT5), and numerous hydrolases have been identified on the brushborder membrane of the enterocyte in humans. Those hydrolases are responsible for the final digestion of nutrients. In recent years, studies have focused on the function and regulation of these genes and the pathological conditions associated with gene mutations.

The literature review in this thesis will focus on the biochemical and molecular characterization of peptide, AA, and monosaccharide transporters, and digestive enzyme proteins in the intestine, and their regulation in mammalian species. Also included is a discussion of the study animal, the black bear, including nutrition, gastrointestinal

physiology, and seasonal changes in metabolism. Although transporter proteins have been studied extensively in domestic animals and lab species, relatively little research has been conducted using wild species, and there is no information to date regarding nutrient transport in the gastrointestinal tract of black bears.

The study reported in this thesis was conducted to provide a descriptive survey of nutrient transporter mRNA in the intestinal tract of black bears. Primers designed for use in the pig and bear intestinal total RNA were used to amplify gene fragments that were used as probes for a Northern blot analysis. Additionally, Real-Time PCR was performed using a cRNA absolute standard curve, to determine the absolute mRNA expression levels of these genes to gain insight as to their relative importance in the bear intestine. This study is the first to report the presence of nutrient transporter genes in the intestinal tract of black bears, and the first to report absolute quantification of transporter and enzyme mRNA by using a cRNA standard curve. These results will potentially aid in improving diet formulation for domestic species and will provide a starting point for research directed at understanding the survival strategies of bears on a molecular level.

Chapter II

Review of Literature

Introduction

Dietary proteins entering the stomach are hydrolyzed through the action of substances secreted by the gastric glands (Pond et al., 1995). The parietal cells secrete HCl in response to the presence of chyme, and the acidic pH environment created by the presence of HCl triggers the activation of pepsin, which is secreted as a zymogen, pepsinogen, by the chief cells. Pepsin acts on proteins to yield proteoses, peptones, and peptides. The end-products of stomach digestion enter the small intestine where pancreatic enzyme digestion (trypsin, chymotrypsin, elastase, etc.) results, to a large extent, in absorbable end-products. Hydrolases are expressed on the brushborder membrane of absorptive, epithelial cells serving to further digest luminal nutrients. The end products of digestion are absorbed by these epithelial cells in the intestine through the action of nutrient transporters located on the brushborder membrane. There are differences in the nutrient requirements of cells throughout the body, and a plethora of transporters are present with varying structures and functions. Transport systems consist of proteins that recognize, bind, and relocate a substrate or multiple substrates across a cell membrane. Transporters have been characterized in endothelial cells and in the apical and basolateral membranes of epithelial cells throughout the body. Among the intestinal transporters that have been well characterized are the peptide transporter, PepT1, the sugar transporters (i.e. SGLT1, Glut2, and Glut5), and the AA transporters (i.e., NBAT, $b^{o,+}AT$, ASCT1, EAAC1, etc.). This review will concentrate on the function and molecular characterization of these genes in the small intestine of mammalian species.

This review will also include a discussion of the study animal, the black bear, including a review of black bear nutrition and gastrointestinal physiology.

Brushborder Membrane Digestion in the Small Intestine

Membrane-bound enzymes expressed in enterocytes serve to further break down the end-products of stomach and pancreatic enzyme digestion. This review will focus on aminopeptidase N (APN) in the small intestine.

Aminopeptidase N (APN) in the Intestine. Aminopeptidases catalyze the removal of AA from the N-terminal end of peptides (Albiston et al., 2004). Aminopeptidases are widespread throughout the body and serve many functions that aid in protein maturation and peptide hormone regulation (Sanderink et al., 1988). Aminopeptidase N (APN, α-aminoacyl-peptide hydrolase (microsomal), EC 3.4.11.2, arylamidase, aminopeptidase M, and alanine aminopeptidase) is a member of the M_1 zinc metalloprotease family, and cleaves AA from the N-terminal end of peptides. Aminopeptidase N is fairly specific for peptides containing an N-terminal L-isomer neutral or basic AA (Sanderink et al., 1988; Luciani et al., 1998). Oligopeptides appear to be the preferred substrate, as APN is less effectual at cleaving residues from dipeptides (Look et al., 1989). Some substrates include neuropeptides (i.e., somatostatin), vasoactive peptides (i.e., angiotensin III), and cytokines (i.e., interleukin 8; Jardinaud et al., 2004). Aminopeptidase N is one of several aminopeptidases classified as having a broad substrate specificity, and like others, is unable to cleave N-terminal proline (Jankiewicz and Bielawski, 2003).

APN Structure and Catalysis Mechanism. APN is a type II membrane-bound protein comprised of 967 AA (150 kDa) and a single transmembrane domain with an N-terminal intracellular domain and a large carboxyl extracellular domain containing the

active site of the enzyme (Olsen et al., 1988). Pig APN is cotranslationally modified with oligosaccharide chains and exhibits a molecular mass of 140 kDa, and side chains are then posttranslationally modified with complex oligosaccharides resulting in the 160 kDa molecular weight protein expressed on the brushborder membrane (Look et al., 1989). Members of the metalloaminopeptidase family use conserved residues within the protein at the carboxyl terminus to create a scaffold for binding of one or two metal ions, specifically, at least one zinc ion for APN (Lowther and Matthews, 2002). Only eight to 10 AA from the N-terminal domain are located intracellularly (Riemann et al., 1999). In most species (rat, pig and human), APN homodimerizes before being glycosylated in the golgi complex, presumably in the endoplasmic reticulum or in another compartment (Look et al., 1989; Danielsen, 1994). Metalloproteases catalyze cleavage by binding metal ions that increase the reactivity of water, stimulating the nucleophilic attack on the carbonyl carbon of the peptide bond, and also stabilizing the transition stage (Holz, 2002). Luciani et al. (1998) used site-directed mutagenesis of the pig APN E^{350} residue to confirm that it belonged to an anionic binding site in the N-terminal binding site of APN, which acts with the zinc ion to form a complex with the free amino group and carbonyl atom of the peptide bond to be cleaved, stabilizing the transition state.

Enzymes in this family are inhibited by compounds that chelate metal ions, such as EDTA, bestatin, and amastatin (Jankiewicz and Bielawski, 2003). APN, thus, serves to break down peptides into free AA and smaller peptides, which are absorbed by free AA transporters, and the peptide transporter, PepT1, respectively.

Tissue and Cellular Distribution of APN. APN activity is greatest in the small intestine, where it makes up approximately 8% of the protein content of the brushborder

membrane (Delmas et al., 1992). A whole-body investigation of APN expression in rats demonstrated highest labeling of APN in the intestine and kidney of adult rats (Jardinaud et al., 2004). Developmentally, APN labeling was not detected until embryo d 20, and labeling increased with age (Jardinaud et al., 2004). It is expressed to a lesser extent in the liver, stomach, lung, fibroblasts, nerve synapses, and colon (Delmas et al., 1992; Jardinaud et al., 2004). APN activity and protein expression increased from the proximal to distal small intestine, parallel to Ezetimibe, an inhibitor of intestinal cholesterol absorption (Kramer et al., 2005), and expression was localized to the brushborder membrane (Jardinaud et al., 2004). Expression of APN is also dependent on cholesterol availability in the lumen (Kramer et al., 2005). Deep apical tubules in the terminal brushborder, a specialized lipid-raft microdomain, serve to connect the brushborder surface to intracellular trafficking, and APN is present in these tubules which disappear when cholesterol levels decline (Hansen et al., 2003).

Other APN Functions. The APN protein is identical to the CD13 antigen and is thought to function in immunity (Albiston et al., 2004). Other roles of APN in the body include regulation of vasoactive peptides and serving as a retrovirus receptor (Albiston et al., 2004). Delmas et al. (1992) discovered that APN was the receptor for transmissible gastroenteritis virus (TGEV) in porcine small-intestinal brushborder membranes. The receptor sites for viruses and toxins are different from the enzymatic site, and enzymatic activity is not required for infectious activity (Albiston et al., 2004). APN is also a target protein for Ezetimibe, an inhibitor of cholesterol absorption (Kramer et al., 2005). APN is thought to play a role in angiogenesis through involvement with endothelial cell

migration and tube formation, and regulation of cell motility and invasion in tumor cells in patients with colon cancer (Hashida et al., 2002).

Summary. Aminopeptidase N is one of many digestive enzymes expressed on the brushborder membrane of enterocytes that is responsible for the final digestion of luminal nutrients. In addition to cleaving AA residues from the N-terminal end of peptides, APN also serves as a regulator of vasoactive peptides, a retrovirus receptor, and may play a role in immune function. The end-products of intestinal APN digestion, small peptides and free amino acids, are then absorbed by the peptide transporter, PepT1, and free amino acid transporters, respectively, also expressed on the brushborder membrane.

Peptide Transporters in the Small Intestine and Kidney

Peptide transporters and free AA transporters are responsible for the uptake of AA in the small intestine and subsequent transport to the bloodstream. The amount of peptides and free AA in the lumen of the small intestine depends on the nature of the ingested protein, with some protein structures, for example, those that are post-translationally modified by glycosylation, being more resistant to hydryolysis (Daniel, 2004). Food-processing thermal effects, including Maillard product formation, can also impact the rate of digestion. The extent of extra- and intracellular hydrolysis of peptides depends on the structure and concentration of peptides at the brushborder membrane. Peptide transport is generally considered to be faster and more efficient than free AA transport (Daniel, 2004). Studies using perfused human intestine demonstrated that dipeptides were absorbed faster than a mixture of the same AA presented in a free form (Daniel, 2004). In terms of energetic efficiency, two or three AA can be transported into the cell by a peptide transporter for the same amount of energy required to transport a

single free AA (Daniel, 2004). In addition, patients suffering from deficiencies in free AA transport were still able to assimilate essential AA, further exemplifying the importance of peptide transport (Adibi, 1997). PepT1 is thought to account for the majority of AA assimilation because of high expression levels, and high capacity, meaning that it can transport a large amount of substrate before becoming saturated and attaining V_{max}. Other evidence pointing to PepT1 as the main route of AA uptake comes from a study in the jejunum of patients suffering from cystinuria, a hyperaminoaciduria resulting from mutations in the $b^{o,+}$ AA transport system (Daniel, 2004). In the intestine of patients that exhibit this disorder, there is malabsorption of cystine, arginine and lysine, resulting in hyperexcretion. However, these patients do not develop a deficiency of lysine, an essential AA, pointing to the possibility that PepT1 transports enough dietary lysine to compensate for a deficiency in free AA transport.

The peptide transporters are members of the Proton-coupled Oligopeptide Transporter (POT) super-family, also called the Peptide Transporter (PTR) family (Daniel and Kottra, 2004). Table 2.1 summarizes the currently identified members of the peptide transporter family. Absorption of AA in the form of peptides was discovered in the 1970's, and in the 1980's it was confirmed that this transport was driven by a proton gradient and by an undiscovered transport protein. In 1994, the first PepT1 mRNA was cloned in the rabbit (Fei et al., 1994), and since then, much research has been conducted to elucidate the structural and functional characteristics of this protein. Peptide transporters transport di- and tripeptides and peptidomimetic drugs from the lumen of the small intestine across the brushborder membrane of the enterocyte, and to the bloodstream via the basolateral membrane (Leibach and Ganapathy, 1996).

Table 2.1 The proton oligopeptide cotransporter family

(Based on Daniel and Kottra, 2004)

Human gene name	Protein	Aliases	Substrates	Transport type/coupling ion	Tissue distribution/cellular expression
SLC15A1	PEPT1	Oligopeptide transporter 1, H^+/peptide transporter 1	Di-, and tripeptides protons	Cotransport, H^+	Intestine and kidney apical membrane, lysosomal membrane
SLC15A2	PEPT2	Oligopeptide transporter 2, H^+/peptide transporter 2	Di-, and tripeptides protons	Cotransport, H^+	Kidney, lung, brain, mammary gland, bronchial epithelium
SLC15A3	hPTR3	Peptide/ histidine transporter 2, human peptide transporter 3, PHT2	Histidine, di- and tripeptides protons	Cotransport, H^+	Lung, spleen, thymus, brain, liver, adrenal gland, heart
SLC15A4	PTR4	Peptide/ histidine transporter 1, human peptide transporter 4, PHT1	Histidine, di- and tripeptides protons	Cotransport, H^+	Brain, retina, placenta

Unlike AA transport, peptide transport requires a proton (H^+) gradient as the primary driving force for transport (Leibach and Ganapathy, 1996). Using the two-microelectrode voltage-clamp technique in cRNA-injected oocytes, and measuring currents for Gly-Sar transport, an inward H^+ current was detected, as well as intracellular acidification (Adibi, 1997).

Originally, it was thought that Na^+ was cotransported with peptides, because of an inwardly directed Na^+ gradient, but it was later found that the Na^+ dependency was the result of the Na^+/H^+ countertransport system on the apical membrane (Daniel, 2004). The proton is cotransported with the peptide into the enterocyte, where it is then transported back out into the lumen by the Na^+/H^+ countertransport system expressed on the brushborder membrane in exchange for a Na^+ (Daniel and Kottra, 2004). The unstirred water layer at the brushborder membrane is an isolated microenvironment free from the influence of the luminal contents and maintains a high extracellular concentration of protons (Adibi, 1997). Three Na^+ are transported out of the cell in exchange for two K^+ by the Na^+/K^+ ATPase pump on the basolateral membrane, resulting in the expenditure of ATP. The Na^+/H^+ countertransport system and the Na^+/K^+ pump serve to maintain the intracellular alkaline environment and intracellular negative membrane potential, respectively (Adibi, 1997). It was hypothesized that H^+ cotransport could be a mechanism to increase Na^+ absorption via the Na^+/H^+ countertransport system on the apical membrane (Adibi, 1997). A small amount of peptides transported into the cell are then transported across the basolateral membrane to the bloodstream by an unidentified basolateral peptide transporter, but the majority of peptides are rapidly cleaved into free

AA by intracellular peptidases, and the AA are then transported out of the cell by basolateral AA transporters.

Peptide transporters are expressed mainly in the intestine and kidney, with expression in the kidney serving to salvage peptide-bound AA, preventing loss in the urine (Leibach and Ganapathy, 1996). The H^+/peptide transporter expressed in the intestine and kidney (PepT1) is low-affinity (high micromolar range)/high-capacity, and the H^+/peptide transporter expressed only in the kidney (PepT2) is high-affinity/low-capacity (Leibach and Ganapathy, 1996).

Cloning and Characterization of PepT1. PepT1 (SLC15) was first cloned by microinjecting messenger RNA isolated from rabbit intestine into *Xenopus* oocytes, resulting in functional expression of the gene (Fei et al., 1994). The predicted rabbit PepT1 protein consists of 707 AA with 12 putative transmembrane domains, with multiple N-glycosylation sites (seven in the human) and consensus sequences for protein phosphorylation by protein kinase A and protein kinase C (Fei et al., 1994). The N- and C- termini are predicted to be located intracellularly. In contrast to the PepT1 characterized for the rabbit and other species, the pig PepT1 is predicted to contain an extracellular amino terminus and an intracellular carboxy terminus (Klang et al., 2005). Klang et al. (2005) found that four different modeling programs yielded different predicted structures of PepT1 for eight species (i.e., 10, 11, 12, or 13 transmembrane domains depending on the program used), thus, all reported PepT1 protein structures are ambiguous. The sequences of other mammalian species are highly similar to the rabbit peptide transporter, with differences in phospohorylation sites. The chicken PepT1 shows less similarity to the mammalian species, with approximately 60% identity to rabbits,

human, mice, sheep, and rats (Chen et al., 2002). The human PepT1 protein consists of 708 AA (80 kDa) with approximately 50% identity to PepT2; however, it does not contain a site for protein kinase A-dependent phosphorylation, but does contain two potential sites for protein kinase C-dependent phosphorylation (Leibach and Ganapathy, 1996; Daniel, 2004). The human PepT1 gene is located on chromosome 13 and consists of 24 exons (Liang et al., 1995). Rabbit and rat PepT1 contain one PKC phosphorylation site and one PKA phosphorylation site (Fei et al., 1994; Miyamoto et al., 1996). Turkey PepT1 contains at least 7 potential N-linked glycosylation sites, one potential site for PKC phosphorylation, and one potential site for PKA-dependent phosphorylation (Van et al., 2005). The sheep PepT1 contains four putative sites for PKC phosphorylation and three for PKA phosphorylation (Pan et al., 2001). A cDNA from a human colon carcinoma cell line (Caco-2) associated with H^+/peptide cotransport was isolated, and consisted of 832 AA and possessed only one transmembrane domain (Leibach and Ganapathy, 1996). It is thought that this protein (HPT-1) may oligomerize to form an active transporter or interact with other proteins to stimulate transport (Leibach and Ganapathy, 1996).

Screening of a human kidney cDNA library led to cloning of the PepT2 mRNA, which is 2,685 bp long, encoding a predicted protein of 729 AA (Leibach and Ganapathy, 1996). PepT2 was found to contain 12 putative transmembrane domains, five potential sites for protein kinase C-dependent phosphorylation and no sites for protein kinase A-dependent phosphorylation (Leibach and Ganapathy, 1996).

A protein domain important for PepT1 function includes a histidine residue (H57) on the extracellular side of the second transmembrane domain (Daniel and Kottra, 2004).

Mutation of this residue led to defective transport. The function of the large extracellular loop between transmembrane domains nine and 10 remains unclear, and only one AA has been demonstrated to show functional importance. The human PepT1 protein shows 81% identity to the rabbit PepT1 protein (Liang et al., 1995), and 83% identity with the rat PepT1 protein (Miyamoto et al., 1996). Amino acids in the transmembrane domains are well conserved among species, while AA in the loops are more variable (Miyamoto et al., 1996). A comparison of the AA sequence between human PepT1 and PepT2 shows 50% identity and 70% similarity, with PepT2 containing 21 more AA than PepT1 (Leibach and Ganapathy, 1996). PepT1 and PepT2 do not exhibit a high degree of homology with other cloned transporter proteins, however; a weak homology exists with the peptide transporters in yeast (*Saccharomyces cerevisiae*) and *Lactococcus lactis.*

PepT1 has been cloned and characterized in multiple species including the rabbit (Fei et al., 1994), rat (Saito et al., 1995), sheep (Pan et al., 2001), chicken (Chen et al., 2002), turkey (Van et al., 2005), human (Liang et al., 1995), pig (Klang et al., 2005), and the cynomolgus monkey (Zhang et al., 2004). Cloning of PepT1 in the monkey is of particular interest because of the importance of the monkey as a preclinical model, and with the use of orally available peptidomimetic drugs as targets for PepT1 (Zhang et al., 2004). It was found that there were some differences in mRNA expression between the human and monkey PepT1 (lower PepT1 mRNA in monkey kidney than in human), but these results do not necessarily correlate to functional protein expression in those tissues. Also, monkey PepT1 appeared to exhibit a higher affinity for two tested drugs, captopril and cefadroxil, than human PepT1. These are important matters to consider when using the monkey as a model for human medicine. It was also discovered that PepT1 from the

mouse and human are modular, with transmembrane domains and loops encoded by different exons, a result of exon shuffling and rearrangements (Daniel, 2004).

The TATA boxes are located 511 and 517 bp upstream from the transcription start site of human PepT1, with the GC boxes located near the start site at -29 bp (Daniel, 2004). This suggests that in humans the GC boxes may be more important in the transcriptional regulation of PepT1 because of the closer location to the transcription start site. In the rat, the TATA and GC sequences were found 22 and 39 bases upstream from the transcriptional start site, respectively (Shiraga et al., 1999). Shiraga et al. (1999) found that the transcriptional start site was a thymidine residue located at -80 bp from the translational start site. Deletional 5′ constructs of PepT1 with a deletion of the promoter from -351 to -171 bp caused a 2.5-fold drop in transport, and a deletion of -50 to -27 bp caused a fourfold drop in transport.

Substrate Specificity. PepT1 recognizes and transports all di- and tripeptides as well as peptidomimetic drugs, such as penicillins and cephalosporins (Brandsch et al., 2004).The substrate specificity of PepT1 was determined by competition experiments in which the ability of peptides containing more than three AA to inhibit uptake of di- and tripeptides was determined (Leibach and Ganapathy, 1996). It was concluded from these experiments that PepT1 accepts only di- and tripeptides as substrates (Leibach and Ganapathy, 1996).

PepT1 and PepT2 both have an affinity for peptides containing a broad range of AA. However, the affinity for substrates is markedly different for the two proteins. Results from functional expression studies have demonstrated that the K_t for glycylsarcosine was 1.0 m*M* for rabbit PepT1, 0.3 m*M* for human PepT1 (Leibach and

Ganapathy, 1996), and 0.94 m*M* for pig PepT1 (Klang et al., 2005), whereas the K_t for human PepT2 was 0.07 m*M* (Leibach and Ganapathy, 1996). In pigs, the concentration of unlabeled peptide that inhibited 50% of ^{3}H-Gly-Sar uptake (IC_{50}) in transfected Chinese hamster ovary cells ranged from 0.004 to 0.53 m*M* for eleven dipeptides and two tripeptides (Klang et al., 2005). In general, the affinity constants for PepT1 range from 200 μ*M* to 10 m*M*, and for PepT2 range from 5 to 500 μ*M* (Rubio-Aliaga and Daniel, 2002). PepT1 and PepT2 exhibit a broad substrate specificity and will potentially transport all 400 different dipeptides and 8000 different tripeptides possible from combining the 20 dietary L-α-AA (Daniel, 1996). The peptide transporters exhibit higher affinities for peptides with a D-enantiomer at the N-terminal position (Rubio-Aliaga and Daniel, 2002). It is thought that water plays an important role in accommodating this broad specificity by shielding the charges of the AA side chains within the substrate binding domain of PepT1, which allows both charged and uncharged substrates to bind at the same domain (Daniel, 2004). It has also been demonstrated that metal ions (i.e. Zn^{2+}, Mn^{2+}, and Cu^{2+}) interact with transporter proteins to enhance peptide absorption (Leibach and Ganapathy, 1996).

The proton to substrate ratio for neutral and cationic AA transported by PepT1 is one, whereas the ratio for charged anionic AA is two (Daniel and Kottra, 2004). Steel et al. (1997) proposed a model for the coupled transport of neutral and charged dipeptides. The H^+ that is coupled to transport, theoretically first binds to a proton binding site on the carrier protein, followed by peptide binding, and then transport across the membrane. The proton binding site contains a histidine residue which mediates proton binding. It is thought that dipeptides containing an acidic residue, such as Gly-Glu, are transported in a

charged form, and one proton binds to its proton binding site, whereas a second proton neutralizes the negative charge on glutamate, and within the cell, a proton from the carboxyl group of the glutamate residue dissociates, resulting in the net transport of 2 H^+. This also accounts for the decline in affinity for acidic substrates at a higher pH, where the acidic residue is not charged, and a higher affinity at a lower pH (Steel et al., 1997). On the other hand, the optimal pH for basic dipeptides is higher. The optimal pH for transport of Gly-Lys is close to the pK of histidine. PepT1 displays a lower affinity for this dipeptide, especially when the lysine residue is located on the carboxyl side, which Steel et al. (1997) proposed occurs because at a lower pH the protonated histidine residue on the peptide binding site might interfere with the binding of substrate extracellularly, and transport could only occur after the pH is raised and the histidine residue is deprotonated. In pigs, di- and tripeptides containing an N-terminal or C-terminal lysine were poor substrates for PepT1, and IC_{50} values were greater than 1.38 m*M* (Klang et al., 2005).

PepT2, on the other hand, transports neutral substrates at a 2:1 proton to substrate ratio and transports charged substrates at a variable stoichiometry. It was also found that peptide transporters exhibit a greater affinity for peptides containing the L-isomers of AA compared to the D-isomers (Leibach and Ganapathy, 1996; Brandsch et al., 2004). It is also essential that if a peptide bond is present, that it be in the *trans* conformation (Brandsch et al., 2004). If the peptide bond is in the *trans* conformation transport may occur because the carbonyl oxygen becomes negatively charged, increasing the affinity for substrates (Daniel, 2004). Charged peptides appear to exhibit a lower affinity for

PepT1 as compared to peptides that are neutral at physiological pH (Brandsch et al., 2004).

Fei et al. (1998) identified the potential substrate binding domain in PepT1 and PepT2 by creating chimeras of the two proteins and determining substrate affinities. They concluded that the substrate binding site was comprised of transmembrane domains seven, eight, and nine of the two transporters. Chimeric proteins containing the first 9 ½ transmembrane domains of PepT1, with a substitution in the remaining domains with PepT2 resulted in low-affinity for Gly-Sar, characteristic of PepT1. However, substitutions in transmembrane domains seven, eight, and nine, and the large extracellular loop between domains nine and 10 with PepT2, resulted in a high-affinity for Gly-Sar, characteristic of PepT2. Other studies have suggested that the histidine residue at the substrate binding domain is located at His-57 and His-87 in human PepT1 and rat PepT2, respectively, on the second transmembrane domain extracellularly (Fei et al., 1997), and the tertiary structure of the protein may be conducive for interaction between the histidyl residue and membrane domains seven, eight, and nine (Fei et al., 1998).

It was found that pretreatment of renal brushborder membrane vesicles with DEPC, a histidine-modifying reagent, abolished peptide transport (Meredith and Boyd, 2000). Fei et al. (1997) found that mutations of human PepT1 His-57 and PepT2 His-87, with asparagine or glutamine completely abolished transport activity, and was, therefore, considered to be absolutely essential. Asparagine and glutamine were chosen because substitutions would have the least effect sterically and charge-wise. Two other conserved PepT1 (His-121 and His-260) and PepT2 (His-142 and His-278) His residues were examined, and mutations did not inhibit peptide transport. Western blots revealed that

protein expression levels of the mutants in HeLa cells were similar to the controls, pointing to a direct binding mechanism responsible for the inhibition (Fei et al., 1997). Modifying peptides containing an amino terminal lysine, by replacing it with a neutral AA substantially increased the affinity, possibly because Pept1 exhibits electrostatic interactions with positively charged residues (Brandsch et al., 2004). It has also been demonstrated that modifications of the amino terminal residue, for example by methylation or acetylation, also decreases the affinity. The affinity for Pro-Gly as compared to Gly-Pro is very much decreased, because of the imino ring on the amino terminus. Histidine and thiol group residues have been shown to play a role in the activity of peptide transporters, with the imidazole side chain of histidine serving as a proton donor/acceptor, and dithiol-disulfide interactions promoting activity of the transporters (Leibach and Ganapathy, 1996).

Binding affinity for charged peptides and peptidomimetic drugs changes when pH is disturbed (Daniel and Kottra, 2004). When the pH is decreased, affinity for the acidic AA increases, and when the pH increases, affinity for the basic AA increases. It is important to point out though, that pH dependence has been studied in vitro. In the intact intestine, an acidic microclimate is maintained at the brushborder membrane, independent of the intestinal luminal contents (Daniel, 2004). Changes within the lumen do not directly affect the unstirred water layer, and thus the results of in vitro studies are difficult to relate to the intact, functioning intestine.

It has been found though, that a peptide bond is not required for substrate recognition. Doring et al. (1998) conducted a study to determine the minimum substrate required for peptide transport, and found that PepT1 and PepT2 recognized and

transported substrates as simple as omega-amino fatty acids with a positive amino and negatively-charged carboxyl head group, which were separated by a minimum of four methylene groups. Removal of the amino or carboxyl head resulted in a similar affinity but no transport. Also, a distance of between 500 and 635 pm between the centers of the functional groups was required to allow for conformational requirements to be met that would allow for binding to the PepT1 substrate binding domain.

Additional features of small peptides and other compounds serve to change the affinity of the substrate and to initiate or prevent substrate binding (Daniel and Kottra, 2004). The N-terminal amino group on a dipeptide is not essential, but provides good substrate affinity. The C-terminal carboxyl group is also not essential, but when present, the sterical configuration is critical. Also, the carbonyl group of the peptide bond closest to the N-terminal end of the peptide increases the affinity, and allows hydrogen bonding to a protein side chain. Knutter et al. (2004) found that modification of the side chains of dipeptides still resulted in transport, but that transport depended on the hydrophobicity of the side chain modification, and the distance between the alpha-carbon and the side chain blocking group. Within the peptide bond, the carbonyl oxygen is the functional element because it can form hydrogen bonds with substrate binding domain of PepT1 (Daniel, 2004). PepT1 transported AA aryl amides in Caco-2 cells and *Xenopus* oocytes, proving that the amino group of a peptide may be replaced with an amide group (Meredith and Boyd, 2000).

Tissue and Cellular Distribution of Peptide Transporters. PepT1 mRNA and protein are expressed primarily in intestinal and renal epithelial cells (Daniel and Kottra, 2004). More recently, an additional site of expression, the bile duct epithelium, was

discovered for PepT1 (Daniel and Kottra, 2004). Peptide transport activity was observed in liver cells, and the PepT1 protein was found in the nuclei of vascular smooth muscle cells. While Northern blot analyses have failed to detect the presence of PepT1 mRNA in the colon, RT-PCR has confirmed the presence of PepT1 in the human colon at fivefold lower levels than in the ileum (Ford et al., 2003). Immunohistochemistry revealed weaker intensity of PepT1 protein in the colon as compared to the ileum, and within the colon, staining was basolateral in colonocytes towards the base of the crypt, and apical towards the colonic luminal surface (Ford et al., 2003). This indicates that the colon may play a role in minimal absorption of dietary and endogenous peptides. Although almost no dietary peptides or AA reach the colon, there is a large supply of endogenous proteins in the colon, possibly serving as substrates for proteolysis by microflora. These results contradict the findings of Merlin et al. (2001), who when using Northerns, RT-PCR, and immunomicroscopy on normal human colon, were unable to detect a PepT1 product in normal human colonic mucosa. There is no explanation by Ford et al. (2003) to account for this discrepancy. Past studies have reported the lack of a PepT1 signal in the colon using Northern blots, a relatively insensitive method, but these two studies are the first to report findings using RT-PCR. In the chicken, Northern blots revealed considerable expression of PepT1 in the ceca, in addition to the small intestine and kidney (Chen et al., 2002). In chickens, mRNA abundance appeared to be highest in the duodenum, with fainter bands in the jejunum and ileum (Chen et al., 1999). In rats, protein expression was highest in the distal small intestine (ileum) as compared to the jejunum (Tanaka et al., 1998). Howard et al. (2004) used RT-PCR to determine PepT1 mRNA distribution in rat intestine, and found that PepT1 mRNA was equally expressed throughout the length of

the small intestine. In contrast to most species, ruminants, such as sheep, express PepT1 mRNA in the stomach, specifically, in the omasum and rumen (Pan et al., 2001). In lactating Holstein cows, mRNA followed a similar pattern of expression (Chen et al., 1999). Sheep PepT1 mRNA was also detected in the small intestine, but not in the liver and kidney. In rabbits, PepT1 mRNA was detected in the brain and liver, while it was not detected there in rats by a Northern blot analysis (Miyamoto et al., 1996).

In situ hybridization has been used to examine cellular distribution and expression of PepT1 mRNA. Freeman et al. (1995) found that PepT1 mRNA was expressed throughout the length of the rabbit small intestine with lighter staining in the colon. PepT1 mRNA was not detected in the stomach, sacculus rotundus, or cecum. PepT1 mRNA staining was restricted to enterocytes, with no expression in the lamina propria or muscularis. Enterocyte staining for PepT1 mRNA was first visible at the crypt-villous axis, and increased rapidly 100 to 200 μ*M* from this point (Freeman et al., 1995). Within the colon, expression was restricted to the surface columnar epithelial cells. Expression of PepT1 in the colon may be advantageous for utilizing the digestive products of the microbes. Compared with other villi, staining of PepT1 mRNA in the follicle-associated epithelium (FAE) superimposed on Peyer's patch follicles, was reduced. This is in keeping with the function of Peyer's patches in mucosal immunity and not in nutrient transport (Freeman et al., 1995). Bockman et al. (1997) found by immunofluorescence microscopy that PepT1 also localized to the nuclei of smooth muscle cells in the wall of arterioles, lysosomes in acinar cells and nuclei of the schwann cells in the unmyelinated nerves in the pancreas, suggesting a role in absorption of peptides across intracellular membranes in organs, following intralysosomal protein degradation.

Immunohistochemical analyses showed that in rats, PepT1 protein was localized to the brushborder membrane of cells lining the villi; no protein was detected in the crypts (Ogihara et al., 1996; Tanaka et al., 1998). Also, PepT1 exhibited an expression gradient increasing from the crypt-villous junction to the villous tip, with the most prominent staining in the jejunum (Ogihara et al., 1996). Groneberg et al. (2001) utilized a novel method of a fluorophore-conjugated dipeptide to examine the histological pattern of transport. After incubation of murine intestinal segments with D-Ala-Lys-AMCA, fluorescence was restricted to the brushborder membrane and cytoplasm of enterocytes, with no accumulation of the reporter in the nucleus, goblet cells, or lamina propria. The intensity increased in cells towards the villous tip, and maximal fluorescence was obtained after 10 min of incubation.

PepT2 is expressed in the kidney tubule, lung, mammary gland, choroids plexus, and glia cells in the nervous system, but not in the intestine (Daniel, 2004). PepT2 mRNA is expressed in kidney tubule cells in the S2 and S3 segments, bronchial epithelial cells, mammary gland, type II pneumocytes, epithelial cells of the choroids plexus, and brain astrocytes (Daniel and Kottra, 2004).

Pharmacological and Clinical Importance of Peptide Transport. In addition to absorbing di- and tripeptides, PepT1 also accepts antibiotics with structural similarities to peptides, called “peptidomimetics”, and participates in their absorption, which is of major therapeutic value (Leibach and Ganapathy, 1996). This allows for the design of pharmacological drugs that possess acceptable oral availability because of transport by PepT1. PepT1 and PepT2 have been found to transport cephalosporins, penicillins, bestatin, valin ester prodrugs of acyclovir and ganciclovir, and inhibitors of angiotensin

converting enzyme (Steffansen et al., 2004). The angiotensisn-converting enzyme inhibitors include captopril, enalapril, and fosinopril (Daniel and Kottra, 2004). DOPA derivatives, sulpiride, and bestatin are also transported by PepT1 (Daniel and Kottra, 2004). A tumor therapy compound, δ-aminolevulinic acid, a medium-chain omega-amino fatty acid with a carbonyl group in the 5-position is also taken up by PepT1 (Rubio-Asiaga and Daniel, 2002). The β-lactam antibiotics (penicillins and cephalosporins) are similar to tripeptides (Rubio-Asiaga and Daniel, 2002). The C-terminal peptide bond is incorporated into the β-lactam ring, and when rotated by 180° displays excellent affinity matching the L-stereochemistry of peptides. Aminopenicillins and aminocephalosporins have high affinities and transport rates because of a free amino group at the N-terminal position. The angiotensin-converting enzyme inhibitors, which are used to treat hypertension and include captopril and enalapril, have a good affinity for PepT1, but a low affinity for PepT2 (Rubio-Asiaga and Daniel, 2002). Aminopeptidase inhibitors, including bestatin, are used as antitumor agents, and are excellent substrates for both peptide transporters. 3,4-Dihydroxy-L-phenylalanine (L-DOPA) and valacyclovir, a L-valyl ester of acyclovir, are derivatized drugs with improved bioavailability because of a peptide-like structure (Rubio-Asiaga and Daniel, 2002).

Small peptides have been considered as a source of AA in feeding solutions for patients with altered intestinal absorptive function (Leibach and Ganapathy, 1996). Synthetic di- and tripeptides offer an advantage over free AA that are unstable or insoluble (Leibach and Ganapathy, 1996). Examples are glutamine and tyrosine, which are both extremely important for humans (Adibi, 1997). Because of the labile nature of glutamine, and insoluble nature of tyrosine, absorption in a small peptide form increases

the availability of these AA to the body (Adibi, 1997). Also, the use of dipeptides reduces the hypertonicity that results from a free AA feeding solution (Adibi, 1997).

Dietary Regulation of PepT1 in the Small Intestine. Peptide transport may be regulated by its own substrates (Adibi, 2003). To investigate this, Gly-Sar was incubated with Caco-2 cells for 24 hr, and transport properties were examined (Thamotharan et al., 1998). The V_{max} increased without any significant change in K_m. Addition of a mixture of the constituent free AA did not stimulate peptide transport, and a 2-h uptake study with Gly-Sar did not elicit upregulation of PepT1. Western blot analyses showed a twofold increase in expression of PepT1 in the apical membrane of Caco-2 cells. Brefeldin treatment abolished transport, showing that increased synthesis and processing by the transGolgi network accounted for increased expression at the apical membrane. A Northern blot analysis confirmed that increased gene expression was occurring, in which PepT1 mRNA exhibited a threefold increase in Caco-2 cells incubated with Gly-Sar. A similar upregulation in transport was observed with cefadroxil. Similar uptake experiments were performed with a naturally occurring dipeptide, Gly-Gln, confirming the physiological relevance of these findings (Walker et al., 1998). Indeed, exposure to 4 m*M* Gly-Gln for 3 d prior to Gly-Sar treatment resulted in a twofold increase in V_{max}, with no change in K_m. The Gly-Gln treatment also upregulated the cellular mRNA and membrane PepT1 protein level twofold. In addition, actinomycin D was added to inhibit RNA transcription in the Caco-2 cells in order to investigate the effect of Gly-Gln on mRNA stability. There was an increase of hPepT1 mRNA half-life by approximately 30% in the Gly-Gln supplemented cells, and this in combination with increased

transcription levels accounted for the total magnitude in increases of PepT1 mRNA in the Caco-2 cells.

Erickson et al. (1995) conducted the first studies to examine mRNA expression levels in rat intestine as a result of feeding a high protein diet. Rats were maintained on a low protein diet (4% casein) for 7 d and transferred to a high protein diet (50% gelatin) for 14 d. Control rats were fed a diet consisting of 17.5% casein for 7 d. PepT1 mRNA was upregulated approximately twofold by the high protein diet. Shiraga et al. (1999) conducted a similar study in which they examined the cellular and molecular mechanisms of dietary regulation in rats. They maintained rats on a 20% casein diet for 1 wk. A group of rats were switched to a protein-free diet, and others were switched to a diet consisting of 50%, 20%, or 5% casein, 20% of a dipeptide, or 10% of a single AA, fed for 3 d. Gly-Sar uptake was increased in the ileal brushborder membrane vesicles 4 d after feeding the 50% casein diet, increasing linearly to 2 min. In contrast, there was a decrease in transport activity after 4 d in the protein-free diet. After 1 min of Gly-Sar uptake, activity in the 50% casein rats was around twofold greater than the protein-free rats. In the 50% casein rats, the increase in activity was accompanied by an increase in V_{max}, but no change in K_m. According to the Northern blot analysis, PepT1 mRNA was increased 2.4-fold in the 50% casein rats, compared to the protein-free rats. According to the western blot and reaction to a 90 kDa protein, PepT1 protein in the ileal brushborder membrane vesicles in 50% casein rats were 2.2-fold greater than the protein-free rats. In the 5% casein rats, mRNA and protein levels did not differ significantly from the protein-free rats. Rats fed the dipeptide Gly-Phe instead of casein, exhibited a 2.6-fold higher activity than the protein-free rats. The rats fed free phenylalanine exhibited a 2.7-fold higher

activity than the protein-free rats, while the glycine rats did not exhibit a significantly different activity. In the Gly-Phe and the phenylalanine rats, the increase in transport was accompanied by an increased V_{max}, with no change in K_m. Compared with the protein-free rats, Northern blots revealed a 2.4-fold increase in PepT1 mRNA for the Gly-Phe rats, and a significantly higher mRNA for the phenylalanine but not the glycine rats. The protein expression of PepT1 displayed similar upregulation.

Regulation by dietary substrate appears to occur by two mechanisms: 1) by increasing mRNA stability and 2) by increasing gene transcription rate (Adibi, 2003). Peptide transport also varies among dietary treatments. Transport is upregulated by high-protein treatments (Shiraga et al., 1999), and interestingly, short-term food deprivation also enhances peptide transport (Thamotharan et al., 1999a). Rats fed increasing levels of protein for 3 d, exhibited both an increased expression of the PepT1 protein, but also an increase in peptide transport activity (Shiraga et al., 1999). Upregulation by a high-protein diet appears to be a mechanism to take advantage of the abundant resource, but it is doubtful that this upregulation would continue if the metabolic cost of maintaining the transporters exceeded the benefits. In chickens, an increase in PepT1 mRNA was observed in the intestine of chickens fed 18 and 24% CP diets with restricted food intake, and a decrease in PepT1 mRNA was observed in chickens fed a 12% CP diet (Chen et al., 2005). In the intestine of birds fed a 24% CP diet ad-libitum, a decrease in PepT1 mRNA abundance was observed during the first 14 d of age, and then rose to d 35, but was still lower than PepT1 mRNA levels in the restricted-intake birds.

Short-term starvation in rats has been shown to increase mRNA and protein expression of PepT1 (Ihara et al., 2000). Rats starved for 4 d exhibited a 179% increase

in mRNA and protein expression. Rats that were fed 50% of the control for 10 d and rats given total parenteral nutrition for 10 d exhibited a 161% and 164% increase in PepT1 mRNA expression, respectively. This dramatic upregulation was in spite of the fact that the mucosal weight decreased in the starved and TPN group by 41 and 50%, respectively, indicating mucosal atrophy. In a different study, the rate of peptide transport in rats increased dramatically after 1 d of fasting (Thamotharan et al., 1999a). The V_{max} of Gly-Gln uptake increased by twofold, without affecting K_m in brushborder membrane vesicles. The abundance of rat PepT1 mRNA and protein increased by threefold in the fasted rats. In a more recent study, Howard et al. (2004) examined the effects of total parenteral nutrition (TPN) and administration of glucagons-like peptide (GLP-2) on the mRNA expression of PepT1 in rat small intestine. TPN for 7 d upregulated PepT1 mRNA in the distal intestine, while proximal (duodenal) mRNA was unchanged. Administration of GLP-2 inhibited the effect of TPN on mRNA expression of PepT1. GLP-2 has been shown to maintain cellular protein synthesis during luminal starvation, and perhaps infusion of GLP-2 reduced the need for upregulation of PepT1. Also, it is thought that during luminal starvation, the need to absorb endogenous protein products increases, and thus, apical membrane transporters, which in this study included NBAT, EAAC1, ASCT2, and PepT1, are upregulated in the distal intestine to maximize assimilation of AA (Howard et al., 2004). Diabetic rats also exhibited increased PepT1 protein expression levels (Gangopadhyay et al., 2002). Rats were made diabetic by an injection of steptozotocin for 96 h, leading to insulin deprivation, during which time the activity of PepT1 increased in the intestinal mucosa. The V_{max} increased, indicating an increase in the number of peptide transporters. According to a western blot analysis,

PepT1 protein expression increased by 60%. The amount of PepT1 mRNA increased by 60% according to a northern blot analysis, but the level of transcription remained unchanged according to a nuclear run-on assay, indicating perhaps an increased stability of mRNA. Interestingly, this increase in expression is more pronounced in the peptide transporter than in the glucose transporters. This is perhaps a mechanism for increased substrate availability for gluconeogenesis to compensate for glucose lost in the urine. Naruhashi et al. (2002) used quantitative RT-PCR to determine mRNA expression levels of PepT1 in fed and starved rat intestine that was divided into eight segments. PepT1 mRNA levels were highest in the distal region of the small intestine, and lowest in the proximal region of the small intestine. In starved rats, mRNA levels increased in all segments compared to the fed rats, with a more pronounced and statistically significant increase in the proximal intestine (first five segments). Transport of cefadroxil increased in the proximal region of the small intestine in starved rats as compared to the fed rats, with a significant increase in the mid-region (segment four). Cefadroxil transport was generally greatest in the distal region (segment eight) of fed rats. In general, starvation decreases the absorptive surface area of the intestine, but it appears that an increase in the membrane population of transporters counteracts this, resulting in maintenance of peptide transport (Adibi, 2003). Upregulation of peptide transport during fasting or starvation appears to be a mechanism to preserve intestinal function and to ready the intestine for anticipated food intake.

Developmental Regulation of PepT1. Peptide transport activity varies with species and age (Leibach and Ganapathy, 1996). Activity of PepT1 is highest at birth and declines with age until adulthood. Rome et al. (2002) examined the ontogenetic

regulation of PepT1 in rats from postnatal d 4 to d 50, using semi-quantitative RT-PCR and immunohistochemistry. PepT1 mRNA was evenly distributed from the proximal to distal small intestine, and this pattern remained unchanged from birth to adulthood. PepT1 mRNA levels decreased in the proximal and middle part of the intestine on d 50 compared with d 21 (weaning), and remained constant in the distal intestine. The PepT1 protein was localized on the brush border membrane of epithelial cells from d 4 to d 50, with an increase in expression from the crypt-villous junction to the villous tip. There was no expression detected in the crypts.

Shen et al. (2001) examined the developmental expression of PepT1 and PepT2 in the rat small intestine, colon, and kidney. Northern blot analyses were used to examine PepT1 mRNA expression in the intestine and kidney, and it was found that PepT1 mRNA was detected at d 20 of the fetus, and expression spiked at birth in all segments of the small intestine, and peaked during d 3 to d 5 after birth. The PepT1 mRNA levels then dropped sharply to about 12% of maximal levels by d 14, and then at weaning, around d 24, the levels rose to 23 to 58% of maximal expression levels. In the adult rat, levels plateaued to about 25% of the expression levels observed at d 3 to d 5. Colonic PepT1 mRNA was detected at d 1 to d 5, and dropped to almost undetectable levels at d 7, and was undetectable afterwards at all days. PepT1 protein levels followed a similar pattern. PepT1 protein was detected at fetal d 20, was maximal at d 3 to d 5 after birth, dropped sharply and then rose to 59 to 88% of maximal expression at weaning, and then plateaued at adulthood to 70% of maximal expression. Protein was detected in the colon at d 1 to d 5, but was not detected afterwards. PepT1 and PepT2 mRNA were detected in the kidney at d 17, but rose gradually to maximal levels at weaning, and then dropped gradually to

adult expression levels of 70% of maximum expression. Protein expression followed a similar pattern, with a pronounced linear increase towards d 14, after which it plateaued. These trends in developmental expression seem to correlate with functional changes in the rat intestine. At birth, the intestine becomes the site of nutrient assimilation, and the animals begin to consume a high-protein milk diet. At weaning, the animal shifts to the adult diet, consisting of predominantly carbohydrates. These results paralleled those of an earlier study, which also demonstrated a peak in PepT1 rat mRNA in the jejunum at d 10, followed by a drastic drop and then plateau to adult levels at d 28 (Miyamoto et al., 1996).

In 2002, Hussain et al. conducted a similar study to investigate the cellular localization of PepT1 during rat development. Immunocytochemical microscopy revealed a major increase in staining between fetal d 18 and day of birth in the duodenum, and right after birth staining spread to the subapical cytoplasm, basal cytoplasm, and basolateral membrane, in addition to the apical membrane. At weaning and adulthood, PepT1 expression was restricted to the brushborder membrane. The cellular signaling pathways underlying the trafficking of this protein still remain unclear. The turkey and chicken, although exhibiting a different mode of embryological development than mammalian species, exhibit a similar developmental regulation of PepT1. Van et al. (2005) observed a 3.3-fold increase in mRNA expression levels of PepT1 in turkey intestinal tissue from 5 d before hatch to day of hatch, which is similar to rats, in which PepT1 mRNA spiked at birth (Shen et al., 2001). Chen et al. (2005) observed a 50-fold increase in intestinal chicken PepT1 mRNA from embryo day 18 to day of hatch in one experiment, and a 14-fold increase in a second experiment, with expression peaking right

before hatch in both experiments. This upregulation before hatch could be a mechanism to prepare the bird for peptide uptake after hatch (Chen et al., 2005).

Hormonal Regulation of PepT1. Hormones have been found to regulate PepT1 expression and activity levels (Daniel, 2004). The first evidence of hormonal regulation of PepT1 came with a study of insulin stimulation (Thamotharan et al., 1999b). Insulin was investigated because of its importance in metabolic regulation throughout the body. Addition of 5-n*M* insulin to Caco-2 cells stimulated Gly-Gln uptake as shown by an increase in the number of PepT1 transporters, no difference in mRNA abundance, and no alteration in K_m (Thamotharan et al., 1999b). Insulin acts by binding to a transmembrane glycoprotein receptor with protein tyrosine kinase activity that becomes stimulated after insulin binds. An inhibitor of this activity, genistein, was added to determine the mechanism of insulin influence on the peptide transporter. This inhibition of protein tyrosine kinase activity did not impact Gly-Gln transport, but did abolish the stimulatory effect of insulin. Insulin stimulation of PepT1 most likely occurs by increasing the number of transporters on the apical membrane as evidenced by increased V_{max} in insulin-treated cells, increased amount of PepT1 in isolated apical membranes, and destruction of insulin stimulation by disrupting microtubules that play a role in membrane trafficking of proteins from the cytoplasm (Thamotharan et al., 1999b). Insulin is not a hormone located in the gut lumen, but during normal physiological conditions, circulating insulin can bind to receptors located on the basolateral membrane of enterocytes (Adibi, 2003). A more recent study (Nielsen et al., 2003) examined the effect of membrane-specific treatment of insulin, and found that previous treatment of the basolateral membrane with 50 ng/mL insulin for 1 h significantly increased Gly-Sar

uptake, whereas treatment of the apical membrane had no effect. This stimulation at the basolateral membrane resulted in an increased V_{max} for Gly-Sar transport, but no change in K_m. Basolateral treatment with insulin also caused an increase in apical leucine transport, but no change in glucose transport. Pretreatment of the cells with brefeldin did not affect Gly-Sar uptake, but pretreatment with colchicines inhibited the effects of insulin, and also reduced uptake of Gly-Sar in the control cells, yielding inconclusive results regarding the mechanism of stimulation by insulin. In this study Caco-2 cells were cultured for 26 d, at which time they are fully differentiated and at which time insulin receptor polarization has occurred and 97% of insulin receptors are located on the basolateral membrane. In non-differentiated cells, there is a larger proportion on the apical membrane, which would yield differing results.

Epidermal growth factor (EGF) increased peptide uptake in Caco-2 cells (Nielsen et al., 2001; Nielsen et al., 2003). The EGF is a peptide growth factor that stimulates growth of many epithelial and non-epithelial cells (Nielsen et al., 2001). Since EGF receptors are present on both the apical and basolateral membranes of Caco-2 cells, Nielsen et al. (2001) examined site-specific regulation, and found that long-term basolateral treatment with EGF resulted in a downregulation of peptide transport of Gly-Sar, with the V_{max} decreasing by 60%, with no change in K_m. Apical treatment resulted in no change in transport. According to western blots and RT-PCR, PepT1 protein levels decreased significantly in the basolateral EGF treated groups, and mRNA levels dropped by 65%, suggesting that EGF downregulates PepT1 at the gene expression level. Cells were exposed to EGF for 26 d at a concentration of 5 ng/mL. After 5 d, a significant downregulation in transport was observed, which plateaued after 15 d of treatment,

demonstrating a long-term regulation effect. Opposite effects were observed when Nielsen et al. (2003) investigated the short-term effects of EGF treatment. Caco-2 cells treated with EGF at the apical membrane did not exhibit a change in hPepT1 Gly-Sar uptake. Treatment with EGF at the basolateral membrane stimulated transport, similar to the effects seen with insulin (Nielsen et al., 2003). Stimulated uptake was seen in 5 min, and plateaued after 20 min. For the stimulated uptake by basolateral treatment with EGF, V_{max} was increased by 50%, with no change in K_m. Basolateral stimulation of the Caco-2 cells with EGF stimulated apical leucine uptake, but not glucose uptake, similar to the effects of insulin treatment. Pretreatment with brefeldin and colchicines did not significantly affect Gly-Sar uptake in EGF treated Caco-2 cells. According to the RT-PCR data, there was no increase in hPepT1 mRNA in the EGF-treated Caco-2 cells. Epidermal growth factor has been shown to increase the small intestinal absorptive surface area, most likely through a pathway that involves redistribution of the brushborder membrane (Nielsen et al., 2003). In vivo, EGF is released from the gastrointestinal glands into the intestinal lumen in large amounts, resulting in constant exposure to the enterocytes. Clearly, EGF exhibits a short-term and long-term effect on peptide transport; however, researchers are unsure of the underlying cellular mechanisms that control EGF regulation of nutrient transport.

Leptin, a hormone secreted by the stomach into the lumen, also upregulates peptide transport in Caco-2 cells, appearing to regulate PepT1 by increasing the number of carrier proteins, but not affecting K_m (Buyse et al., 2001). Leptin, which is secreted by both adipocytes and by the stomach, has been found to be released into the stomach and reaches the intestine in a nondegraded form, suggesting that it may play a role in small

intestinal functioning. The effect of leptin addition to Caco-2 cells elicited similar responses as observed with insulin. The addition of 100 n*M* leptin to both the apical and basolateral side of the membrane resulted in a twofold increase in transport of cephalexin. Addition to only the apical membrane elicited an identical response, and addition to only the basolateral membrane had no effect on cephalexin transport. Similar results were obtained when examining Gly-Sar transport. Gly-Sar transport increased twofold with 1 n*M* leptin, and fourfold with 2 n*M* leptin. Western blot analyses showed that apical addition of leptin increased the amount of membrane PepT1 and reduced the intracellular amount of PepT1. Brefeldin A treatment did not affect transport, whereas colchicines treatment reduced leptin-induced stimulation of Gly-Sar transport. This study was repeated with perfusion studies in rat jejunum in vivo to confirm the physiological importance of these findings. Similar results were observed with a twofold increase in plasma cephalexin after jejunal perfusion of cephalexin. Similar to the hypothesized effects of insulin, it is thought that stimulation of peptide transport by leptin may involve increased trafficking of cytoplasmic PepT1 proteins to the apical membrane. This is supported by an increase in V_{max} of Gly-Sar transport, an increase in membrane-bound PepT1 compared to intracellular PepT1, and decreased stimulation after treatment with colchicines (Buyse et al., 2001). After ingestion of food, leptin may serve to stimulate AA assimilation, and serve to balance fat and protein intake.

On the other hand, thyroid hormone (T_3) treatment in Caco-2 cells reduced PepT1 V_{max} for Gly-Sar transport, as well as PepT1 mRNA by 25%, while the K_m value remained the same (Ashida et al., 2002). PepT2 protein expression was also decreased in cells receiving T_3 pretreatment. These effects were shown to be specific for peptide

transport, because threonine uptake was not inhibited by T_3 treatment. Thyroid hormone plays an important role in growth and development of all body tissues, including the intestinal epithelium. Thyroid hormone enters cells and binds to a receptor in the nucleus and appears to regulate metabolic activities by transcriptional regulation, although a thyroid hormone-responsive element has not been found in the PepT1 promoter region of the rat and mouse. The mechanism of thyroid hormone regulation on PepT1 is unknown, but is thought to involve transcriptional regulation, as evidenced by a decrease in PepT1 mRNA in T_3 treated Caco-2 cells.

Diurnal Regulation of PepT1. PepT1 mRNA and protein is also regulated diurnally (Pan et al., 2002). Rats were placed on a 12 h light schedule (0800 to 2000), during which time they were allowed free access to water and a commercial diet. Peptide transport of Gly-Sar was greater during the dark cycle, than during the light cycle. Protein expression of PepT1 was highest at 2000 and lowest at 0800, with mRNA levels following a similar trend. This diurnal rhythm was restricted to intestine, and did not occur in the kidney. This appears to correlate well with the eating habits of rodents in the wild, which occurs during the night. Pan et al. (2003) then examined the effects of nutrient intake on the diurnal rhythm of PepT1 in the small intestine of fed and fasted rats, and found that 2 to 4 d of fasting abolished the diurnal rhythm of PepT1 protein. Interestingly, PepT1 mRNA maintained a diurnal rhythm during the fasting state, which suggests that PepT1 is trancriptionally regulated by other factors (Pan et al., 2003). Uptake of an oral antibiotic Ceftibuten (CETB) was shown to exhibit a diurnal rhythm in fed rats which was abolished in fasted rats, similar to the pattern of PepT1 expression (Pan et al., 2003).

Regulation of PepT1 During Disease. PepT1 exhibits an amazing resiliency in the intestine by maintaining expression and activity levels in spite of intestinal damage. PepT1, which is expressed in very small quantities in the colon compared to the proximal intestine (Ford et al., 2003), is upregulated in the colon of patients suffering from short-bowel syndrome (SBS) (Ziegler et al., 2002). A molecular analysis of PepT1 mRNA and protein demonstrated that in SBS patients PepT1 mRNA and protein were highly expressed in epithelial cells in the colon (fivefold increase), perhaps serving as a mechanism to maximize absorption of dietary AA in patients with a restricted capacity (Ziegler et al., 2002). This same pattern of expression is also exhibited in patients suffering from chronic ulcerative colitis and Crohn's disease (Merlin et al., 2001). In colonic mucosa from diseased patients PepT1 mRNA and protein was detected. Also, N-formyl-methionyl-leucyl-phenylalanine (fMLP) a major proinflammatory peptide of the human colonic lumen is found at higher levels in the colon, where bacterial loads are higher compared to the small intestine, and where PepT1 is minimally expressed. However, during a diseased state, PepT1 expression increases in the colon, transports fMLP, and stimulates expression of immune genes (MHC-1; Merlin et al., 2001).

Bacterial infections caused by endotoxin administration in rats, were shown to regulate expression and activity of PepT1 in the intestinal tract (Shu et al., 2002). However, in contrast to the other types of diseases discussed, endotoxin treatment served to downregulate PepT1 expresssion, most likely through the action of proinflammatory cytokines. PepT1 mRNA and protein in LPS treated rats exhibited identical decreased expression (32 to 62% of controls). The mRNA expression levels of SGLT1 and GLUT2 were also decreased by LPS treatment. In this study, mRNA levels of cytokines (IL-1β

and TNF-α) were increased by LPS treatment, suggesting that the decrease in PepT1 is related to cytokine release. A glucocorticoid, dexamethasone, was administered to LPS treated rats, and served to counteract the effects on PepT1 expression, suggesting a role of interference with LPS-induced cytokine production. Sekikawa et al. (2003) found that infection with the nematode *Nippostrongylus brasiliensis* in rats downregulated mRNA and/or protein expression levels of GLUT5, PepT1, LAT2, and SGLT1 7 to 14 d after infection. This downregulation of nutrient transporters could account for the malnutrition that ensues in patients with a nematode infection (Sekikawa et al., 2003).

The effect of 5-fluorouracil, an anti-tumour drug has been investigated in brushborder membrane vesicle peptide transport in rats (Tanaka et al., 1998). This drug has been shown to cause damage to the intestinal epithelium, resulting in altered absorptive function (Adibi, 2003). Tanaka et al. (1998) observed no significant difference in the kinetics of Gly-Sar transport in ileal brushborder membrane vesicles between 5-FU treated rats and the control rats. After 3 d of 5-FU treatment, the level of PepT1 protein expression decreased to 18% of the initial level. On the other hand, the level of PepT1 mRNA in 5-FU treated rats increased to 230% of the control value, while sucrase activity, glucose uptake, and glycine uptake was decreased. It was hypothesized that PepT1 shows resilience to mucosal damage by increasing the expression of mRNA to ensure that peptide transport function is maintained. A histochemical analysis showed that 5-FU caused damage to the tips of the villous and the ileum was most sensitive to mucosal injury.

Barbot et al. (2003) examined the effects of *Cryptosporidium parvum*, a cause of diarrhea, on PepT1 in rat intestine from d 4 to d 50. Rats were gastrically infused with *C.*

parvum on d 4, and the parasite disappeared on d 21. On d 10, the parasitic infection was greatest in the ileum than in the proximal small intestine. Villous atrophy occurred throughout the small intestine, but was most pronounced in the ileum, with villous morphology returning to normal on d 21. PepT1 mRNA levels increased on d 10, during the peak of the infection, and returned to normal levels after removal of the parasite (d 21). In the control animals, PepT1 mRNA was evenly distributed throughout the small intestine from d 4 to d 50. Also, immunohistochemical staining revealed expression of PepT1 protein from d 4 to d 50 on the brushborder membrane throughout the small intestine, but during the peak of infection (d 10 through d 12), PepT1 was also detected intracellularly, after which it disappeared. This intracellular staining was not detected in the control animals. Similar to other studies, here it was shown that PepT1 is indeed resistant to intestinal injury, and may be transcriptionally regulated, or perhaps regulated by increased mRNA stability. It is not clear how the parasite may regulate PepT1; it may interfere with trafficking of the protein from the cytoplasm to the membrane (Barbot et al., 2003).

Motohashi et al. (2001) examined the effects of intestinal transplants on PepT1 expression in rats. Intestinal isografts (tissue obtained from a donor immunologically identical to the recipient) in rats expressed increased amounts of PepT1 protein, while mRNA levels remained unchanged. In allografts (non-immunologically similar), PepT1 mRNA and protein decreased. PepT1 has been shown to be resistant to injury, and in the syngeneic small intestinal transplanted rats, it appeared that increased levels of PepT1 protein in intestinal epithelial cells was due to increased stability of the protein and not increased synthesis of mRNA (Motohashi et al., 2001). In the allografts, rejection

occurred as evidenced by villous flattening, and epithelial apoptosis. In those rats, decreased expression of PepT1 was most likely the result of decreased biosynthesis of the carrier protein and not the actual rejection, per se (Motohashi et al., 2001).

Intracellular Signaling Regulation of PepT1. PepT1 activity is inhibited by activators of protein kinase C (PKC), the mechansim of which involves phosphorylation of the PepT1 protein (Leibach and Ganapathy, 1996). The PepT1 protein, in multiple species, contains two putative sites for protein kinase C phosphorylation, supporting the theory that regulation of the PepT1 protein by protein kinase C involves direct phosporylation. PKC activity decreases PepT1 V_{max}, without decreasing its affinity (Daniel, 2004). Cholera toxin and *E. coli* enterotoxins induce high levels of cyclic adenosine monophosphate (cAMP) in Caco-2 cells, inhibiting PepT1 activity (Muller et al., 1996). The mechanisms involving PepT1 regulation and cellular signaling mechanisms are important for understanding how PepT1 influences drug therapy, enteral feeding, and disease states (Adibi, 2003).

Peptide Transporter Inhibitors. Inhibitors of peptide transport have been identified based on affinities one hundredfold higher than normal substrates, which compete for binding (Knutter et al., 2001). Lys (Z(NO2))-Pro was developed as a competitive inhibitor of Gly-Sar uptake that was not transported, as demonstrated by the lack of the compound in Caco-2 cells after an uptake study. The K_t value for Gly-Sar increased twofold in the presence of the inhibitor, and the inhibitor was capable of binding to the intra- and extracellular binding site of PepT1. This demonstrated that adding the Z group to the ε-amino group of lysine turns a transportable dipeptide, Lys-Pro, into a non-transported substrate, by preventing the conformational change required

for translocation of the transporter. Identification of PepT1 inhibitors may provide insight into the discovery of the substrate binding domain on the PepT1 protein. Muller et al. (1996) demonstrated that treating Caco-2 cells for 2 h with cholera toxin significantly inhibited Gly-Sar uptake by 20%, and after 4 h, uptake inhibition plateaued at 40%. It is thought that this inhibition is cAMP dependent, and results in increases in the cAMP levels in the enterocyte, and is activated through the action of PKC. It was hypothesized that protein kinase A (PKA) also played a role, but human PepT1 protein does not contain the putative PKA site found in the rabbit PepT1 protein. Peptide transport is inhibited by cAMP and PKC, and because it contains a putative site for PKC phosphorylation, it is probable that PepT1 is regulated by posttranslational modifications.

Other Peptide Transporters. Two peptide transporters, PHT1 (SLC15A4) and PHT2 (SLC15A3) were cloned in mammalian species, with less than 30% sequence identity to the other peptide transporters (Daniel and Kottra, 2004). PHT1 was identified in rat brain, retina, and placenta, and is a peptide and histidine transporter requiring cotransport with a proton. The PHT1 mRNA is expressed at low levels throughout the rat and human gastrointestinal tract, as demonstrated by RT-PCR, however, the ability of this protein to transport peptides is still unclear (Herrera-Ruiz et al., 2001). The rat PHT1 protein consists of a predicted 572 AA and 12 transmembrane domains (Yamashita et al., 1997). In situ hybridization revealed that PHT1 is widely expressed throughout the brain, especially in the hippocampus, cerebellum, pontine nucleus, and choroid plexus. This peptide transporter could serve to transport peptide neuromodulators and remove degraded neuropeptides.

PHT2, which is expressed primarily in the lymphatic system, was identified in rat immunocytes, and is a peptide and histidine transporter (Daniel and Kottra, 2004). The rat PHT2 protein consists of 582 AA with 12 putative transmembrane domains, with four potential sites for PKC dependent phophorylation, and two sites for PKA dependent phosphorylation (Sakata et al., 2001). PHT2 requires cotransport with a proton, and is expressed in the lung, spleen, thymus, and to a lesser extent, in the brain, liver, adrenal gland, and heart. In situ hybridization revealed that within the intestine, rPHT2 is expressed in the solitary follicles, Peyer's patches, and lamina propria, where lymphocyte, macrophages, and leukocytes are located, suggesting an immunity-related role in the intestine. Human PHT2 consists of 528 AA, four putative N-linked glycosylation sites, and multiple phosphorylation sites for protein kinase A and C (Daniel and Kottra, 2004).

Summary. Transport of AA in the form of di- and tripeptides is mediated by peptide transporters, expressed on the brushborder membrane of epithelial cells in the intestine and kidney. In addition to dietary peptides, peptide transporters also recognize, bind, and transport peptidomimetic drugs and other compounds with a peptide-like structure. PepT1, the low-affinity/high-capacity transporter is expressed in both the intestine and kidney, whereas PepT2, the high-affinity/low-capacity transporter, is restricted to the kidney. Amino acid transport in the form of peptides is generally considered to be faster and more energetically efficient than free amino acid transport, and expression tends to be more evenly distributed among intestinal segments. Peptide transport requires cotransport with H^+, and PepT1 is potentially capable of transporting all 400 different dipeptides and 8,000 different tripeptides possible from combining the

20 dietary amino acids. Peptide transporter gene regulation has been researched extensively, and currently it is accepted that PepT1 is regulated by diet, developmental stage, diurnal phase, hormones, and disease. The mechanisms underlying changes in gene expression are still unclear, but most likely involve changes in transcription rate, mRNA stability, protein synthesis rate, and protein trafficking.

AA Transporters

Transport systems for AA exist throughout the body, with both broad and narrow substrate specificities, and some specificity overlap among transporters. AA transporters are responsible for the transport of free AA, and play important roles in addition to their function in the small intestine. Amino acid transporters transport neurotransmitter AA from the synaptic cleft, and transport AA across the blood-brain barrier in the central nervous system (Wagner et al., 2001). Amino acid transporters also supply amino acids to the fetus from the maternal side in the placenta and play a role in detoxification of fetal blood. Many AA transporters have narrow substrate specificities that allow them to play an important role in providing specific required AA for protein synthesis, for example glutathione. The functional differences of these AA transporters have caused them to be classified into different families based on function (substrate), mechanism (ion coupling) and structure. Originally transporters were classified into systems based on transport activity, which did not allow for the differentiation of individual transporters because of overlapping activities and substrate specificities. In recent years, molecular cloning technology has allowed individual transporter genes to be identified and cloned, providing insight into structural and functional similarities among transport families. The ASC (zwitterionic), EAAT (excitatory), CAT (cationic), and HAT (heterodimeric) AA

transporter families have been well characterized in multiple species and are thought to account for the majority of free AA absorption. The literature is replete with numerous cloned AA transporters representing a wide range of transport systems. This review will emphasize the transporters responsible for transport in the small intestine. Table 2.2 represents AA transporter systems, families, and individual cloned transporters organized by human gene name. In an intestinal epithelial cell, the B^oAT1 transporter mediates the Na^+-dependent transport of neutral AA across the apical membrane, creating a transmembrane gradient. The $b^{o,+}AT$-NBAT transporter, functioning as a Na^+-independent obligatory exchanger, mediates the uptake of cationic AA in exchange for neutral AA, in effect recycling substrate for the B^oAT1 transporter. The EAAT3 transporter is responsible for the Na^+-dependent transport of anionic AA in exchange for a K^+. The peptide transporter PepT1 mediates the H^+-dependent uptake of all di- and tripeptides. On the basolateral membrane, the y^+LAT1-4F2hC heterodimer transports cationic AA out of the cell in exchange for Na^+ and neutral AA. The LAT2-4F2hC transporter also functions as an exchanger on the basolateral membrane, exchanging neutral AA in a Na^+-independent manner. A currently unidentified anionic AA transporter mediates the efflux of anionic AA. The Na^+-ATPase pump serves to reestablish the extracellular to intracellular Na^+ gradient, and a Na^+/H^+ countertransporter on the apical membrane restores the H^+ gradient.

Table 2.2 AA transport systems and families (Based on Palacin and Hediger, 2003; Kanai and Hediger, 2004, and Broer et al., 2004)

Human gene name	Protein	Aliases	Substrates	Transport type/coupling ion	Tissue distribution/cellular expression
SLC3A1	rBAT	NBAT, D2	Heterodimerizes with light subunit $b^{o,+}AT$ to form system $b^{o,+}$	Exchanger	Apical membrane of epithelial cells in kidney and small intestine; also in liver and pancreas.
SLC3A2	4F2hC	CD98hC	Transport systems L, y^+L, Xc- and asc; heterodimerizes with light subunits SLC7A5-8 and SLC7A10-11	Exchanger	Ubiquitous. Basolateral membrane of epithelial cells.
SLC7A1	CAT-1	ATRC1, ERR, REC1	Cationic L-AA	Facilitated transporter, non-obligatory exchanger	Ubiquitous except for liver/plasma and intracellular membranes. Basolateral membrane of epithelial cells.
SLC7A2	CAT-2(A or B)	TEA, ATRC2	Cationic L-AA	Facilitated transporter	CAT-2A: liver, skeletal muscle, pancreas; CAT-2B: inducible in many cell types.
SLC7A3	CAT-3	ATRC3	Cationic L-AA	Facilitated transporter	Thymus, ovary, testis, brain (neurons) plasma membrane.
SLC7A4	CAT-4		Unknown	Orphan transporter	Brain, testis, placenta/plasma and intracellular membranes.
SLC7A5	LAT1	Assoc. with 4F2hC	Large neutral L-AA, T3, T4, L-DOPA, BCH (system L)	Exchanger	Brain, ovary, testis, placenta, blood-brain barrier, fetal liver, activated lymphocytes, and tumour cells.

Human gene name	Protein	Aliases	Substrates	Transport type/coupling ion	Tissue distribution/cellular expression
SLC7A6	y^+LAT2	Assoc. with 4F2hC	Na^+ indep.: cationic AA; Na^+ depend.: large neutral L-AA (system y^+L)	Exchanger (prefers intracell. cationic AA for extracell. neutral AA plus Na^+	Brain, small intestine, testis, parotid, heart, kidney, lung, liver, basolateral membrane in epithelial cells
SLC7A7	y^+LAT1	Assoc. with 4F2hC	Na^+ indep.: cationic AA; Na^+ depend.: large neutral L-AA (system y^+L)	Exchanger (prefers intracell. cationic AA for extracell. neutral AA plus Na^+	Small intestine, kidney, leukocytes, placenta, lung/basolateral in epithelial cells
SLC7A8	LAT2	Assoc. with 4F2hC	Neutral L-AA (large and small), BCH (system L)	Exchanger (similar intra- and extra-cellul. selectivities. Lower intracell. apparent affinity.	Small intestine, kidney, brain, placenta, ovary, testis, skeletal muscle/ basolateral membrane in epithelial cells
SLC7A9	$b^{o,+}$AT	Assoc. with rBAT	Cationic AA, large neutral AA (system $b^{o,+)}$	Exchanger, prefers extracell. cationic AA for intracell. neutral AA	Small intestine, kidney, lung, placenta, brain, liver/apical membrane in epithelial cells
SLC7A10	Asc-1	Assoc. with 4F2hC	Small neutral L- and D-AA (Na^+ indep.: system asc)	Exchanger	(Human) brain, heart, placenta, skeletal muscle, and kidney
SLC7A--	Asc-2	Assoc with ?	Small neutral AA (Na^+ indep.: system asc)	Facilitated transporter; exchanger?	(Mouse) kidney, placenta, spleen, lung and skeletal muscle/plasma membrane, non-polarized
SLC1A1	EAAT3	EAAC1, system X^-_{AG}	L-Glu, D/L-Asp	Cotransporter (Na^+, H^+, and K^+)	Brain (neurons), intestine, kidney, liver, and heart

Human gene name	Protein	Aliases	Substrate	Transport type/coupling ion	Tissue distribution/cellular expression
SLC1A2	GLT-1	EAAT2, system X^-_{AG}	L-Glu, D/L-Asp	Cotransporter (Na^+, H^+, and K^+)	Brain (astrocytes), liver.
SLC1A3	GLAST	EAAT1, system X^-_{AG}	L-Glu, D/L-Asp	Cotransporter (Na^+, H^+, and K^+)	Brain (astrocytes), heart, skeletal muscle, placenta
SLC1A4	ASCT1	AAAT, system ASC	L-Ala, L-Ser, L-Thr, L-Cys, and L-Gln	Cotransporter (Na^+), exchanger (AA)	Widespread
SLC1A5	ASCT2	AAAT, system ASC, $hATB^o$	L-Ala, L-Ser, L-Thr, L-Cys, and L-Gln	Cotransporter (Na^+), exchanger (AA)	Lung, skeletal muscle, large intestine, kidney, testis, and adipose tissue
SLC1A6	EAAT4	System X^-_{AG}	L-Glu, D/L-Asp	Cotransporter (Na^+, H^+, and K^+)	Cerebellum (Purkinje cells)
SLC1A7	EAAT5	System X^-_{AG}	L-Glu, D/L-Asp	Cotransporter (Na^+, H^+, and K^+)	Retina
SLC6A19	B^oAT1	System B^o	Neutral AA	Cotransporter (Na^+)	Kidney and small intestine, apical membrane

AA Transporters and Cellular Nutrient Signaling

In addition to their role in AA uptake, AA transporters also serve to initiate amino acid dependent cell signaling (Hyde et al., 2003). Initiation of cell signaling may be the result of changes in substrate concentration or flux, or to regulate the availability of AA to nutrient receptors (Hyde et al., 2003). The mechanism by which this occurs can involve a transition in the conformation of the transporter protein, or binding of substrate to the transporter may stimulate cell signaling. Transporters are probably capable of detecting the intracellular concentration of AA at the plasma membrane, and this information is transduced to signaling molecules including G-proteins. A second mechanism by which cell signaling occurs is that the actual transport of the AA along with the cotransported solute, evokes secondary changes within the cell, including changes in the pH, ion concentrations (Na^+, K^+, H^+), membrane potential, and cell volume, which then triggers cell signaling. A third mechanism involves transport of the extracellular AA across the plasma membrane where it then binds to an intracellular receptor that directly initiates cell signaling. The signaling capacity is dependent on intracellular concentrations of AA, thus, transporter expression (V_{max}) and K_m should impact the sensor activities. A fourth mechanism involves an extracellular receptor that senses extracellular AA concentrations and activity of the transporters, mediating initiation of cell signaling.

Na^+-Dependent, Neutral AA Transporters, System B^o

System B^o, which has been investigated in Caco-2 cells and brushborder membrane vesicles, is a Na^+-dependent transporter responsible for the uptake of most

neutral AA, and is the major apical glutamine transporter (Avissar et al., 2004). It has been known for some time that the apical transport of neutral AA in the kidney and intestine occurs by system B^o, but the transporters had yet to be identified. For some time it was thought that ASCT2, a brushborder membrane transporter, was responsible for this transport, but the transport characteristics of ASCT2 were not confirmed to be identical to the system (i.e., whether ASCT2 could mediate non-obligatory exchange transport; Avissar et al., 2004). Recently, Broer et al. (2004) reported the molecular cloning of B^oAT1 (SLC6A19) from system B^o in mouse kidney and intestine. The mB^oAT1 cDNA consists of 1,904 bp, and the protein consists of 634 AA, 12 putative transmembrane domains, and the amino and carboxyl termini are located intracellularly (Broer et al., 2004). RT-PCR and in situ hybridization showed that mRNA expression was restricted to mouse kidney and small intestine. Within the kidney mRNA was localized to the proximal tubule, and within the intestine, mRNA was localized to the enterocytes on the villi, with highest expression towards the tip of the villous. Uptake studies in oocytes revealed a membrane-potential and pH dependent transport, independent of chloride. Uptake of radioactively labeled leucine was significantly inhibited by an excess of all other neutral AA, suggesting that all L- neutral AA are recognized and transported by this protein. System B^o (apical membrane of enterocyte) and system Gly (central nervous system and erythrocytes) account for the majority of glycine plasma membrane transport (Tunnicliff, 2003). The B^oAT1 also appears to be a low-affinity transporter, exhibiting a K_m of about 630 μ*M* for leucine. This transporter has been classified as a member of the SLC6 family of AA transporters. Mutations in this system are thought to be responsible

for hartnup disorder, an autosomal recessive disorder in which patients exhibit defective neutral AA transport, resulting in neutral hyperaminoaciduria (Seow et al., 2004).

Heterodimeric AA Transporters (HATs)

The heterodimeric AA transporters (HATs) consist of a light chain protein that heterodimerizes with a heavy chain protein by disulfide bonds and non-covalent linkages. Currently, two heavy chain proteins and nine light chain proteins have been identified (Palacin et al., 2005). NBAT and 4F2hC represent the two heavy chain proteins. LAT1, LAT2, y^+LAT1, y^+LAT2, asc1, and xCT form heterodimers with the heavy chain protein 4F2hC. The $b^{o,+}$AT light chain protein heterodimerizes with NBAT, and the asc2 and AGT-1 associate with an unidentified heavy chain protein.

Glycoprotein Associated AA Transporters (gpaATs). Many transport systems consist of two or more proteins that associate to form an active complex. In most cases these complexes consist of a light-chain catalytic protein, and a glycosylated heavy-chain protein. The functional cell-surface expression of these transporters depends on coexpression with the type II glycoprotein, of which two have been identified, 4F2hC and NBAT (rBAT) (Verrey et al., 2000). The light-chain protein and heavy-chain protein associate by disulfide bonds, but the function of the disulfide bonds remains unknown (Wagner et al., 2001). The disulfide bonds could serve to stabilize the structure on the plasma membrane or could play a role with associated proteins. The discovery of the gpaAT transporters came about through expression cloning of NBAT in *Xenopus laevis* oocytes (Bertran et al., 1993).

The $b^{o,+}$ Family of Glycoprotein-Associated AA Transporters. The glycoprotein-associated AA transporters are also referred to as the gpaAT family, and also by the

human gene name, SLC7 (Verrey et al., 2003). The $b^{o,+}$ AA transporters facilitate the Na^+-independent transport of neutral or basic AA into the cells in exchange for intracellular neutral AA. The finding that transport of neutral AA evoked an outward current of positive charge, and the transport of basic AA evoked an inward movement of positive charge led to the realization that this system functioned as an obligatory exchanger (Chillaron et al., 1996; Wagner et al., 2001). The light or "catalytic" chain of the heterodimeric AA transporters (HAT; SLC7 family) are highly hydrophobic proteins that associate with a type II glycoprotein (heavy chain) by disulfide bonds to make up the $b^{o,+}$ system (Palacin et al., 2005). The light catalytic subunit contributes the AA transport activity to the heterodimer (Palacin et al., 2005). The heavy chains, NBAT (rBAT, D2) and 4F2hc form the human gene family SLC3. One light chain, $b^{o,+}$AT, the basic and neutral AA transporter, associates with NBAT to form the $b^{o,+}$ transport system, and is then routed to the brushborder membrane of the kidney proximal tubule and intestine.

NBAT, discovered before the light-chain counterpart, was found to contain only a single membrane domain, and, therefore, it was implied that it must be associated with an unidentified AA transport protein to function as a transporter. NBAT was discovered by expression cloning, simultaneously by three research groups (Tate et al., 1992; Wells and Hediger, 1992; Bertran et al., 1993). The $b^{o,+}$AT transporter (at the time identified as "BAT1") was discovered by screening of a mouse cDNA library and subcloning the cDNA into a mammalian expression vector and transfection into COS-7 cells (Chairoungdua et al., 1999). The primary function of the heavy chain, NBAT, is the movement of the light chain to the apical membrane. Mutations in NBAT result in defective trafficking to the apical membrane (Wagner et al., 2001). The current

hypothesis of this pathway suggests that $b^{o,+}$AT and NBAT enter the endoplasmic reticulum independently, and the two proteins heterodimerize, forming disulfide bonds, experience changes in conformation, after which the complex is released from the endoplasmic reticulum (Palacin and Kanai, 2004). The complex undergoes N-glycosylation in the Golgi, and then reaches the brushborder membrane of epithelial cells. Studies have demonstrated that the stability of the NBAT protein is increased by heterodimerization with $b^{o,+}$AT, but the stability of $b^{o,+}$AT is independent of NBAT. Because of this and the knowledge that mutations in $b^{o,+}$AT result in an inactive transporter, it was hypothesized that $b^{o,+}$AT is the catalytic subunit, and is properly inserted and folded within the endoplasmic reticulum membrane without the presence of NBAT (Wagner et al., 2001). Also, upon abolishing disulfide bonds, the expression of the heterodimer at the apical membrane was not completely eliminated, demonstrating the presence of noncovalent interactions between the light and heavy subunits (Palacin and Kanai, 2004).

Coimmunoprecipitation showed that all $b^{o,+}$AT in renal brushborder preparations associated with NBAT, but not all NBAT associated with $b^{o,+}$AT, suggesting the presence of an additional light subunit for NBAT, currently unidentified (Fernandez et al., 2002). In HeLa and Madin-Darby canine kidney (MDCK) $b^{o,+}$AT transfected cells, Reig et al. (2002) demonstrated that the $b^{o,+}$AT protein, in the absence of NBAT, assumes an active conformation with full transport activity. However, in enterocytes, coexpression with NBAT is required for trafficking of the transporter to the brushborder membrane. Also, maturation of N-glycosylation of NBAT, occurring with the processing of the protein from the endoplasmic reticulum to the golgi complex, is dependent on the presence of

$b^{o,+}$AT. Studies with protein expression in MDCK cells demonstrated that NBAT and $b^{o,+}$AT remained intracellular unless coexpressed, after which they appeared at the apical cell surface (Bauch and Verrey, 2002). In the absence of $b^{o,+}$AT, NBAT was rapidly degraded, and in its presence it was glycosylated and was stable over an 8-h period.

An asymmetery in substrate affinity has also been reported for this heterodimer (Reig et al., 2002). The reconstituted system $b^{o,+}$ in HeLA cells showed a high affinity for arginine and leucine from the outside of the cell, and a low-affinity of leucine from inside. A unidirectional transporter on the apical membrane maintains a high intracellular concentration of neutral AA, resulting in a transmembrane concentration gradient that drives obligatory exchange (Bauch et al., 2003). Similar results were found in chicken intestinal brushborder membrane vesicles (Torras-Llort et al., 2001) and with other heteromeric AA transporters expressed in oocytes (Meier et al., 2002). This system is advantageous in the kidney, where system $b^{o,+}$ is solely responsible for apical reabsorption of cystine in the renal proximal tubule (Fernandez et al., 2002), and being a high-affinity carrier, ensures uptake of all cystine.

A deficiency in the $b^{o,+}$ system results in cystinuria, a disorder characterized by an accumulation of cystine in the kidney, ultimately resulting in kidney failure. NBAT and $b^{o,+}$AT mutations result in 80% of the reported cystinuria cases (Palacin et al., 2005). However, cystinuria does not result in malnutrition (Palacin et al., 2005). It is thought that absorption of cystine in the form of small chain peptides by PepT1 prevents malnutrition. Two types of cystinuria have been classified: type I and nontype I, with type I being transmitted as an autosomal recessive disorder, and nontype I being

transmitted dominantly. Therefore, type I heterozygotes are silent, and nontype I heterozygotes exhibit clinical symptoms.

SLC3 Heavy Chain Protein, 4F2hC. The SLC3 HAT family of AA transporters also includes the heavy chain 4F2hC protein (Palacin and Kanai, 2004). The 4F2hC protein is a type II glycoprotein consisting of a single transmembrane domain with an intracellular amino terminus, a glycosylated extracellular carboxyl terminus, and a molecular mass of approximately 85 kDa (Deves and Boyd, 1998). The rat 4F2hC protein consists of approximately 527 AA, a molecular mass of approximately 60 kDa, and shares 26% sequence similarity to the rat renal NBAT protein (Yao et al., 1998). Heterodimeric complexes of 4F2hC are localized to the basolateral membrane of epithelial cells (Palacin and Kanai, 2004), and is widely distributed throughout mouse tissues (Deves and Boyd, 1998). The transporters that heterodimerize with 4F2hC for expression are members of systems L, y^+L, x_c^-, and asc. It is believed that 4F2hC controls the signaling that routes the complex to the basolateral membrane. The 4F2hC protein is involved in many cellular functions including cell activation, growth, and adhesion. Genetic defects of 4F2hC are lethal. Expression of this protein is upregulated in cancers, and anti-4F2hC antibodies have been shown to suppress cancer cell growth, but the underlying mechanisms behind this pathway are unknown.

Putative Structure of $b^{o,+}$ Glycoprotein-Associated AA Transporters. The heterodimeric AA transporter consists of a light chain ($b^{o,+}$AT) with 12 transmembrane domains associated with the heavy chain (NBAT) through a disulfide linkage between the third transmembrane domain and the single domain of NBAT, with the carboxyl and amino termini of the light chain located intracellularly (Verrey et al., 2003). The heavy

chain proteins consist of a single transmembrane domain with an extracellular carboxyl terminus, an intracellular N-terminus, and a large extracellular domain (Palacin and Kanai, 2004). The large carboxy terminal extracellular domain putatively resembles the glycosidases and hence, most likely forms a globular tertiary structure (Verrey et al., 2000). The human NBAT protein consists of 685 AA residues (85 kDa) and the 4F2hC protein consists of 529 residues. The mouse NBAT protein contains four putative N-glycosylation sites at residues 123, 234, 345, and 349 in the extracellular location, and a conserved leucine zipper motif in the C-terminal end of the protein (Segawa et al., 1997). Also in the mouse, four NBAT mRNA species were detected corresponding to sizes of 2.4, 4.2, 5.4, and 8.0 kb in the kidney. The 4.2 kb transcript was the only species detected in the brain, and the intestine hybridized to all transcripts except for the 5.4 kb transcript. In the intestine, the 2.4 kb transcript was the dominant species. The cysteines that form disulfide bonds with the heavy-chain protein are located in the loop between transmembrane domains three and four (Verrey et al., 2000). It has been suggested that the cysteine residues on the carboxyl terminus of NBAT play an important role in the functionality of the $b^{o,+}$ transport system. Peter et al. (2000) found that NBAT mutants with the cysteine at position 664 replaced by arginine showed significantly lower arginine and cystine transport when injected into oocytes. This cysteine residue and residues 671 and 683 are conserved among the human, rat, rabbit, and mouse, and these residues are thought to contribute to the stabilization of the heterodimeric complex (Peter et al., 2000). The mouse and human $b^{o,+}$AT protein consists of 487 AA (approximately 50 kDa), with 12 predicted transmembrane domains and a conserved cysteine residue in the putative extracellular loop between domains three and four (Chairoungdua et al., 1999; Wagner et

al., 2001). A potential tyrosine kinase-dependent phosphorylation site was predicted at residue 99, protein kinase C-dependent phosphorylation sites were predicted at residues 5, 51, 169, 345, and 399, and a potential cAMP-dependent phosphorylation site was located at residue 350.

Substrates of the $b^{o,+}$ System. Coexpression of NBAT and $b^{o,+}$AT induces a Na^+-independent, high affinity transport of L-cystine and cationic AA, and a lower affinity for neutral AA, occurring by an exchange mechanism. The $b^{o,+}$ is the primary transport system for cystine apical reabsorption in the kidney (Palacin and Kanai, 2004). This system is also responsible for ornithine reabsorption (Wagner et al., 2001). Defects in this system cause an inherited defect, known as cystinuria, a type of aminoaciduria. The $b^{o,+}$ system serves to mediate the exchange of dibasic AA and cystine for neutral AA. Luminal cystine and cationic AA are transported because of a high extracellular affinity, while intracellular neutral AA are transported out of the cell to the lumen because of a high intracellular concentration. These neutral AA transported out of the cell can then be cycled back into the cell by system B/B^o (Bauch et al., 2003). This high intracellular accumulation of neutral AA occurs by a B^o-type Na^+-cotransporter recently identified as the B^oAT1 in mouse intestine (Broer et al., 2004). Evidence to support the role of B^oAT1 in being the primary transporter providing an intracellular accumulation of neutral AA, is that the preferred substrate is leucine, which is also the preferred efflux substrate for the $b^{o,+}$ heterodimer (Broer et al., 2004). Until cloning and characterization of B^oAT1, it was thought that ASCT2 was the transporter responsible for B^o transport at the apical membrane. However, it does not recognize phenylalanine or tryptophan, which are known to be B^o substrates and are recognized by B^oAT1, and ASCT2 is an obligatory

exchanger, which does not allow for the net flux and accumulation of neutral AA inside the cell.

Tissue Distribution of $b^{o,+}$ Transporters. The $b^{o,+}$AT and NBAT transporter genes are expressed in the apical membrane of epithelial cells in small intestine, kidney, liver, placenta, lung, and pancreas (Verrey et al., 2000; Wagner et al., 2001; Palacin and Kanai, 2004). NBAT is expressed in the kidney and liver as two mRNA representing alternative polyadenylation sites (Palacin and Kanai, 2004). Expression of the NBAT protein is restricted to the apical membrane of the intestinal epithelia and kidney proximal tubule, but NBAT mRNA is detected in liver, pancreas and brain. NBAT mRNA was also detected in kidney, intestine, pancreas, heart, adrenal gland, brain stem, and spinal cord (Wagner et al., 2001). Light and electron microscopic immunoperoxidase labeling of site-directed antibodies was used to determine localization of NBAT protein in the intestine and kidney, and in addition to being expressed on the apical membrane of epithelial cells, NBAT was also found within enteroendocrine cells and submucosal neurons (Pickel et al., 1993). The labeled enteroendocrine cells were interspersed between enterocytes, and were located in intermediate portions of the villi between the apex and basal crypts, and were most numerous (several per villous) towards the ileum of the small intestine. Within the neurons, labeling was localized to dense core vesicles in axon terminals, suggesting that NBAT plays a role in vesicular uptake of substrate into enteric neurons. Also, within enterocytes, in addition to brushborder membrane staining, there was also localization within the rough endoplasmic reticulum and Golgi apparatus, indicating its site of synthesis and processing. In comparison to other regions stained,

apical membrane protein was stained to a lesser extent, suggesting a rapid turnover of protein in enterocytes.

Ontogenetic regulation of NBAT in rats was examined using semi-quantitative RT-PCR, and NBAT mRNA showed decreased expression from proximal to distal small intestine, and this pattern remained unchanging from postnatal d 4 to 50 (Rome et al., 2002). Also, NBAT protein was localized mainly to the apical membrane, and also to a lesser extent, in the cytoplasm, and there was an increased gradient of expression from the crypt-villous junction to the tip of the villous. There was no protein detected in the crypt cells.

Howard et al. (2004) found that NBAT mRNA exhibited equal mRNA abundance throughout the length of the rat small intestine, similar to PepT1. It has been observed that NBAT and $b^{o,+}$AT show an opposite pattern of expression gradients in the apical membrane of the kidney proximal tubule. The highest expression of $b^{o,+}$AT being in the proximal region of the tubule and lowest expression in the distal region, and a maximum of NBAT in the distal portion of the tubule and lowest expression in the proximal, suggesting that the proteins may bind to other light or heavy chain proteins to allow for expression of the transporter (Bauch and Verrey, 2002; Fernandez et al., 2002). The $b^{o,+}$ heterodimer is expressed in decreasing amounts from the proximal to distal kidney proximal tubule, consistent with the $b^{o,+}$AT protein, and because more than 90% of cystine reabsorption occurs in the proximal region of the tubule, this heterodimer is thought to account for the majority of cystine reabsorption in the kidney (Fernandez et al., 2002).

Clearly, NBAT is a multifunctional protein that may serve other roles in addition to the transport of AA. Segawa et al. (1997) also looked at developmental changes of NBAT in mice, and found that NBAT mRNA was not detected in the kidney by Northern blot in 2-d old mice, and was first visible in 4-d old animals, and subsequently increased in expression levels to normal adult levels. Using immunofluorescence microscopy and expression in MDCK cells, Bauch et al. (2003) found that the NBAT-$b^{o,+}$AT complex followed a similar expression pattern in the kidney proximal tubule as y^+LAT1-4F2hC and LAT2-4F2hC, suggesting a cooperation to function in reabsorption of all cationic AA and cystine. Expression of only the $b^{o,+}$AT complex led to reabsorption of L-arginine and L-leucine, and coexpression of the basolateral transporter y^+LAT1-4F2hC led to not only an increase in L-arginine reabsorption, but also a secretion of L-leucine, rather than absorption. The change in L-leucine directional transport indicates the function of some AA as exchangers. Also, when coexpressed with y^+LAT2-4F2hC, there was an increase in cystine reabsorption. These results demonstrate that transporters (exchangers and unidirectional) expressed in parallel serve to drive net AA absorption.

Homology of $b^{o,+}$ System Among Species. Currently, AA sequences are available for NBAT from the human, rat, mouse, dog, opossum, and rabbit, which share 69 to 80% identity (Palacin and Kanai, 2004). Six sequences for 4F2hc are available, and include human, rat, mouse, Chinese hamster, zebrafish, and sea lamprey, and share 31 to 77% identity. Also, the $b^{o,+}$AT transporters exhibits striking identity to the system L transporters LAT1 (43%) and LAT2 (43%) (Chairoungdua, 1999).

Physiological Importance of Obligatory Exchangers. The functions of AA transporters that serve as exchangers are dependent on AA concentrations intra- and

extracellularly, ionic concentrations, and the membrane potential (Verrey et al., 2000). The normal molar ratio of AA exchange for these transporters is 1:1, indicating that a net flux of AA can not be achieved. All evidence argues that the glycoprotein-associated AA transporters all function only as obligatory exchangers. However, these obligatory exchangers can cooperate with unidirectional transporters expressed on the same membrane by transporting out substrates that are substrates of the unidirectional transporter against the exchanged AA. The concentration gradient of the intracellular recycled AA can then provide a driving force for uphill transport of the exchanged AA. For example, the $b^{o,+}AT$ complex transports extracellular cationic AA in exchange for intracellular neutral AA, which then serve as substrates for the unidirectional $B^{o,+}$ system on the apical membrane. Thus, the obligatory exchangers can increase the AA specificity range for transport across the apical and basolateral membrane by providing recycled substrate for the unidirectional system expressed on both membranes. This also brings up the matter of transport asymmetry and the driving force for transport. Sources of transport asymmetry for the $b^{o,+}AT$ transporter includes a high extracellular affinity for cationic AA that drives transport both by a membrane potential and by the concentration gradient of the intracellular neutral AA. In the case of cystine, for which $b^{o,+}AT$ exhibits a lower affinity, there is a chemical sink leading to a concentration gradient, in which transported cystine is reduced to two cysteine and a concentration gradient of the exchanged AA is developed.

Summary. The $b^{o,+}$ system is a Na^+-independent amino acid transport system expressed on the brushborder membrane of epithelial cells in the intestine. The active form of the transporter consists of a light-chain protein, $b^{o,+}AT$ linked by disulfide bonds

to a heavy-chain protein, NBAT. The $b^{o,+}$ transport system functions as an obligatory exchanger. It mediates the exchange of an intracellular neutral amino acid for an extracellular neutral or basic amino acid, without the direct expenditure of ATP. Accumulation of those intracellular neutral amino acids occurs through the action of the Na^+-dependent unidirectional brushborder membrane transporter, B^oAT. The intracellular neutral amino acids tend to be non-essential; therefore, this system ensures that $b^{o,+}$ is able to capture essential cationic amino acids and cystine in the lumen and transport them into the cell for use by the body. The $b^{o,+}$ system exhibits a high extracellular affinity for cationic amino acids, preventing an endless loop of neutral amino acid transport by this exchanger.

The Cationic AA Transporters (CATs), System y^+

The SLC7 family is divided into two families: 1) the already discussed glycoprotein-associated AA transporters, and 2) the cationic AA transporters (Verrey et al., 2003). Despite the sequence identity between the CATs and $b^{o,+}AT$, there have been no reported interactions between the CATs and either of the two identified heavy chain proteins (Wagner et al., 2001). Transporters within the CAT family transport substrate by deriving energy from a differential *trans*-stimulation by intracellular substrates. Nitric oxide synthesis may also be regulated by the cationic AA transporters by controlling the rate of L-arginine uptake. The substrates for the CAT transporters are more specific than for the glycoprotein-associated AA transporters.

The mouse cationic AA transporter (mCAT-1) was the first identified member of the cationic AA transporter family (human gene family name SLC7), and was first identified as a receptor for murine ecotropic leukaemia virus (Verrey et al., 2003). Other

CAT members, CAT1, CAT2, CAT3, and CAT4 have been identified in mammals. CAT transporters are thought to have 14 transmembrane domains, with the first 12 domains very similar to the $b^{o,+}$AT protein, and two helices on the carboxyl terminus (Wagner et al., 2001).

CAT-1 Transport Properties. CAT-1 (SLC7A1, ATRC1) is a high-affinity, low-capacity transporter whose expresssion is widespread throughout the body, except for the liver (Verrey et al., 2003). The CAT-1 transporter was first identified as a receptor for murine ecotropic leukaemia virus (Verrey et al., 2003). Nonexpression in the liver is supported by evidence that murine ecotropic leukaemia virus does not infect this organ (Deves and Boyd, 1998). CAT-1 mRNA has been reported as two transcriptional products, 7 and 7.9 kb, resulting from polyadenylation (Deves and Boyd, 1998). The mouse CAT-1 protein consists of 622 AA and an approximate molecular mass of 67 kDa (Deves and Boyd, 1998). CAT-1 is a pH and Na^+-independent transporter (Kizhatil and Albritton, 2003). The highest expression levels have been found in the testis, and to a lesser extent, in the bone marrow, brain, stomach, spleen, kidney, lung, ovary, uterus, large and small intestine, thymus, heart, skeletal muscle, and skin (Deves and Boyd, 1998). CAT-1 expression is localized to the basolateral membrane of epithelial cells, but is expressed in the plasma membrane and intracellular vesicles in glioblastoma cells (Wolf et al., 2002). Kizhatil and Albritton (2003) found using immunofluorescence microscopy that CAT-1 localized as clusters in the basolateral membrane of Madin-Darby canine kidney and human embryonic kidney, confirming that CAT-1 protein expression is restricted to specific membrane microdomains. Different proportions of CAT-1 clusters in different microdomains could be indicative of differences in

physiological function. CAT-1 is the major transporter for system y^+ in most cells, because homozygous CAT-1 knockout in mice was lethal 1 d after birth (Kizhatil and Albritton, 2003). Expression of CAT-1 is regulated by cell proliferation, hormones, cytokines, and growth factors, and, similar to other transporters, mRNA is increased during nutrient restriction (Verrey et al., 2003). Activity of this protein also appears to be posttranslationally regulated by activation of PKC in human endothelial cells, and also by cytoskeletal protein interaction. Transport by CAT-1 is sensitive to *trans*-stimulation, which appears to be controlled by a large intracellular loop between membrane domains 4 and 10, which also determines the substrate affinity. The CAT proteins play an important role in transporting arginine into pools of cationic AA from which endothelial and inducible nitric oxide synthase can receive substrate (Verrey et al., 2003).

CAT-2 Transport Properties. Two alternative splice products of CAT-2 (SLC7A2), CAT-2A and CAT-2B, are expressed quite differently (Verrey et al., 2003). CAT-2A, a low-affinity transporter, is most highly expressed in the liver, but is also present in skeletal muscle, cardiomyocytes, cardiac microvascular cells, vascular smooth muscle, and pancreas. In contrast, CAT-2B requires induction of expression, usually after cytokine or lipopolysaccharide treatment, and follows the same expression pattern as CAT-1. CAT-2B is also induced with the inducible isoform of nitric oxide synthase. CAT-2B gene knockouts, although apparently normal phenotypically, do exhibit decreased nitric oxide production in peritoneal macrophages, demonstrating that CAT-2B is critical for sufficient inducible nitric oxide synthase substrate supply.

CAT-3 Transport Properties. CAT-3 (SLC7A3) expression is found in developing mouse embryo tissues, and in the central neurons of adult mice and rats

(Verrey et al., 2003). CAT-3 is also expressed in human thymus, uterus, testis, mammary gland, brain, ovary, and stomach.

CAT-4 Transport Properties. CAT-4 (SLC7A4) has been found in brain, testis, and placenta, and is restricted to the plasma membrane and intracellular vesicles in glioblastoma (Verrey et al., 2003). Its function has yet to be identified.

Putative Structure of Cationic AA Transporters, System y^+. The CAT proteins contain 14 putative transmembrane domains and two confirmed glycosylation sites that differ in position between isoforms and between species (Verrey et al., 2003). By contrast, the glycoprotein-associated AA transporters contain two less transmembrane domains, are not glycosylated, and associate with a glycoprotein to induce surface expression.

Summary. The cationic amino acid transporters (CATs) are a group of high-affinity/low-capacity Na^+-independent transporters with widespread expression throughout the body. CAT-1 is expressed on the basolateral membrane of intestinal epithelial cells, and functions as an obligatory exchanger, similar to the $b^{o,+}$ system, although no heterodimerization with a heavy chain protein has been observed in the CATs. While brushborder membrane transporters serve to transport amino acids from the lumen of the small intestine to the inside of the enterocyte, the basolateral membrane transporters function to transport amino acids from inside the enterocyte across the basolateral membrane to the bloodstream.

The Heterodimeric Neutral AA Transporters, System L

System L is responsible for the Na^+-independent transport of large branched and aromatic neutral AA (Rajan et al., 2000). Members of this family form heterodimers with

the heavy chain protein 4F2hC. System L transport is widespread and found in most types of cells.

LAT-1 Transport Properties. LAT1 (SLC7A5), a system L transporter, associates with the heavy chain protein 4F2hC to mediate the Na^+-independent transport of large, neutral AA on the basolateral membranes of nonpolarized cells (Verrey et al., 2003). Like NBAT, 4F2hC functions to traffic the complex to the plasma membrane. LAT-1 was first isolated from rat C6 glioma cells by expression cloning in *Xenopus* oocytes (Kanai et al., 1998). The LAT-1 cDNA encodes a putative 512 AA protein, with 12 transmembrane domains, and no glycosylation sites. The human LAT-1 protein consists of 507 AA (Prasad et al., 1999). This transporter exhibits a high affinity for branched chain and aromatic AA, with the affinity being up to 100-fold higher for extracellular AA than for intracellular AA (Verrey et al., 2003). Because of this, the extracellular AA concentration limits the rate of uptake. LAT-1 is also capable of recognizing and transporting D-isomers of leucine, methionine, and phenylalanine (Kanai et al., 1998). The heterodimeric complex functions as an exchanger that does not transport by facilitated diffusion, and uptake is *trans*-stimulated by intracellular AA, with a 1:1 ratio. It is thought that this complex functions to equilibrate concentrations of AA across the plasma membrane rather than to cause net uptake. The current transport theory suggests that a Na^+-dependent transporter protein transports neutral AA across the apical membrane, after which they are exchanged for extracellular neutral AA via the LAT-1 transporter.

LAT-1 is also expressed in cancer tissues, suggesting that it functions in growing cells. LAT-1 mRNA was found in most tissues including brain, spleen, thymus, heart, lung, blood-brain barrier, leukocytes, placenta, testis, colon and human tumour cell lines

(Kanai et al., 1998; Wagner et al., 2001). In addition to AA, LAT-1 also transports thyroid hormone, but with a much lower affinity (Wagner et al., 2001).

LAT-2 Transport Properties. LAT-2 cDNA was first isolated from a rabbit intestinal cDNA library, and consists of 535 AA, 12 putative transmembrane domains, with both the amino and carboxy termini located intracellularly (Rajan et al., 2000). The putative structure does not contain any N-linked glycosylation sites, but it does contain one PKC dependent phosphorylation site, three sites for PKA dependent phosphorylation, and one site for tyrosine kinase-dependent phosphorylation. The transport properties of LAT-2 (SLC7A8) are very similar to LAT-1 in that it associates with 4F2hC to form a heterodimeric complex which is trafficked to the basolateral membrane, where it serves as an exchanger to equilibrate the concentration of neutral AA across the membrane. LAT-2 exhibits a much broader substrate specificity, accepting small netural AA (L-alanine, L-glycine, L-cystine, and L-serine) and glutamine, as well as the large neutral AA (Rajan et al., 2000). Compared to LAT-1, LAT-2 is a relatively low-affinity transporter, with K_t values for AA much higher (severalfold) than LAT-1. It exhibits a lower affinity for intracellular AA than for extracellular AA. Also, in comparison to LAT-1, LAT-2 is stimulated by an acidic pH, and glutamine transport activity increases almost 100% when the pH is decreased from 8.5 to 6.5 (Rajan et al., 2000). LAT-2 also serves as an exporter of cystine (Wagner et al., 2001).

LAT-2 is primarily expressed in the basolateral membrane of epithelial cells in the proximal kidney tubule and small intestine. LAT-2 is also expressed in the placenta, ovary, brain, liver, spleen, prostate, testis, skeletal muscle, heart, and lung (Wagner et al., 2001). Bauch et al. (2003) found by immunofluorescence microscopy in MDCK cells that

LAT2 and 4F2hC colocalized to the basolateral membrane, similar to their localization in mouse kidney and small intestine (Rossier et al., 1999).

Summary. System L AA transporters heterodimerize with the 4F2hC protein to form an active transport complex. Transport is Na^+-independent and occurs by an exchange mechanism serving to equilibrate the concentration of neutral amino acids across the membrane. LAT-2 is expressed intestinally, and is localized to the basolateral membrane of absorptive, epithelial cells. Similar to the mode of transport on the brushborder membrane, net uptake across the basolateral membrane occurs through the action of Na^+-dependent unidirectional transporters working in cooperation with the exchangers.

The Heteromeric Neutral AA Transporters, System y^+L

The y^+L system mediates the Na^+-independent transport of cationic AA and the Na^+-dependent transport of neutral AA (Verrey et al., 2003) via a heterodimeric complex formed with the 4F2hC protein. In polarized epithelial cells, system y^+L is expressed on the basolateral membrane and is responsible for the efflux of dibasic AA in exchange for the influx of large neutral AA and a Na^+ (Wagner et al., 2001). Transport in the absence of Na^+ also occurs where intracellular dibasic AA are exchanged for extracellular dibasic AA.

y^+LAT1 Transport Properties. The y^+LAT1 (SLCA7) protein is expressed with 4F2hC to mediate the transport of neutral AA and cotransport with Na^+, with high affinity, and an exchange occurs for intracellular cationic AA, resulting in an efflux of cationic AA (Verrey et al., 2003). The y^+LAT1 is an obligatory exchanger, and transports dibasic AA Na^+-independently, and neutral AA Na^+-dependently (Wagner et al., 2001).

When Na^+ is not present, protons may be cotransported, which increase the affinity of neutral AA that are also transported in a 1:1 ratio. However, cotransport with protons does not occur under normal physiological conditions. The y^+LAT1 protein is expressed on the basolateral membrane of epithelial cells in kidney proximal tubule and small intestine, and follows a decrease in expression along the length of the kidney proximal tubule. The y^+LAT1 transporter is also expressed in leukocytes, lung, erythrocytes, and placenta (Wagner et al., 2001). The human y^+LAT1 protein consists of 511 AA, and a putative molecular mass of approximately 56 kDa. Defects of this protein cause the hereditary disorder lysinuric protein intolerance (LPI). Bauch et al. (2003) found that in the absence of exogenous 4F2hC in MDCK cells, very little y^+LAT1 appeared at the basolateral membrane. Coexpression with $b^{o,+}$AT and NBAT in the MDCK cells, led to an increase in basolateral radioactively labeled L-arginine and a decrease in intracellular arginine, indicating that y^+LAT1-4F2hC mediates the basolateral transport of arginine out of the cell. Mutations of y^+LAT1 result in lysinuric protein intolerance (LPI), in which patients experience poor absorption of dibasic AA, especially lysine, and large amounts are found in the urine (Palacin et al., 2005). Because of this, circulating plasma concentrations of basic AA are low, which has important implications for the urea cycle, in which arginine and ornithine are important intermediates.

y^+LAT2 Transport Properties. In contrast to the y^+LAT1 protein, y^+LAT2 (SLC7A6) expression is widespread and expressed in both nonepithelial and epithelial tissues (Verrey et al., 2003). It is expressed in brain, small intestine, testis, parotids, heart, kidney, lung, and liver (Wagner et al., 2001). Also functioning as a heterodimeric complex with 4F2hC, it exchanges neutral and cationic AA across the basolateral

membrane at a ratio of 1:1, and the transport of neutral AA is coupled to Na^+. The y^+LAT2 protein consists of 515 AA with an approximate 56 kDa molecular mass (Wagner et al., 2001). The influx of neutral and cationic AA is very efficient, but the efflux of cationic AA is more efficient than neutral AA. The uptake of leucine and glutamine is good, but these substrates are not easily released. The physiological role of this protein has not yet been clearly defined.

Summary. System y^+L mediates the Na^+-independent transport of cationic AA and the Na^+-dependent transport of neutral amino acids through a heterodimeric complex formed with the 4F2hC protein. Both characterized members of this family, y^+LAT1 and y^+LAT2 are expressed in the intestine, with localization on the basolateral membrane. This system works in collaboration with the $b^{o,+}$ and L system to drive net uptake of cationic amino acids in the intestine. Mutations of the y^+L system result in lysinuric protein intolerance, where large amounts of lysine are excreted in the urine.

The Heteromeric Neutral AA Transporters, System asc

System asc is a Na^+-independent transport system for small, neutral AA (Wagner et al., 2001). System asc functions as a heterodimer, with asc-1 associating with the 4F2hC protein, and asc-2 associating with an unknown heavy chain protein. The asc-1 protein is expressed in the small intestine. This system has not been well characterized and the physiological importance of the asc transporters is unknown.

asc-1 Transport Properties. The asc-1 transporter (SLC7A10) associates with the 4F2hC protein to mediate Na^+-independent transport of small neutral AA including L-glycine, L-alanine, L-serine, L-threonine, L-cystine, α-aminoisobutyric acid, and β-alanine. In addition, asc-1 also transports D-serine with high affinity, and similar to other

member of system asc, it functions as an obligatory exchanger. The asc-1 transporter is most similar structurally to the L-system transporter LAT-2 (66% identity), but unlike LAT-2, which transports all neutral AA, asc-1 only transports small neutral AA. The asc-1 protein consists of 523 AA (Wagner et al., 2001). The asc-1 mRNA is expressed in kidney, small intestine, placenta, lung, and brain. The physiological role of this protein has not been clearly defined, but it is thought to serve as a regulator of synaptic transmission, because it transports D-serine, an endogenous modulator of NMDA-type glutamate receptors.

asc-2 Transport Properties. Like other asc-type transporters, asc-2 exhibits Na^+-independent transport, but unlike asc-1, it does not associate with 4F2hC and is stereoselective and does not transport some of the high affinity substrates, including β-alanine (Verrey et al., 2003). The asc-2 is expressed in kidney, placenta, lung, spleen, and skeletal muscle. Western blots have demonstrated the presence of multiple bands under nonreducing conditions, and a single band of low molecular mass under reducing conditions, showing that it is indeed linked to an as of yet unidentified heavy chain protein by a disulfide bond.

Summary. Unlike the other nutrient transport systems described in this review, the asc AA transport system is not well characterized and the function of the transporters is unclear. The asc-1 protein is expressed in the small intestine, and associates with the 4F2hC protein to mediate the Na^+-independent obligatory exchange of neutral AA.

The Glutamate/Neutral AA Transporters, System X^-_{AG}

Apical absorption of anionic AA in the intestine and kidney occurs through Na^+-dependent transport by system X^-_{AG}. Members of this family of high-affinity glutamate

transporters have similar putative structures, but have varying transport mechanisms and physiological functions (Kanai and Hediger, 2004). These transporters mediate the high-affinity transport of L-glutamate, L-aspartate, and D-aspartate, along with cotransport of 3 Na^+ and 1 H^+, and countertransport of 1 K^+. This coupling method allows for the effective transport of glutamate uphill against a steep electrochemical potential gradient. This also plays a major role in preventing glutatmate toxicity in neurons, through maintenance of extracellular glutamate concentrations at low levels. Glutamate, as an abundant neurotransmitter, is very influential within the central nervous system, and overactivation of glutamate receptors can result in excitotoxicity and even cell death (Seal and Amara, 1999). Also, as a neurotoxin, system X^-_{AG} is necessary for clearance of excitatory AA. L-glutamate and K^+ extracellular concentrations increase during seizures and ischemia, and it appears that the efflux of L-glutamate results from a reversal in the carriers. Therefore, this represents a target for strategies to treat these patients by designing inhibitors of reverse transport. Transport is driven by free energy stored in the form of electrochemical potential transmembrane gradients of the coupling ions. The binding of glutamate to the glutamate transporters is rapid, and is responsible for the quick clearance of glutamate from the synaptic cleft (Kanai and Hediger, 2004). Also characteristic of the high-affinity glutamate transporters is a substrate-gated anion conductance (Kanai and Hediger, 2004). The EAAT family consists of five family members, EAAT1, EAAT2, EAAT3, EAAT4, and EAAT5, and the X^-_{AG} system also includes two related members of the ASC family, ASCT1 and ASCT2 (Seal and Amara, 1999).

System X^-_{AG} Putative Structures. Members of the high-affinity glutamate transport family contain eight predicted transmembrane domains, a large extracellular loop between transmembrane domains three and four, and a loop between domains seven and eight (Kanai and Hediger, 2004). The seventh domain contains two AA residues, Tyr 403 and Glu 404 that are critical for the coupling of K^+ that serves to relocate the transporter from the intracellular side of the membrane to the extracellular side of the membrane after transport.

EAAT3 Transport Properties. EAAT3 (SLC1A1, EAAC1) is a high-affinity glutamate transporter first isolated in 1992 (Kanai and Hediger, 2004) by expression cloning in *Xenopus oocytes*. It is expressed in the apical membrane of kidney proximal tubules, and in the neurons of the brain hippocampus, cerebral cortex, olfactory bulb, striatum, superior colliculus and thalamus (Kanai and Hediger, 2004). Mouse EAAT3 gene knockouts developed dicarboxylic aminoaciduria, an inborn error of glutamate/aspartate transport across epithelial cells of kidney and intestine, showing that EAAT3 plays a role in reabsorption of glutamate from the kidney proximal tubules (Peghini et al., 1997).

EAAT3 is also capable of minimal substrate exchange, in addition to glutamate uptake (Kanai and Hediger, 2004). EAAT3 expression was shown to be downregulated by PKC in *Xenopus* oocytes, and among the other high-affinity glutamate transporters EAAT3 appears to be the only one regulated by intracellular trafficking. One group examined ontogenetic regulation of EAAT3 expression in rats, and EAAT3 mRNA was detected in highest amounts towards the distal region of the small intestine, and this remained unchanged from postnatal d 4 through d 50 (Rome et al., 2002). EAAC1

mRNA increased from d 4 to d 21 in all segments of the small intestine. EAAT3 protein localized to the brushborder membrane of enterocytes in the crypts and at the base of the villi, a distribution quite different from other nutrient transporters, suggesting that EAAT3 may play a role in providing fuel to rapidly dividing cells in the crypts, rather than providing glutamate to the rest of the body. The protein expression was similar in all three segments of the small intestine at all ages. Erickson et al. (1996) found that EAAT3 exhibited an mRNA expression gradient in the small intestine, with expression levels being highest in the ileum. In addition, EAAT3 mRNA levels increased in response to the feeding of a high protein diet, very similar to PepT1. EAAT3 has also been thought to be able to bind to AA on both the cytoplasmic and extracellular surface to function as an AA sensor (Hyde et al., 2003). Howard et al. (2004) found a similar expression pattern in rats, with mRNA abundance being highest in the ileum. They also found that total parenteral nutrition (TPN) for 7 d upregulated mRNA expression of EAAT3 in the ileum, and infusion of glucagons-like peptide 2 (GLP-2) abolished this effect. EAAT3 is an apical transporter, and similar to ASCT2, NBAT, and PepT1 in this study, it was thought that upregulation of apical transporters in the distal intestine could maximize assimilation of AA, presumably from endogenous protein secretions (Howard et al., 2004).

EAAT1 Transport Properties. EAAT1 (SLC1A3, GLAST-1) is a glial type high affinity glutamate transporter expressed in astrocytes, cerebellar Bergmann glia, heart, skeletal muscle, and placenta (Kanai and Hediger, 2004). In situ hybridization revealed mRNA localization within the cerebellar Purkinje cell layer in association with the Bergmann glia (Seal and Amara, 1999). This transporter plays an important role in intracellular signaling by interacting with glutamate to regulate glial signal transduction

and morphology (Hyde et al., 2003). Upregulation of EAAT1 mRNA expression occurs following the provision of excess glutamate. Providing excess glutamate to cultured astrocytes extracellularly, increased the EAAT1 V_{max}.

EAAT2 Transport Properties. The high-affinity glutamate transporter EAAT2 (SLC1A2, GLT1) is expressed in the astrocytes of brain cerebral cortex and hippocampus (Kanai and Hediger, 2004). Knockout of the EAAT2 gene in mice resulted in seizures and neuronal degradation in the hippocampus, further emphasizing the role of EAAT2 in neuroprotection (Tanaka et al., 1997).

EAAT4 Transport Properties. EAAT4 (SLC21A6) is a high-affinity glutamate transporter expressed primarily in cerebellar Purkinje cells on postsynaptic dendritic spines (Seal and Amara, 1999; Kanai and Hediger, 2004). EAAT4 and EAAT5 were identified from a human cDNA library using sequences that were conserved among transporters within the family (Seal and Amara, 1999).

EAAT5 Transport Properties. EAAT5 is an excitatory AA transporter that is expressed in the retina, and by immunocytochemical studies was found to be associated with rod photoreceptors and bipolar cells (Pow and Barnett, 2000). The investigators concluded that in rat retina, EAAT5 functions as a photoreceptor and bipolar cell glutamate transporter. Transport by EAAT5 exhibits an associated chloride conductance. The expression of the protein was examined at a series of time points including day of birth, postnatal d 7, 14, and 21, and labeling was found to be developmentally regulated, with the weakest staining occurring around postnatal d 7, and strong labeling of photoreceptors and a heterogeneous population of bipolar cells at d 21.

Summary. System X^-_{AG} mediates the Na^+-dependent transport of anionic AA across the brushborder membrane of absorptive, epithelial cells in the intestine and kidney. These transporters mediate the transport of L-glutamate, L-aspartate, and D-aspartate along with cotransport of three Na^+, one H^+, and countertransport of one K^+. This coupling method allows for transport to occur against a steep electrochemical potential gradient. The EAAT3 protein is expressed in the small intestine and is the primary transporter responsible for anionic AA uptake across the brushborder membrane.

The ASC Family of AA Transporters

The ASC family of transporters is a ubiquitous system that mediates Na^+-dependent transport of small zwitterionic AA, excluding branched-chain AA (Pinilla-Tenas et al., 2003). ASC transporters exhibit a high-affinity for alanine, serine, cystine, and threonine, and function as exchangers, independent of K^+ and H^+ (Kanai and Hediger, 2004).

Na^+-Dependent Neutral AA Exchanger, ASCT1. The cDNA encoding the ASCT1 protein was first isolated from human hippocampal libraries in two labs (Arriza et al., 1993; Shafqat et al., 1993). Shafqat et al. (1993) named this protein SATT, but the protein is more commonly referred to as ASCT1. The ASCT1 cDNA encodes for a 529 AA protein, with 39 to 44% sequence identity to the excitatory AA family (EAAT). ASCT1 is a Na^+-dependent transporter that functions as an exchanger, presumably through the action of an antiport system, in which substrate AA are obligatorily associated with the Na^+ movement in both directions (Bussolati et al., 1992). ASCT1, therefore, does not mediate a significant net AA flux (Zerangue and Kavanaugh, 1996). An aspect of ASCT1 (SLC1A4, SATT) function, similar to the excitatory AA

transporters, is that it stimulates a substrate-gated flux of chloride that is not coupled to AA transport (Zerangue and Kavanaugh, 1996). In contrast to the glutamate transporters, however, ASCT1 does not result in the countertransport of K^+ and a net AA flux. Transport by ASCT1 is electroneutral, shown by the fact that ^{3}H-alanine injected into oocytes resulted in a large flux but no net current flows, despite the fact that Na^+ is cotransported into the cell and K^+ is not countertransported. This symmetry must result from the electroneutral exchange of extracellular and intracellular neutral AA. By this mechanism Na^+ is cotranspoted into the cell with a neutral AA in exchange for an extracellular AA.

The physiological implications of the electroneutral obligatory exchange mechanism of ASCT1 are that AA can accumulate in the cell, and because ASCT1 is an exchanger it can result in the uptake of a particular, maybe valuable substrate, when the transmembrane gradient for the intracellular AA is established (Zerangue and Kavanaugh, 1996). This could be important in erythrocytes for maintaining AA flow among organs. It has been suggested that Na^+, instead of acting as the driving force, instead acts as an allosteric modulator, which is bidirectionally coupled to transport (Pinilla et al., 2001).

ASCT1 Substrate Specificity. ASCT1 exhibits a high affinity for alanine, serine, cysteine, and threonine, and at a low pH (< 6), cystine and the acidic AA, glutamate and aspartate, inhibit alanine transport (Malandro and Kilburg, 1996; Pinilla et al., 2001). However, the specific affinities for extra- vs. intracellular substrates have not been reported. Pinilla-Tenas et al. (2003) demonstrated that ASCT1 transported proline and hydroxyproline to the inside of *Xenopus laevis* oocytes, and glutamate inhibited ASCT1-

mediated alanine uptake at high substrate concentrations, although it itself did not serve as a substrate. This indicated that perhaps there is a binding domain on the exchanger for glutamate that mediates transport. Pinilla-Tenas et al. (2003) also demonstrated that proline and hydroxyproline also serve as substrates for ASCT1, with ASCT1 exhibiting an affinity for proline three times lower than that for alanine, and half-V_{max} for proline than for alanine. They found that ASCT1-mediated uptake of proline was Na^+-dependent, saturatable, transported against a concentration gradient, and was inhibited by alanine, cysteine, threonine and hydroxyproline, all substrates for ASCT1. The K_t for proline was 704+/- 86 μ*M*, which was three times higher than alanine, and the K_t for hydroxyproline was 33.2 +/- 4.3 μ*M*, showing that hydroxylation of proline increases transporter affinity for this substrate.

Tissue and Cellular Distribution of ASCT1. ASCT1 mRNA was recently reported in rat small intestine by RT-PCR and exhibited an expression gradient that increased from the duodenum to the ileum by 20 to 80% (Howard et al., 2004). Rats were given total parenteral nutrition (TPN) for 7 d, and during this time ASCT1 mRNA was significantly upregulated in the proximal intestine (duodenum) while mRNA in the distal small intestine was unchanged (Howard et al., 2004). In a separate treatment, TPN rats were also coinfused with glucagon-like peptide 2 (GLP-2), which has been shown to prevent mucosal atrophy during TPN. In those rats, mRNA levels of ASCT1 decreased in both the proximal and distal small intestine, relative to the control animals, which were maintained on an orally fed commercial diet. ASCT1 expression in Caco-2 cells is localized to the basolateral membrane (Howard et al., 2000). Thus, it is thought that during luminal nutrient deprivation, basolateral transporters are upregulated to feed the

enterocyte from the circulation, and that after GLP-2 is administered, mucosal protein synthesis is maintained, reducing the need for AA absorption (Howard et al, 2004).

ASCT1 plays an important role in the transport of L-serine in brain cells. Through the use of in situ hybridization and immunohistochemistry, Sakai et al. (2003) found that at early developmental stages of the mouse brain, neuroepithelial cells in the ventricular zone expressed ASCT1 mRNA and protein. During late embryonic development and the neonatal stage, ASCT1 was downregulated in neuronal populations, and expression was high in radial glial cells and astrocytes. In adult brain astrocytes, ASCT1 was localized to the cell membrane. Expression of the protein and similar distribution of 3PGDH, a serine biosynthetic enzyme, along with high serine concentrations in glial cells, suggests a role of ASCT1 in releasing glial L-serine to adjacent cells, as well as in the transport of AA at the blood-brain barrier during fetal development. In rat brain from embryonic d 14 through postnatal d 18, ASCT1 was localized to the cerebral cortex, hippocampus, and cerebullum as determined by immmunocytochemical analysis (Weiss et al., 2001). ASCT1 appeared to be localized to brain regions that rely on glutamate as a neurotransmitter, and coincides with the expression of rat EAAT3. ASCT1 transporters on the neurons may function in transporting alanine, which can be converted to glutamate and, hence, may help regulate the glutamine-glutamate cycle. In addition, low extracellular pH serves to increase the affinity of ASCT1 for anionic AA, and in response to the localized acidification it may contribute to glutamate toxicity.

Na^+-Dependent Neutral AA Exchanger, ASCT2. ASCT2 is a Na^+-dependent transporter that functions as an exchanger, similar to ASCT1 (Bode, 2001). In addition to the above-mentioned substrates of the ASC transporters, ASCT2 (SLC1A5, AAAT, or

hATB^{o}) also recognizes and transports glutamine and asparagine with high-affinity, and glutamate, methionine, leucine, and glycine with low-affinity (Kanai and Hediger, 2004). It was thought for some time that ASCT2 and ATB^{o} were different genes with the same system activity, but results from further research led to the conclusion that they were simply isoforms of the same gene isolated from different species (Bode, 2001). The ASCT2 protein was found to be expressed in the brushborder membrane of epithelial cells in the intestine and kidney proximal tubules (Kanai and Hediger, 2004). The ASCT2 mRNA is also expressed in placenta, lung, pancreas, skeletal muscle, human colon carcinoma cells lines, and glia (Bode, 2001). The ASCT2 transporter was thought to account for the majority of glutamine uptake in human liver-derived fibroblasts, demonstrating that expression may not be limited to epithelial cells (Bode, 2001). It was thought that ASCT2 was responsible for system B^{o} activity at the brushborder membrane of enterocytes (Avissar et al., 2004), but recently the B^{o}AT1 gene was cloned and characterized from this system. However, ASCT2 mRNA and protein changes in response to 2-wk growth hormone treatment in 70% enterectomized rabbits, paralleled changes in system B^{o} activity, and exhibited a similar tissue and cellular distribution (lowest expression in the duodenum and highest expression in the colon; Avissar et al., 2004). ASCT2 protein and mRNA were decreased after massive enterectomy in rabbits by 50% in the small intestine and colon. Growth hormone treatment then increased ASCT2 mRNA abundance in the ileum, and increased system B^{o} activity. Therefore, ASCT2 may be a member of system B^{o}, in addition to the newly discovered B^{o}AT1, but this has not been confirmed experimentally.

Summary. The ASC system mediates the Na^+-dependent electroneutral exchange of small, neutral AA (i.e., alanine, serine, threonine, and cystine) across a membrane. The ASCT1 and ASCT2 transporters are expressed ubiquitously, and are both detected in the intestine. The ASCT1 transporter is localized to the basolateral membrane, and the ASCT2 transporter is localized to the brushborder membrane. The advantage of an electroneutral exchange system is that AA accumulate in the cell, and ASCT1 can transport a crucial substrate into the cell when the transmembrane gradient for the intracellular AA is established. It has been suggested that the coupled Na^+ acts as an allosteric modulator instead of as the driving force for transport.

The AA transport systems discussed in this review exhibit a broad range of substrate specificities and transport mechanisms. This diversity among transporters ensures maximal uptake of AA across a wide range of physiological conditions and cell types, and guarantees that essential AA are assimilated by the cell. It has been demonstrated that in the intestine, luminal amino acids are transported faster and more efficiently in the form of di- and tripeptides by the H^+-dependent low-affinity/high-capacity transporter, PepT1. The high-affinity/low-capacity amino acid transporters exhibit a narrower range of substrate specificity, and in the intestine, perhaps function to scavenge the remaining AA.

Monosaccharide Transport in the Small Intestine

Essentially all of carbohydrate digestion occurs in the intestine (Pond et al., 1995). Salivary amylase will act to a minimal extent on starch and dextrins in the diet to yield maltose, and carbohydrates leaving the stomach are acted upon by pancreatic amylase secreted into the lumen of the small intestine, which also acts on starch and

dextrins to yield maltose. The remainder of carbohydrate digestion is carried out by brushborder membrane hydrolases expressed in intestinal epithelial cells, which include but is not limited to, sucrase, lactase, maltase, and sucrase-isomaltase, to yield the absorbable end-products, D-glucose, D-galactose, and D-fructose. These three monosaccharides are absorbed by different routes. Glucose and galactose are transported across the brushborder membrane by the Na^+-dependent transporter SGLT1, while fructose is transported by the GLUT5 protein by facilitated diffusion (Bird et al., 1996). Currently, SGLT1 is the only identified protein responsible for glucose and galactose absorption (Wright and Turk, 2004). It is hypothesized that a second low-affinity/high-capacity glucose transporter exists on the brushborder membrane (Wright et al., 2003), and even GLUT2 has been implicated in the conditional transport of glucose via a translocation to the brushborder membrane (Mithieux, 2005), but these results have been inconclusive. Once inside the enterocyte, the three monosaccharides are transported across the basolateral membrane to the bloodstream by facilitated diffusion by the GLUT2 transporter (Bird et al., 1996). The GLUT2 transporter is the only identified protein responsible for the exit of monosaccharides from the cell. In GLUT2 null mice, however, there was no deficiency in glucose absorption (Wright et al., 2003). An alternate pathway for basolateral glucose transport was proposed that involved vesicle trafficking, but further studies are needed to elucidate this pathway (Wright et al., 2003). This review will concentrate on the glucose transporter, SGLT1.

The Na^+/Glucose Intestinal Transporter, SGLT1. SGLT1 (SLC5A1) was the first cotransporter protein identified in the SLC5 Na^+/glucose cotransport family (Wright and Turk, 2004). It is a high-affinity/low-capacity Na^+/glucose transporter expressed in

the intestine and kidney (Ferraris, 2001). The affinity values for D-glucose were 0.3 m*M* in rabbits, and 0.8 m*M* in humans (Steel and Hediger, 1998). SGLT1 is expressed predominantly in the apical membrane of small intestinal enterocytes, and is responsible for the transport of D-glucose and D-galactose from the lumen across the brushborder membrane (Wright et al., 1994). SGLT1 also functions as Na^+ uniporters, water channels, urea channels, and cotransporters of water and urea (Loo et al., 1996; Wright and Turk, 2004). Loo et al. (1996) demonstrated that approximately 260 molecules of water are coupled to each glucose molecule transported by SGLT1, accounting for approximately 5 L of water absorption per day in a human. Mutations in the SGLT1 gene have been linked to glucose-galactose malabsorption, a rare autosomal recessive disease (Wright and Turk, 2004).

SGLT1 Transport Mechanism. According to the proposed model for glucose transport, one glucose unit is cotransported across the brushborder membrane with 2 Na^+ by SGLT1 (Bird et al., 1996). The SGLT1 protein is negatively charged and exhibits a transport mechanism in which the ions bind first and pre-steady-state kinetics result from orientation of the empty protein at the membrane, and ion binding or dissociation (Steel and Hediger, 1998). After ion and substrate binding to the carrier protein at the luminal surface of the membrane, a conformational change occurs, in which the carrier rotates and is reoriented to the cytoplasmic side of the membrane (Wright et al., 2003). After rotation, glucose dissociates from the carrier, and then the 2 Na^+ enter the cytosol. The low affinity on the cytosolic sites of SGLT1 for glucose and Na^+, as well as the electrochemical gradient of Na^+, promotes the dissociation, after which SGLT1 is re-

oriented back to the luminal surface of the brushborder membrane. The transporter turnover rate is 1,000 x sec at 37°C (Wright et al., 2003).

SGLT1 Protein Structure. In 1989, Wright et al. reported the cloning and characterization of rabbit SGLT1. The rabbit SGLT1 gene encoded for a 662 AA protein (approximately 73 kDa), with 11 putative transmembrane domains, and a large hydrophilic loop between membrane domains 7 and 8, and between 10 and 11, and two potential N-linked glycosylation sites (Wright et al., 1989). More recent analysis by freeze-fracture electron microscopy suggests that the SGLT1 putative protein structure contains 14 transmembrane domains, with the amino and carboxyl termini facing the extracellular side of the membrane, and an extracellular hydrophilic loop between transmembrane domains 6 and 7 with one N-linked glycan (Wright et al., 2003; Wright and Turk, 2004).

Tissue and Cellular Distribution of SGLT1. On a cellular level, SGLT1 mRNA was limited to the villi, with no labeling in the crypts (Ramsanahie et al., 2004). Villi were labeled in the mid and upper thirds of the villi, with very little label in the tips (Shirazi-Beechey et al., 1994; Ramsanahie et al., 2004). Immunofluorescence staining revealed the presence of SGLT1 protein on the apical membranes in the jejunum and diffuse staining in the cytoplasm (Ramsanahie et al., 2004). SGLT1 is also expressed to a lesser extent in the plasma membranes in the trachea, kidney, and heart (Wright and Turk, 2004). Shirazi-Beechey et al. (1994) suggested the presence of a 58 kDa non-glycosylated SGLT1 precursor protein in enterocytes leaving the crypt. As enterocytes migrate out of the crypt, the expression of this protein declines, while the expression of the 75 kDa mature glycosylated SGLT1 appears and begins to increase. They suggested

the presence of a sugar receptor in the crypt cells that stimulate synthesis of the precursor protein in response to the presence of luminal sugars.

Regulation of SGLT1. SGLT1 is regulated by luminal sugar contents (Dyer et al., 1997b). In lambs, weaning coincides with development of the rumen, which ferments carbohydrates and limits their entry into the small intestine. The onset of weaning (5 wk) also parallels a significant decrease in active glucose transport and SGLT1 protein expression in lamb brushborder membrane vesicles. Lambs maintained on a milk-replacer at 5 wk maintained active transport of glucose and SGLT1 protein expression, demonstrating that luminal sugar contents regulate the expression of SGLT1 (Dyer et al., 1997b).

SGLT1 expression is also regulated by fructose in the diet (Goda, 2000). Rats fed a fructose-containing diet exhibited significant upregulation of SGLT1 and GLUT5 mRNA levels (Goda, 2000). SGLT1 expression and activity is also induced by 3-0-methyl α-D-glucopyranoside and methyl α-D-glucopyranoside, demonstrating that metabolizable substrates are not necessary for regulation (Shirazi-Beechey et al., 1994). Dyer et al. (1997a) found that SGLT1 activity and protein expression was decreased in dysfunctional small intestine exposed to a limited supply of nutrients. This downregulation of SGLT1 was independent of intestinal damage, because villin and lactase abundances were unchanged between the damaged and healthy tissues, demonstrating that SGLT1 is regulated by the presence of luminal nutrients. The dietary carbohydrate induced changes in SGLT1 expression occurred initially in the crypt cells, as demonstrated by phlorizin-binding in mouse intestine (Ferraris and Diamond, 1993). The upregulation of SGLT1 transporter numbers after a switch to a high-carbohydrate

diet was significant in the crypt cells at 12 h after the dietary switch, and only after 36 h did the villous transporter numbers exhibit a significant increase. They concluded that crypt cells are programmed by dietary carbohydrate levels, and that changes in the villi occur only after changes in the crypt cells, and migration of the crypt cells to the villous tips (Ferraris and Diamond, 1993).

Diamond et al. (1984) sought to determine the effect of diet history prior to starvation, and found that in fasted mice previously fed a carbohydrate-free diet, glucose uptake increased by 50 to 100%. In fasted mice previously fed a high-carbohydrate diet, glucose uptake decreased (Diamond et al., 1984). In postnatal rabbits exposed to a restricted protein-energy diet for 12 h, SGLT1 transport increased, with a twofold increase in V_{max}, as well as an increase in the lipid-to-protein ratio in the brushborder membrane and change in the lipid components, even though the static and dynamic fluidity of the membrane was unchanged (Butzner et al., 1990). Fasting or malnutrition reduces the exposure of luminal nutrients to the brushborder membrane, which causes mucosal atrophy, but increases glucose transport per milligram of intestine (Ferraris and Carey, 2000). The magnitude of the response is dependent on the age of the animal and the nature of the food deprivation (Ferraris and Carey, 2000). The mechanism underlying the response to changes in luminal contents is unknown (Ferraris and Carey, 2000).

Expression of monosaccharide transporters is regulated differently in humans with type II diabetes (Dyer et al., 2002). Na^+-dependent glucose transport in the brushborder membrane vesicles of patients with noninsulin-dependent diabetes mellitus was 3.3-fold higher compared to healthy patients. SGLT1, GLUT5, and GLUT2 mRNA levels increased threefold, and protein levels of SGLT1 and GLUT5 increased 4.3 and

4.1-fold, respectively. During diabetes there appears to be a mechanism to maximize the absorption of monosaccharides.

Diurnal SGLT1 expression is affected by glucagons-like peptide-2 (GLP-2) (Ramsanahie et al., 2004). Effects were examined at 3 h (ZT3) or 9 h (ZT9) after light onset, the interval where SGLT1 expression is induced. The SGLT1 mRNA increase between the two groups was eightfold. GLP-2 treatment did not alter diurnal increases in mRNA; however, SGLT1 protein increased in both the ZT3 and ZT9 group, with a threefold increase in the ZT9 group. A histological analysis revealed increased villous height, crypt depth, and proliferation in the GLP-2 treated rats. The villi from GLP-2 treated rats contained label for SGLT1 mRNA along the entire length, while villi from the control animals were labeled in the mid and upper thirds, with little labeling in the tips. Immunofluorescence revealed staining restricted to the brushborder membrane in GLP-2 treated rats, while in control rats more staining was observed in the cytoplasm. Similar results were obtained in a study examining the diurnal rhythmicity of SGLT1 mRNA expression and V_{max} in rats at four different circadian light periods (Tavakkolizadeh et al., 2001). Rat intestinal glucose uptake increased to maximal activity shortly before the onset of the dark period when rats begin to feed. This was evidenced by an increase in V_{max}, but no change in K_m, and an increase in mRNA expression levels of SGLT1 that peaked right before the onset of the dark period, in anticipation of the onset of feeding (Tavakkolizadeh et al., 2001). In situ hybridization revealed a more intense staining in enterocytes on the villi right before the dark period, suggesting increased transcription in all cells expressing SGLT1.

Rhoads et al. (1998) examined the underlying molecular mechanisms controlling SGLT1 expression in response to nutritional status, and also observed a distinct circadian rhythm in SGLT1 mRNA and transcription, even in the absence of dietary manipulation. They also discovered a hepatic nuclear factor-1 (HNF-1) element in the 5′ flanking region of rat SGLT1 that formed different complexes with nuclear extracts under a distinct circadian rhythm. The diurnal change in mRNA expression was paralleled by a change in the HNF-1 isoform complement at the HNF-1 5′ site. In the early light phase when transcription was high (1000 to 1100), nuclear extracts formed an AM complex with HNF-1α. In the late light phase when SGLT1 transcription was lower (1600 to 1630), the PM complex was formed with both HNF-1α and HNF-1β. It is thought that diurnal HNF-1 β binding is a mechanism allowing the animal to adapt to environmental signals (Rhoads et al., 1998). Rhoads et al. (1998) hypothesized that during the rest phase of nocturnal animals, SGLT1 transcription peaks and mRNA accumulates. Transcription rate then slows, but increased mRNA stability allows accumulation of the SGLT1 transporter. Protein translation and membrane trafficking lead to peak activity of SGLT1 late in the morning, preparing the animal for feeding. During feeding at night, mRNA destabilizes and abundance decreases, and SGLT1 activity decreases to baseline as a result of protein turnover and enterocytes sloughing off into the lumen (Rhoads et al., 1998). This diurnal rhythmicity of SGLT1 expression could be an adaptation to the feeding routine established by the photoperiod, and not so much a function of intrinsic clock signals (Rhoads et al., 1998).

Thyroid hormone (T_3) regulation of SGLT1 expression was examined in Caco-2 cells (Matosin-Matekalo et al., 1998). T_3 treatment of Caco-2 cells stimulated a tenfold

increase in glucose uptake, and an increase in SGLT1 mRNA levels. The V_{max} increased sixfold, but the protein abundance remained unchanged. Only cells exposed to glucose, but not a glucose analogue, could respond to T_3 treatment, which is different than in vivo, where glucose analogues are capable of inducing expression of SGLT1 in the crypt cells.

Epidermal growth factor (EGF) plays a regulatory role in proliferation and differentiation of epidermal tissue (Bird et al., 1996). In the gut, EGF exerts effects on the mucosal growth and epithelial cell proliferation, and has also been implicated in stimulating nutrient uptake. EGF exhibited a dose-dependent stimulation on glucose uptake in the jejunum of mice, with a 30% increase in active glucose transport in three age groups (Bird et al., 1994). Transforming growth factor alpha (TGFα) is a homologue of EGF that binds to a common receptor and functions similarly to EGF (Bird et al., 1996). However, in contrast to EGF, TGFα does not appear to induce a stimulatory effect on glucose transport (Bird et al., 1996). The differences in the abilities of the two hormones to stimulate glucose uptake could be related to events that occur after ligand-receptor binding (Bird et al., 1996).

Active transport of glucose is also stimulated by treatment with peptide tyrosine tyrosine (PYY), a straight-chained, 36-amino-acid brain-gut peptide. Peptide YY plays an important role in inhibiting pancreatic exocrine secretions, gastric acid secretion, and in decreasing intestinal motility post-prandially (Bird et al., 1996). Mice treated with PYY exhibited increased jejunal glucose transport, which occurred as a result of an upregulation in the number of SGLT1 proteins on the apical membrane (Bird et al., 1996).

Summary. To date, SGLT1 is the only transporter identified on the brushborder membrane of enterocytes responsible for the uptake of D-glucose and D-galactose. The SGLT1 protein mediates the Na^+-dependent uptake of glucose and galactose, and GLUT5 transports fructose across the brushborder membrane by facilitated diffusion. The SGLT1 transporter also functions as a Na^+ uniporter, water channel, and urea channel. Mutations of this gene result in glucose-galactose malabsorption, a rare autosomal recessive disease. Glucose is a non-essential nutrient, as demonstrated by the fact that patients with this disorder are still able to thrive. Similar to the peptide and amino acid transporters, SGLT1 is regulated by its own substrates, as well as fasting or malnutrition, diurnal phase, hormones, and disease.

Summary

In summary, a broad range of enzymes and nutrient transporters are expressed in epithelial and endothelial cells throughout the body, exhibiting varying structure and function to accommodate the nutritional needs of the intestine and other organs. Much research has been directed at understanding the dietary, diurnal, hormonal, developmental, and disease regulation of peptide, AA, and monosaccharide transporters in the intestine. This knowledge is of great interest pharmaceutically, therapeutically, and economically. Improved bioavailability of drugs, improved feeding solutions for patients with damaged intestine, and enhanced diet formulation for livestock are a few of the potential applications of the knowledge gained from these studies.

In recent years, it has become increasingly accepted that peptide transport accounts for the majority of AA assimilation in the intestine because of a relatively rapid rate of transport and greater energetic efficiency compared to free AA transport.

However, a lack of quantitative information on expression of PepT1 relative to free AA transporters in the intestine has left a cloud of doubt surrounding this assertion.

Transporter genes have been studied extensively in common laboratory species and domestic animals, but few studies have been directed at understanding nutrient assimilation in wild species. Understanding the physiological processes associated with nutrient absorption in wild animals potentially increases our understanding of the survival strategies of these animals, and hence, our ability to manage for the animal based on nutritional needs. This has huge potential in a zoo-type atmosphere where the primary goal is to improve reproduction, a parameter greatly impacted by the nutritional status of the animal. This review will conclude with a discussion of black bear nutrition, gastrointestinal physiology, and the metabolic changes that occur during hibernation.

Black Bear Nutrition

Black bears are omnivorous, and tend to be opportunistic feeders, in that they will eat what is readily abundant or available in the wild. Thus, their diet tends to change seasonally with natural variations in food abundance (Eagle and Pelton, 1983; Graber and White, 1983; Hellgren et al., 1989). Age, sex, and reproductive status may also cause shifts to the diet (Bacon and Burghardt, 1983). Eagle and Pelton (1983) found that, in general, plant material comprised the majority (80%) of bear scat in the Great Smoky Mountain National Park, with animal matter (12%) making up most of the remainder. The remainder was considered debris (i.e., twigs, leaves, and rock chips). The diet in spring was composed mainly of herbaceous material (i.e. squawroot, serviceberry, huckleberry, and blueberry) high in protein, while the remainder was made up of carrion and insects, with the animal matter content increasing from early to late spring (Eagle and

Pelton, 1983). In southeastern North Carolina, plant material also represented the majority of the diet, with corn as a major diet component during each active month (Landers et al., 1979). In spring, the bears fed primarily on young vegetative material, and during the summer shifted to fruits (i.e. huckleberries, blueberries and blackberries; Landers et al., 1979). In North Carolina, white-tailed deer represented the most common vertebrate food (Landers et al., 1979). The peak in protein intake during spring paralleled the replacement of lean muscle mass lost during hibernation and lactation. In the summer, soft mast (i.e., blackberry, huckleberry, and blueberry) comprised approximately 60% of the diet, representing a period of maintenance for the animals (Eagle and Pelton, 1983). In the fall, the bears switched to a high-fat, high-carbohydrate diet characterized by acorns, other nuts, and herbaceous matter, to aid in accumulating fat stores necessary for hibernation (Landers et al., 1979; Eagle and Pelton, 1983).

Black Bear Gastrointestinal Physiology

The gastrointestinal tract of the black bear is surprisingly simplistic, and has some unusual features (See Figure 2.1).The length of the intestinal tract is relatively short (< 10 m) and exhibits no differentiating morphological characteristics throughout the length of the tract, as are observed in other species. Upon opening the intestinal tract longitudinally, there is usually a marked difference in the visual appearance and texture of the tissue. In the colon of other species such as the pig or chicken, where substantial fermentation occurs, there is usually a distinct odor and change in the consistency of the digesta. The bear lacks a cecum, an essential feature of other species in which vegetation comprises a large portion of the diet. Jones and Zollman (1997) hypothesized that the lack of a cecum is an adaptation to prevent bacterial overgrowth during hibernation, as

evidenced by a lack of bacterial metabolites in the plasma, but other hibernating mammals, such as the alpine marmot and ground squirrel, do have a cecum and do not exhibit bacterial translocation or overgrowth during hibernation (Barnes, 1970; Hume et al., 2002). The bear's body temperature during hibernation remains close to the values observed for active animals, while most hibernators, such as the ground squirrel, drop their temperatures close to the ambient level during hibernation, so perhaps bacterial overgrowth is more of a concern in the bear because of the elevated body temperature during hibernation. Bunnell and Hamilton (1983) hypothesized that black bears are able to rapidly gain weight during the hyperphagic period as a function of a short, unspecialized gut that allows rapid digestion, but inefficient processing of low-quality food items, as a tradeoff to enhance survival.

Figure 2.1 Black bear gastrointestinal tract.

Metabolic Changes during Hibernation

Natural variations in food abundance necessitate a physiological mechanism to facilitate survival during periods of decreased food availability. Bears hibernate during the winter when ambient temperatures drop and when there is not enough food to meet their metabolic demands. It has been suggested though, that the cues to hibernate are driven by molecular genetics and not so much by food availability or environmental conditions. This is evidenced by dramatic physiological changes occurring in hibernating mammals without environmental cues (Carey et al., 2003), and by the inability of bears to hibernate in the summer when housed in the cold and dark (Nelson, 1980). Also, in two different populations of ground squirrels housed in the same room, different annual hibernation and reproduction cycles were observed in different individuals, and even after 3 yr synchronization did not occur (Blake, 1972).

In late summer and early fall, bears undergo a period of hyperphagia, in which their food intake increases dramatically (from 5,000 to 8000 kcal/d during spring and summer to 15,000 to 20,000 kcal/d during hyperphagia), and they consume a lot of high-fat food items, such as acorns, that aid in accumulating the fat stores necessary to hibernate (Nelsen et al., 1983). Brody and Pelton (1988) suggested that during hyperphagia, intake of both fat and carbohydrate increase dramatically, while protein assimilation decreases, and that this is under hormonal influence. Nelson (1973) estimated that black bears gain 1 kg BW/d and experience a total BWG of up to 40%. In late fall, black bears undergo a period of hibernation in which their body temperature and metabolic rates drop, and the bear does not eat, drink, urinate or defecate during a 5 to 6

mo period (Nelson et al., 1983). It has been reported that black bear denning activity is related to geographic location and climate (Smith, 1986). In places with mild winters such as Arkansas, bears tracked with radio-collars were prone to periodic arousals during hibernation (Smith, 1986). Bridges et al. (2004) used remote cameras at bear den sites in Virginia to investigate denning behavior and observed extra-den activity during hibernation, including a bear descending from a tree, a bear eating snow, and a sow carrying a cub from the den. Based on those two studies, it appears that bears do exhibit infrequent periods of arousal during the denning period.

In contrast to other mammals that hibernate at body temperatures closer to the ambient temperature (close to 0°C), the body temperature of the black bear remains close to normal (a drop from 38 to 34°C) throughout hibernation (Carey et al., 2003). It is because of this high core body temperature that bears are not considered to be "true" hibernators (Nelson, 1973). Harlow et al. (2004) found that hibernating bears exhibited the same spikes in body temperature four times a day, as students exercising in the cold, suggesting that bears exhibit muscle activity during hibernation, accounting for the retention of muscle strength, without arousal from sleep and elevation of core body temperature.

During the hibernation period, parturition and lactation occurs, which involves a tremendous expenditure of metabolic energy. Based on body fat utilization calculations, bears expend approximately 4,000 kcal/d during hibernation (Nelson et al., 1983) and lose approximately 300 g BW/d (Nelson, 1973), amounting to approximately 25 to 30% total BW lost during the denning period (Nelson et al., 1973). In hibernating bears, metabolism shifts from carbohydrates to lipid metabolism (Nelson et al., 1983). Lean

muscle mass is retained, and bears subsist off of fat stores. Lipid reserves, which provide water and energy, account for the approximately 15 to 25% BW loss during hibernation (Nelson et al., 1975). Two indicators for this include a low blood ratio of urea: creatinine, and elevated serum free fatty acid levels (Nelson, 1980).

During hibernation, nitrogen metabolism is tightly controlled – AA are diverted to protein synthesis rather than degradation, and urea is cycled to the gut where it is taken up and metabolized by the intestinal microflora (Nelson et al., 1975; Ahlquist et al., 1976). Approximately 100 mL of urine is filtered daily by the kidneys during hibernation as compared to 1 to 2 L during the active state (Nelson et al., 1973; Nelson, 1980). The urea taken up by the microflora is hydrolyzed to NH_3, and according to Masone (1984), the NH_3 is taken to the liver where the N and glycerol combine to synthesize AA. It is unlikely that NH_3 is absorbed in the intestine and taken to the liver; it is more likely that microbial protein synthesis occurs in the gut lumen, after which degradation occurs and subsequent uptake of the constituent amino acids (Pond et al., 1995). The availability of these amino acids to the animal depends on the site of ureolytic activity in the gut in relation to the site of maximal absorptive activity. A minimal amount of AA enter the urea cycle during this time – instead glycerol released from lipid metabolism, and N released from AA catabolism are used to synthesize alanine, which is then used to synthesize glucose (Ahlquist et al., 1976; Nelson et al., 1983).

It has been suggested that the use of lipids as the sole source for metabolism does not result in the formation of ketones, as evidenced by analysis of blood and analysis of the first urine after hibernation (Ahlquist et al., 1976; Nelson et al., 1979; Nelson, 1980;). The bear exhibits a respiratory quotient (RQ) of approximately 0.6 during hibernation,

indicative of lipid metabolism and carbon recycling (Nelson et al., 1973; Castellini and Rea, 1992). It was theorized that ketosis is prevented by glycerol metabolism, and in the winter, triglyceride turnover inhibits fatty acid entry into the ketone synthetic pathway (Nelson et al. 1983).

Although researchers cited in this review have concluded that lipid metabolism constitutes the sole source of energy during hibernation in bears, it is highly unlikely that the animal subsists off of fat alone. Fat as the sole source of energy is insufficient to replenish the oxaloacetate needed to combine with acetyl CoA to form citrate during the Krebs cycle (Berg et al., 2002). Mammals are unable to convert acetyl CoA into oxaloacetate, and although oxaloacetate is formed as an intermediate during the Krebs cycle, it must be replenished by the carboxylation of pyruvate to maintain normal operation of the cycle. A modest amount of glucose is needed to accommodate this reaction, and is most likely generated by gluconeogenesis of amino acids.

Hisa et al. (1998) quantified seasonal changes in plasma nitrogenous compounds in the European brown bear and hypothesized that during hibernation, bears obtained essential AA through degradation of collagen in the connective tissues and through degradation of muscle, as evidenced by the increase in the cross-linked carboxyterminal telopeptide of type I collagen and 3-methylhistidine in the plasma of captive denning bears. This suggests that bears are unable to subsist off of fat stores alone, and that there is indeed some loss of lean muscle mass. Concentrations of phenylalanine, lysine, and methionine, all essential AA, were increased in the plasma during hibernation of European brown bears, and it is doubtful that the bear can synthesize these AA *de novo*, since glycerol cannot be used as a carbon skeleton for essential AA synthesis (Hissa et

al., 1998). Lundberg et al. (1976) found a three- to fivefold increase in labeled albumin turnover during hibernation, constant levels of serum albumin, and more labeled leucine was incorporated into plasma proteins during hibernation as compared to the active state, suggesting induction of a protein anabolic pathway to outcompete other pathways for use of AA. It was hypothesized that increased protein synthesis could be a mechanism to prevent essential AA from being catabolized to carbon dioxide, water, and urea (Lundberg et al., 1976). This may also meet the demands for lipolytic and gluconeogenic enzymes needed during hibernation, and protein turnover in turn results in thermogenesis, which may be a key factor in regulating body temperature during hibernation (Lundberg et al., 1976).

Intestinal Mucosal Structure and Function During Hibernation

Maintenance of intestinal function during hibernation has been investigated in 13-lined ground squirrels, a mammalian species considered to be a "true hibernator" because its body temperature drops very close to the ambient temperature during hibernation (Carey, 1990). Carey (1990) examined mucosal weight and protein content, villous height, and mucosal surface area in the jejunum of captive ground squirrels representing all seasons. All of these parameters were greatly reduced in hibernating animals, but interestingly, absorption of 3-OMG, a non-metabolizable glucose analogue, and net Na^+ flux was greater in hibernating squirrel jejunum bathed in 37°C, when expressed per unit of mucosal surface area. This demonstrates that intestinal function is preserved, perhaps as a compensatory response to degradation of the mucosa (Cary, 1990). The short-circuit responses to L-alanine and D-glucose was greater in hibernators than active squirrels without normalization to surface area, and it was suggested that nutrient transporters are

expressed in greater numbers to elicit this response during hibernation (Carey, 1990). However, tissues bathed at 7°C, the body temperature of a hibernating ground squirrel, exhibited a decrease in transport of 3-OMG and Na^+. The response at a higher temperature may represent the physiological response to the infrequent arousals exhibited by ground squirrels during hibernation.

A follow-up study was conducted to investigate the kinetics of sugar and AA transport in the hibernating and active ground squirrels, and again, at normal body temperature, hibernators exhibited greater 3-OMG and total proline uptake/mg of intestinal weight, and this increase was associated with a higher V_{max}, with no change in the apparent affinity. Additionally, in the hibernators, there was a shift to a higher Na^+-dependent proline transporter, than in the active animals (Carey and Sills, 1992). It was hypothesized that the increase in V_{max} was the result of an increase in the number of enterocytes on the villi that express nutrient transporters (Carey and Sills, 1992). At 10 m*M* substrate concentrations, the ratio of 3-OMG to proline uptake decreased threefold in the hibernators, which could confer an advantage to the animal, in being able to scavenge any remaining AA in the lumen as well as AA from extruded cells. This is a minimal amount of substrate, but perhaps enough to contribute to the AA balance of the animal (Carey and Sills, 1992).

Studies were conducted to investigate the mechanism behind upregulation of nutrient transport during hibernation, especially under conditions of mucosal atrophy (Carey and Sills, 1996). While Carey and Martin (1996) observed reduced enterocyte proliferation and migration rates in hibernating squirrels, Carey and Sills (1996) found that microvilli heights in jejunal enterocytes were similar between hibernators and active

ground squirrels; however, the density of microvilli was greater in the hibernators, providing insight as to how intestinal function is maintained during hibernation. It was suggested that during hibernation, most genes will achieve steady-state levels of mRNA and protein abundance, but a few proteins crucial to survival will be upregulated, whereas less important genes and ones that inhibit hibernation will be downregulated (Carey et al., 2003). In support of this theory, it was observed that mRNA of Na^+/K^+ ATPase, and mRNA and protein abundance of SGLT1, sucrase-isomaltase, and amino-oligopeptidase (aminopeptidase N) was similar between active ground squirrels and hibernating squirrels (Carey and Martin, 1996). The V_{max} for glucose uptake was increased 1.5-fold in hibernators, while K_t remained unchanged (Carey and Sills, 1996).

Summary. The information reported here further demonstrates that nutrient transporters are regulated by luminal contents, and that the complete absence of nutrients in the lumen for an extended period of time appears to upregulate transporters, perhaps to serve as a mechanism to ready the intestinal epithelium for upcoming food ingestion. None of these hibernation studies address peptide transport, so it is unknown if PepT1 expression would be regulated the same way as glucose and AA transporters. Given the relative importance of peptide transport in the intestine, and previous studies of peptide transport in fasted animals, it is probable that PepT1 would be upregulated during hibernation to ensure maximal absorption of AA during spring emergence.

It is likely that the black bear would exhibit a different pattern of intestinal epithelial atrophy and regulation of gene expression during hibernation because of the maintenance of a core body temperature similar to the active state, in contrast to ground squirrels whose temperature drops to the ambient temperature during hibernation. The

results from studies reported here demonstrated that induction of transporter expression and epithelial cell proliferation increased when ground squirrel intestine was warmed to active state, representing infrequent bouts of arousal during hibernation. That the bear exhibits a warm body temperature during hibernation may be indicative of maintenance of intestinal integrity and function during the 6-mo hibernation period. Efforts to understand nutrient transporter expression and seasonal regulation in the intestine of black bears could improve our ability to formulate management decisions based on natural food abundances and spring emergence conditions after hibernation.

Chapter III

DISTRIBUTION AND RELATIVE ABUNDANCE OF NUTRIENT TRANSPORTER mRNA IN THE GASTROINTESTINAL TRACT OF BLACK BEARS

ABSTRACT

Black bears are omnivorous, and tend to be opportunistic feeders, in that they will eat what is readily abundant or available. The end-products of intestinal digestion are absorbed by the body through the action of transporter proteins expressed on the brushborder membrane of small intestinal epithelial cells. The goal of this study was to increase the understanding of the physiological processes associated with nutrient assimilation by black bears. Distribution and relative abundance of mRNA of a peptide transporter (PepT1), a glucose transporter (SGLT1), two AA transporters (NBAT, $b^{o,+}AT$), and a digestive enzyme, aminopeptidase N (APN) in the intestinal tract of black bears were investigated. Ten bears were used for this study. For tissue collection, the intestine was removed from the animal and divided into five sections. Each collected section was opened longitudinally, rinsed in ice-cold PBS, and the mucosal scrapings were stored at -80°C. Total RNA was extracted and quantified spectrophotometrically. Abundance of PepT1, SGLT1, NBAT, $b^{o,+}AT$, and APN mRNA was determined by performing Northern blots, using bear cDNA probes. Northern blot data were quantified by densitometric analysis, with the abundance of each gene expressed relative to GAPDH. Abundance of PepT1 ($P < 0.05$), APN ($P < 0.05$), and SGLT1 ($P < 0.0001$)

changed quadratically from the proximal to the distal intestine with abundance being greatest in the midregion. Abundance of $b^{o,+}$AT mRNA increased linearly ($P < 0.05$) from the proximal to distal intestine. Abundance of NBAT mRNA did not change among intestinal segments. The absolute number of molecules of mRNA/ng of total RNA for each gene was determined using Real-Time PCR. Similar to the Northern blot results, abundance of PepT1 ($P < 0.0003$), SGLT1 ($P < 0.0003$), and APN ($P < 0.02$) changed quadratically from the proximal to distal intestine with abundance being greatest in the mid-region, and $b^{o,+}$AT mRNA increased linearly ($P < 0.0001$) from the proximal to distal intestine. NBAT mRNA abundance also increased linearly ($P < 0.0001$) from proximal to distal intestine. PepT1 mRNA was present at tenfold or greater levels than AA transporter mRNA in all segments of the intestine, suggesting that di- and tripeptides constitute the major form in which AA are absorbed. NBAT and $b^{o,+}$AT mRNA abundance was greater towards the distal portion of the intestine, suggesting their importance in salvaging remaining unabsorbed AA. These results indicate that the mRNA of nutrient transporters examined and APN are differentially expressed throughout the gastrointestinal tract of black bears, suggesting their involvement in nutrient assimilation.

Key Words: Nutrient Transporter, Black Bear, PepT1, Gastrointestinal Tract, Northern Blot, Real-Time PCR.

Introduction

Black bears are omnivorous, and tend to be opportunistic feeders, in that they will eat what is readily abundant or available. Their diets tend to change seasonally in relation to changes in food abundance. When natural foodstuffs are in abundant supply, these usually constitute what is consumed by bears to meet their nutritional needs. In addition to natural foodstuffs, supplemental foodstuffs may play an important role as nutrient sources for the black bear (Gray, 2001). Included among supplemental foodstuffs would be corn from cornfields, fruit from fruit trees, vegetables and berries from gardens, garbage, wild feed, etc. Humans often supplement foodstuffs by creating feeding sites where corn grain or bakery wastes are made available. Understanding how a black bear responds physiologically to the demands imposed by natural variations in food supply and by the consumption of supplemental foodstuffs may provide insight for the management of bear populations.

The end-products of intestinal digestion are absorbed by the body through the action of transporter proteins expressed on the brushborder membrane of small intestinal epithelial cells. Many factors can influence the process of nutrient absorption by an animal including age, health, diet, reproductive status, presence of anti-nutrient factors, and so on. The goal of this study was to increase the understanding of the physiological processes associated with nutrient assimilation by black bears.

With some regularity, it becomes necessary for wildlife personnel to euthanize problematic bears that are classified as a “nuisance”. Bears that are classified as a “nuisance” are either captured and transported to a remote area for release, or upon

repeated capture are euthanized. Bears used in this study were nuisance bears captured and transported from locations within Virginia or West Virginia to the Virginia Tech Center for Ursid Research.

Materials and Methods

Animals and Intestinal Tissue Sampling. The following procedure was reviewed and approved by the Virginia Tech Animal Care Committee. Ten black bears were used for this experiment. Bears were nuisance bears trapped by either the Virginia Department of Game and Inland Fisheries or the West Virginia Department of Natural Resources. One bear was captured in late summer (August to September, 2003), and nine bears were captured during late spring (May of 2004). Following capture, the bears were transported to the Virginia Tech Center for Ursid Research and held without food, but access to water, for no more than 24 h. Bears were first anesthetized by use of a dart gun and a dart containing Ketamine/Rompun. The Ketamine/Rompun was mixed at a 2:1 ratio and at a concentration of 300 mg/mL, and dosed at 1 mL/44 kg BW. Following induction of anesthesia, which was defined as the point at which the bear did not respond to visual or sensory stimuli, a lethal dose of sodium pentabarbital or saturated potassium chloride (KCl) was administered via the femoral vein of the left rear leg. The pentobarbital was mixed at a concentration of 390 mg/mL and dosed at 86 mg/kg. The saturated KCl was mixed at a concentration of 0.35 g/mL and dosed at 54 mg/kg. When the animal was pronounced dead, the abdominal cavity was quickly opened using a knife, and the entire gastrointestinal tract was removed and placed on a metal tray that was maintained on ice. A piece of the left lateral liver lobe, a piece of stomach, and a lobe from the left kidney was also collected. The intestinal tract was tied off at the rectum with a rubber band, and

was spread out on a metal tray supported by an ice tray. The first and last meters of the intestine were removed and labeled sections 1 and 5, respectively, and the middle section of the intestine was further divided into three sections, labeled 2, 3, and 4, from proximal to distal. Each section was opened longitudinally with a pair of scissors and rinsed in a series of three phosphate-buffered saline (PBS; NaH_2PO_4: 1.47 m*M*, Na_2HPO_4: 8.09 m*M*, and NaCl: 145 m*M*) washes to rinse out the digesta. Autoclaved glass slides were then used to scrape the mucosa from the inner surface of the intestinal sections. Three 0.25 g, two 1.0 g, and one 5.0 g aliquots were then weighed out onto aluminum foil pieces with forceps, folded up, and dropped into a tank of liquid N. The sections of liver, kidney, and stomach were also wrapped in aluminum foil and placed in liquid N. The tissue pieces and intestinal samples were later transferred to a -80°C freezer for storage until analysis.

Materials. All reagents and supplies were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise stated. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Sephadex quick-spin columns were purchased from Roche (Indianapolis, IN). The nick translation labeling kit was purchased from Invitrogen (Carlsbad, CA). The ^{32}P was purchased from Perkin-Elmer Life Sciences (Boston, MA). All chemicals were of a molecular biology grade. All protocols were carried out following the manufacturer's protocols.

Total RNA Isolation. Total RNA was isolated from each sample using the TRI-REAGENT manufacturer's protocol (Molecular Research Center, Inc., Cincinatti, OH). Briefly, the 0.25 g aliquots of tissue were transferred directly from the freezer to 12 mL falcon polypropylene tubes containing 3 mL of cold TRI-REAGENT. The samples were homogenized using a Janke and Kunkel Ultra-Turrax T25 homogenizer at a speed of

9500 min^{-1}. Samples were homogenized for 30 sec while held on ice, and incubated on ice for 10 sec, with this protocol repeated, until no particles remained. The homogenates were held on ice for at least 5 min before being transferred to two 2-mL microcentrifuge tubes (1.5 mL to each tube) containing 300 µL (20% of original homogenate volume) of chloroform. The samples were vortexed for 15 sec and held for 10 min at room temperature. The samples were then centrifuged at 12,000 x g for 15 min at 4°C. All centrifugation steps were carried out in a Micromax Therma IEC microcentrifuge (Thermo Electron Corporation, Waltham, MA). After centrifugation, the upper clear phase was removed (approximately 60% of original homogenate volume), and transferred to a new 2 mL microcentrifuge tube containing 750 µL of isopropanol (50% of original homogenate volume). The samples were vortexed for 5 sec and held at room temperature for 5 min before centrifugation at 12,000 x g for 8 min at 4°C. After centrifugation, the supernatent was poured off and 1.5 mL of 100% ethanol (100% of original homogenate) were added, and pipeted up and down several times to wash the RNA pellet. The samples were immediately centrifuged at 7,500 x g for 5 min at 4°C. After centrifugation, the supernatant was poured off, and the tubes were inverted on a KimWipe® to airdry for 5 min. The pellets were resuspended in 70 µL of diethyl pyrocarbonate (DEPC) - treated water, the two tubes were combined, and the total RNA was quantified spectrophotometrically (Hitachi Instrument Inc, Japan, Model U-2000) at 260/280 nm. The 260:280 ratio for each sample was between 1.7 and 1.8. Isolated RNA samples were then stored at -80°C. Integrity of RNA was tested by running 5 µg of total RNA on a 1% denaturing agarose gel.

Construction of Bear cDNA Probes. Bear-specific cDNA probes were designed using pig-specific primers (see Table 3.2) and total RNA from bear intestinal samples, to perform reverse transcriptase polymerase chain reaction (RT-PCR).Two-step RT-PCR was performed using Promega reagents (Madison, WI) in a PTC-200 Peltier DNA Engine (MJ Research, Reno, NV), following the manufacturer's protocols. Total RNA from each bear sample was used in a reverse transcription reaction (IMProm – II Reverse Transcriptase, Promega) to generate cDNA. Pig-specific primers for a peptide transporter (PepT1), a sugar transporter (SGLT1), three AA transporters (NBAT, ASCT1, and $b^{o,+}AT$), a digestive enzyme (aminopeptidase N), and a housekeeping gene (GAPDH) were used in a polymerase chain reaction (PCR) to generate bear specific PCR products. Pig-specific primers for EAAT3, LAT1, ASCT2, GLUT2, GLUT5, SGLT1 and Aminopeptidase A (APA) were also used for PCR, but failed to yield a product. Primers were redesigned for SGLT1 using Primer3 (Whitehead Institute for Biomedical Research) based on a region conserved among all mammalian species, and primers were synthesized by Sigma Genosys (Woodlands, TX), and PCR was then successful (See Table 3.2 for all successful primer pairs). The following PCR conditions were used:

1. 95°C for 5 min
2. 94°C for 1 min
3. 54°C for 1 min
4. 72°C for 1 min
5. Repeat steps 2 through 4, 35 times
6. 73°C for 10 min

PCR products were run on a 2% agarose gel, excised for purification using the Qiagen Quick-Gel Extraction Kit (Valencia, CA), and ligated into the pGEM®-T easy vector (Promega, Madison, WI). Vectors were then introduced into *Escherichia coli* competent cells using a BTX-Harvard Apparatus ECM Electro Cell Manipulation System (Holliston, MA), and cells were then plated out overnight with isopropyl thiogalactoside (IPTG) and X-GAL for color selection. Cells containing the vector with the PCR insert were selected by picking white clones, which were added to 2.5 mL LB medium containing 100 μg/mL ampicillin and subsequently placed in a New Brunswick Innova shaker (Edison, NJ) at 37°C overnight. The vector DNA was purified by using the Qiagen Qiaprep spin miniprep kit (Valencia, CA), and an EcoRI digestion was performed to confirm the presence of the insert. Purified plasmid samples were then sequenced at the Virginia Bioinformatics Institute at Virginia Tech, and the DNA sequences of the bear PCR products were determined. Sequences were blasted (NCBI Blast) to confirm the identity of the gene. The Qiagen High Speed Midi Kit (Valencia, CA) was then used to purify and obtain high yields of the plasmid samples following the manufacturer's protocol. The purified plasmids were then labeled by nick translation for use in the Northern hybridizations.

Northern Blot Analysis. Ten micrograms of total RNA from each sample were size-fractionated on a denaturing 1% agarose gel containing 2.2 mol/L of formaldehyde. RNA samples were stained with ethidium bromide, and an RNA ladder was used to determine mRNA size (Promega, 0.24 to 9.5kb). An upward-capillary membrane transfer occurred overnight in NaCl-sodium citrate (20X SSC; NaCl: 3 *M*, sodium citrate: 0.3 *M,* pH 7.0) using Immobilon – Ny^+ membrane (Millipore, Inc., Billerica, MA), the nylon

membrane was rinsed in 2X SSC (NaCl: 300 m*M*, sodium citrate: 30 m*M*, pH 7.0) solution for 15 min, air-dried for 2 h, and RNA was cross-linked to the membrane by UV light at 2 X 0.30 J/cm^2 in a BIOS Bioslink UV crosslinker (New Haven, CT).

The cross-linked membranes were pre-hybridized in a glass bottle in a HYBAID hybridization oven (Thermo Hybaid, Waltham, MA) at 42°C for 4 h in a 50 mL solution containing 50% deionized formamide, 5X Denhardts Solution (0.1% ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS), 5X SSPE (NaCl: 750 m*M*, NaH_2PO_4: 50 m*M*, EDTA: 5 m*M*, pH 7.4), 10 mg/mL baker's yeast tRNA, and DEPC-treated water.

During prehybridization the purified plasmid samples containing the bear-specific PCR products for each of the genes were labeled with (Alpha-^{32}P)dCTP by nick translation using DNA polymerase I/DNase I for 2 h at 15°C, after which 2 µL of a stop solution containing EDTA was added to the reaction. Labeled samples were then centrifuged in a sephadex G-50 quick spin column (Roche, Pleasanton, CA) to remove unincorporated nucleotides. Two microliters of the purified probe were added to 4 mL of scintillation cocktail and was tested on a Beckman LS 3801 scintillation counter (Fullerton, CA) for specific activity. Specific activities were greater or equal to 1 X 10^6 dpms per microliter of probe. Probes were denatured by boiling for 20 min before addition to the hybridization solution.

Hybridization solutions were identical to prehybridization solutions, but with the addition of the radioactively labeled probes at a concentration of 10 ng of plasmid DNA/mL of hybridization solution. For each membrane, a probe for GAPDH was added

in addition to the probe for the gene of interest to normalize results. Hybridization was performed for 18 h at 42°C in the HYBAID oven.

Following hybridization, membranes were washed twice in 5X SSPE (NaCl: 750 m*M*, NaH_2PO_4: 50 m*M*, EDTA: 5 m*M*, pH 7.4) and 0.5% SDS for 15 min at 42°C and twice in 1X SSPE (NaCl: 150 m*M*, NaH_2PO_4: 10 m*M*, EDTA 1 m*M*, pH 7.4) and 0.5% SDS for 15 min at 42°C. Membranes were then tested for radioactivity with a Model 3 survey meter (Ludlum Measurements, Sweetwater, TX). If readings were below 1 K, membranes were wrapped in saran wrap and exposed to Kodak phosphor imaging screens for 1 h in the dark. If a high level of radioactivity (greater than 1K) existed, membranes were washed at high stringency in 0.1X SSPE (NaCl: 15 m*M*, NaH_2PO_4: 1 m*M*, EDTA 0.1 m*M*, pH 7.4) and 1% SDS for 15 min at 65°C, and then developed for 1 h.

After exposure to the membranes, phosphor image screens were scanned with a Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA) with PDQUEST software, version 6.2.1 (Bio-Rad laboratories, Hercules, CA). For images with high background, membranes were washed at high stringency and redeveloped for 1 h. For images with indistinguishable bands, membranes were reexposed to phosphor-image film for 8 h and rescanned.

Densitometric Analysis. The images were analyzed using the volume tools of Quantity One Quantification Software (Bio-Rad Laboratories, Hercules, CA) to compute the density of the hybridization bands. The bands for GAPDH were used to correct for any differences in RNA loading amount, since GAPDH is expressed constitutively among cells. Densities were normalized against background, and mRNA abundance was expressed as a ratio of the normalized gene of interest to normalized GAPDH.

Real-Time PCR. The next objective of the study was to determine the number of molecules of mRNA present for each gene of interest/ng of total RNA starting template, using Real-Time PCR. An absolute standard curve for each gene was generated based on modification of the protocol of Fronhoffs et al. (2002).

Primer Design. Primers were designed for each gene within bear PCR sequences. Primers were designed using the Primer Express software (NIH, Bethesda, MD), optimized for use with Applied Biosystems Real-Time PCR Systems (Foster City, CA). See table 3.3 for list of primer pairs.

Linearization of Plasmid Template. Plasmids containing cloned bear cDNAs were linearized opposite a T7 or SP6 promoter depending on the orientation of the insert sequence, using a restriction digest. The Pst1 restriction enzyme was used to linearize opposite to the T7 promoter and SacII was used to linearize opposite to the SP6 promoter. Restriction digests were carried out at 37°C for 4 h in 100 µL reactions, and were terminated by heat inactivation. Digestion was confirmed by running 4 µL of a 100 µL reaction on a 2% agarose gel. Digested DNA were purified using the Roche PCR purification kit (Pleasanton, CA), and DNA was eluted with 30 µL of elution buffer. The concentration of eluted DNA was quantified spectrophotometrically at 260/280 nm.

In Vitro Transcription of Bear cDNA Templates. In vitro transcription was performed on linearized plasmids using the Ambion Megascript T7 or SP6 in vitro transcription kit (Austin, TX). Reactions were performed following the manufacturer's protocol, and were carried out at 37°C for 16 h. DNase I was added to the reaction, and cRNA was incubated for 15 min at 37°C. The cRNA was precipitated by adding 30 µL lithium chloride and 15 µL of water to the reaction, and holding for 2 h at -20°C. Samples

were then centrifuged at 4°C for 10 min at maximum speed (21,000 x g), the supernatant was poured off, and 1 mL of 70% ethanol was added, and samples were centrifuged once more to precipitate the RNA. The cRNA pellets were solubilized in 20 µL of water, and quantified using a ribogreen assay.

Ribogreen Assay. A Ribosomal RNA standard curve of 1, 0.8, 0.6, 0.4, and 0.2 µg/mL was generated in the presence of TE buffer, and to which 100 µL of 200-fold diluted Ribogreen Reagent was added. Standards were generated in duplicate. All cRNA samples were diluted 200-fold in water, and then further diluted 100-fold or 50-fold in the presence of TE buffer, after which 100 µL of 200-fold diluted Ribogreen Reagent was added. Standards and unknowns were 200 µL. Samples were added to a CoStar 3695 96-well black plate and placed in the FLUOstar OPTIMA fluorescence multiplate microplate reader (BMG LABTECH, Germany). Samples were incubated in the dark at room temperature for 5 min before initiating the Ribogreen High Range Assay. The 100-fold and 50-fold cRNA dilution readings were averaged to obtain a final RNA concentration. Concentration values agreed with those obtained spectrophotometrically.

Creation of Absolute Standard Curve. The number of molecules per µL (N) was calculated using the following equation, using a molecular mass constant derived from Avogadro's constant:

$$N = \frac{\text{Concentration of cRNA } (\mu g/\mu L) \quad x \quad (183.5 \times 10^{13})}{\text{PCR fragment size (bp)}}$$

A dilution series of 10^{11} to 10^{4} molecules per microliter was performed in the presence of yeast tRNA at 10 µg/mL.

Reverse Transcription. The dilution series for each standard curve was reverse-transcribed in parallel with bear intestinal total RNA samples using the Applied

Biosystems High Capacity cDNA Archive Kit (Foster City, CA). Each reverse transcription reaction contained 2,000 ng of RNA at a concentration of 100 ng/μL, and an equal volume of each standard curve dilution cRNA (10 μL) was added to its respective reaction. Following reverse transcription, cDNA were was diluted 1:30 with DEPC-treated water, and the 1:30 dilutions were used for real-time PCR reactions.

Real-Time PCR. Real-Time PCR was performed on an Applied Biosystems 7300 machine (Foster City, CA) using the absolute quantification method. Plates, plate-covers, and tubes were all purchased from Applied Biosystems. Bear intestinal cDNA samples from 5 bears were run in triplicate on a plate, and a standard curve for one gene was run in duplicate on the same plate. For each 25 μL PCR reaction, 2 μL of the cDNA 1:30 dilutions, 12.5 μL of SYBR green master mix (Applied Biosystems), 9.5 μL of water, and 0.5 μL of the forward and reverse primer at a 5 μ*M* concentration were added. PCR was performed under the following conditions:

1. 50°C for 10 min
2. 95°C for 1 min
3. 60°C for 1 min
4. Steps 2 and 3 repeated for 40 cycles

Statistical Analysis

Triplicate samples were automatically averaged by the Applied Biosystems software. Values were exported to Microsoft Excel and segment means were calculated. The data were evaluated using an analysis of variance with the MIXED procedure of SAS, (SAS Inst., Cary, NC, 1997). The model consisted of the transporter genes and APN as the dependent variables, intestinal segment as the independent variable, and bear

was included as a random variable. A linear, quadratic, and cubic contrast were performed for each gene.

RESULTS

Distribution and Relative Abundance of PepT1, APN, SGLT1, $b^{o,+}$AT, ASCT1, and NBAT mRNA in the Gastrointestinal Tract of Bears

According to the Northern blot analysis, abundance of PepT1 (Figure 3.1; $P < 0.05$), APN (Figure 3.2; $P < 0.05$), and SGLT1 (Figure 3.3; $P < 0.0001$) changed quadratically from the proximal to the distal intestine with abundance being greatest in the midregion of the intestine. Abundance of $b^{o,+}$AT mRNA increased linearly ($P < 0.05$) from the proximal to distal intestine (Figure 3.4). Abundance of NBAT mRNA was not different among intestinal segments (Figure 3.5). ASCT1 mRNA was not detected. RT-PCR yielded similar findings, with the exception of ASCT1, and these data are presented in Figures 3.6 and 3.7. Data for PepT1, SGLT1, APN, NBAT, and $b^{o,+}$AT are presented in Figure 3.6. Data for PepT1 are excluded from Figure 3.7 so that quantities of other genes are visualized better. Similar to the Northern blot results, abundance of PepT1 ($P < 0.0003$), SGLT1 ($P < 0.0003$), and APN ($P < 0.02$) changed quadratically from the proximal to distal intestine with abundance being greatest in the midregion of the intestine, and $b^{o,+}$AT mRNA increased linearly ($P < 0.0001$) from the proximal to distal intestine. NBAT mRNA abundance also increased linearly ($P < 0.0001$) from proximal to distal intestine. Quantitative RT-PCR was used because it allows for the determination of absolute quantities of mRNA in a tissue to allow for comparison of expression levels among genes. The mRNA of PepT1 was present in the intestine at a range of 95,200 to 994,000 molecules per ng of total RNA. The mRNA of $b^{o,+}$AT was present at a range of

4,300 to 31,000 molecules, and the mRNA of NBAT was present at a range of 9,600 to 43,800 molecules per ng of total RNA. The mRNA of SGLT1 was present at a range of 20,500 to 170,000 molecules, and mRNA of APN was present at a range of 76,900 to 235,000 molecules per ng of total RNA. ASCT1 mRNA was detected by RT-PCR, but at a range of 2 to 6 molecules per ng of total RNA, which most likely represents background levels (Figure 3.8). PepT1 mRNA was present at tenfold or greater levels than AA transporter genes in all segments of the intestine, suggesting its importance in amino acid assimilation.

DISCUSSION

These results demonstrate that mRNA for a peptide transporter (PepT1), a glucose transporter (SGLT1), a digestive enzyme (aminopeptidase N), and three AA transporters ($b^{o,+}$AT, ASCT1, and NBAT) are present and differentially distributed in the gastrointestinal tract of black bears. That PepT1, SGLT1, and APN were expressed highest in the midportion of the gut suggests that this region (sections 3 and 4) is comparable to the jejunum described in other species, and that this is the region of maximal nutrient absorption. In other species, maximal nutrient absorption occurs in the jejunum, and absorption is mediated by intestinal nutrient transporters (Pond et al., 1995). PepT1 mRNA was present at tenfold or greater levels than AA transporter genes in all segments of the intestine, suggesting that di- and tripeptides constitute the major form in which AA are absorbed. NBAT and $b^{o,+}$AT mRNA abundance was greater towards the distal portion of the intestine, suggesting their importance in salvaging remaining unabsorbed AA. When taking into account the kinetic characteristics of these transporters, these data are logical. PepT1 is a low-affinity/high-capacity transporter that

operates well under high substrate concentration loads and can transport a large amount before it reaches V_{max}. It is, therefore, advantageous for the bear to express high levels of PepT1 throughout the intestinal tract to maximize AA assimilation. The free AA transporters, on the other hand, are high-affinity/low-capacity transporters, meaning that they operate well under low substrate concentration loads, and transport considerably less before attaining V_{max}. Thus, it is advantageous for the bear to express these transporters at the distal end of the tract, where the lowest substrate concentrations exist, so that the transporters can essentially "grab" any remaining AA to maximize absorption before the AA leave the body.

SGLT1 is a high affinity/low capacity transporter and is the sole carrier protein identified thus far on the apical membrane of enterocytes responsible for glucose and galactose absorption in mammals. The mRNA expression levels of SGLT1 correspond to its transport kinetics and perhaps suggest the importance of certain nutrients in the diet. Glucose is not an essential nutrient, and patients suffering from glucose and galactose malabsorption as a result of mutations in the SGLT1 gene are still able to thrive (Wright et al., 2003). The necessity of having AA provided in the diet is emphasized by the wide array of free AA transporters expressed on the brushborder membrane, and the high expression level and fast and efficient absorption of AA in the form of peptides by PepT1. During most of the year, carbohydrates likely comprise a large portion of the bear diet, but considering that glucose is not an essential nutrient in humans, it is practical to express a single high-affinity/low-capacity glucose transporter at moderate levels in the intestinal tract to preserve energy. This also provides insight for understanding the relative contribution of nutrients in the diet – it is known that monosaccharide transport

capacity does not substantially exceed the physiological demands posed on the animal (Henning et al., 1994).

Aminopeptidase N mRNA followed an expected expression pattern, with highest expression levels in the midregion of the intestine, and a pronounced increase in expression in the mid to distal intestine, where PepT1 mRNA expression peaked. The action of APN at the brushborder membrane yields both free AA and smaller peptides for absorption, thus, a pronounced increase in mRNA expression levels in the mid to distal intestine parallels the increase in PepT1 and AA transporter mRNA. This expression pattern for APN is similar to that reported elsewhere, with APN protein increasing from the proximal to distal small intestine in rabbit brushborder membrane vesicles (Kramer et al., 2005).

Results from previous studies have demonstrated that $b^{o,+}$AT and NBAT heterodimerize to form the active $b^{o,+}$ transport system, and that all of $b^{o,+}$AT associates with NBAT, but not all NBAT associates with $b^{o,+}$AT, suggesting the presence of other light subunits (Fernandez et al., 2002). This could explain why more mRNA molecules of NBAT were detected in all segments of the intestine compared to the $b^{o,+}$AT. The patterns of mRNA expression were identical, but perhaps more mRNA of NBAT is transcribed to associate with additional light subunit proteins. However, it is unknown if $b^{o,+}$AT performs other biological functions in addition to AA transport. It is important to point out that these speculations relate strictly to mRNA abundance, and protein expression and activity data are needed to completely understand the expression and function of $b^{o,+}$AT and NBAT. If there are other light subunits to which NBAT associates to account for the increase in mRNA expression levels of NBAT mRNA relative to its

known light subunit, future studies should attempt to elucidate the other light subunit proteins to further our understanding of AA transport.

ASCT1 mRNA was determined to be present at less than six molecules/ng of total RNA in each segment of the intestine, and was not detected by Northern blot analysis. There were no reports of ASCT1 in the intestine, most likely because Northerns were used to quantify mRNA abundance, until recently when ASCT1 was reported in rat intestine by RT-PCR, a sensitive method for detecting an mRNA species (Howard et al., 2004). Expression at such low levels would suggest that the ASCT1 protein is posttranslationally modified, possibly by glycosylation, and has increased activity to compensate for the amount of protein present. Because of the sensitivity of RT-PCR it is likely that the ASCT1 mRNA levels reported here represent background levels, similar to what would be observed for genes known not to be expressed in the intestine. Quantification of ASCT2, the other cloned ASC protein in the intestine, by RT-PCR will contribute to our knowledge of this transport system. Also, information is needed on the protein expression of ASCT1 in the intestine. It would seem metabolically disadvantageous for the animal to transcribe mRNA for a gene and to not produce a functional protein. With low levels of mRNA, a corresponding number of translational events must occur, resulting in low levels of a protein. Posttranslational modification, such as N-glycosylation, can alter intracellular targeting, protein folding, and protein stability, factors that influence the activity of the carrier protein (Motohashi et al., 2001).

In general, transporter and APN mRNA were expressed at higher levels in the mid to distal intestine which was surprising. In other species, such as the pig, mRNA expression levels of transporters tend to be higher in the proximal intestine. Also, when

the bears were sampled, the colon was included in segment 5 of the intestine. In other species, there is a very minimal absorption of nutrients in the colon, an observation supported by immunohistochemistry data in which staining for nutrient transporters tends to be less intense in the large intestine. Intestinal histology of most species illustrates the lack of villi in the colon, resulting in a decreased surface area for absorption, although polarized epithelial cells are present. Therefore, it is very surprising in the bear that segment 5, which included the colon, exhibited such high mRNA abundance of the transporter genes, in comparison to the proximal intestine. In bears, the transit time of ingested feed in the intestine tends to be relatively fast (Pritchard and Robbins, 1990), but can also be dependent on the type of food being ingested; but if food spends less time in the body, then maintenance of high expression levels of these genes throughout the entire length of the intestinal tract ensures maximal absorption to take advantage of all incoming nutrients in spite of the rapid transit time. Interestingly, bears are only 5% less efficient at digesting plant protein than ruminants (Pritchard and Robbins, 1990), which indicates that the rapid passage rate does not result in a substantial decrease in digestive efficiency. More information about absolute mRNA abundance of digestive enzymes and nutrient transporters in the intestine and intestinal histology of other species will confirm whether bears are able to efficiently assimilate nutrients because of maximized absorptive surface area and gene expression.

A greater understanding of the physiological processes associated with nutrient assimilation in the black bear increases our understanding of the survival strategies of this animal, and hence, our ability to manage for the animal. For example, there is an alternative interpretation of the RT-PCR data. In an animal like the bear, struggling to

survive in the wild, it would be beneficial to preferentially express the peptide transporter, which is known to be a faster and more energetically efficient means to absorb AA, in order to conserve as much energy as possible. In order to implement sound and practical management decisions for wildlife, there must be a greater understanding of how the animal is able to utilize different foods. Up to this point, researchers have examined only the quantity and quality of feedstuffs without really investigating how the animal absorbs the dietary nutrients.

There is much to be learned from the black bear, which exhibit fascinating physiological phenomena. However, the limited availability of black bears that can be used for research purposes constrained the design of this project. Because of the opportunistic method of sampling bears, the project lacked a controlled design. Ideally, a study would consist of treatments and a control group maintained in a controlled environment over a fixed period of time on a specific, measurable diet. In this study, there was no experimental variable for comparison. It was a descriptive survey of known transporter gene expression in the bear intestine. The variation in transporter expression among bears was surprisingly low considering the diversity in the sample population, confirming the physiological significance of these findings. Future research should take advantage of captive bear research facilities to explore the factors regulating nutrient transporter gene expression in the black bear.

In conclusion, these results demonstrate that nutrient transporters are differentially distributed throughout the intestinal tract of the black bear and support the argument that peptide transport accounts for the majority of AA assimilation in the animal. Future research should attempt to confirm this assumption in other species through the use of

quantitative RT-PCR and to further investigate how transporter expression levels are regulated in the bear seasonally. This study is the first to report quantification of nutrient transporter mRNA using an absolute cRNA standard curve. It has been suggested that the ability of the bear to extract nutrients from the diet may be related to its ability to quickly adapt to seasonal variations in food abundance (Bunnell and Hamilton, 1983). This adaptation may be linked to expression of nutrient transporters in the intestine. It is also very important to point out that mRNA provides us with only part of the story; protein expression and activity data are needed to better understand the "big picture" of what is occurring in the intestine. The mRNA expression data does not necessarily correlate to protein expression, and studies have demonstrated that mRNA expression tends to be more widespread than expression of the functional protein. Furthermore, these results contribute to our knowledge of nutrient assimilation and will potentially aid in enhancing diet formulation, as well as understanding how bears utilize nutrients. In summary, information that will aid in formulating management decisions based on habitat quality and spring emergence conditions following hibernation.

Table 3.1. Black bear sample size and demographic information

Bear	Sex	Age	Weight (kg)
1	Male	18 mo	53.1
2	Female	2 yr	81.6
3	Male	4 yr	116.0
4	Male	25 yr	205.0
5	Male	4 yr	138.3
6	Male	7 yr	186.0
7	Male	3 yr	89.0
8	Male	3 yr	102.0
9	Male	4 yr	79.0
10	Male	2 yr	68.0

Table 3.2 PCR primer sequences for Northern blot analysis probes

Gene name	Primer sequence	Species
PepT1 (Sense)	GTCTTGAACTTCCCCAGCCA	Human
PepT1 (Antisense)	AAGCATCTTCTTCATCGTGGTCAATG	Human
NBAT (Sense)	ATGCCCAAGGAGGTGCTGTT	Pig
NBAT (Antisense)	GGTGTGGTTGGGGATGAAATC	Pig
ASCT1 (Sense)	CACGGTTGTGAATGTGGAAG	Pig
ASCT1 (Antisense)	TTCCTTGGATTCAGGGTCTG	Pig
$b^{o,+}$AT (Sense)	AGACTTGTTTATGTGGCGGG	Pig
$b^{o,+}$AT (Antisense)	GGAGGCTGAGCTTGTTACTC	Pig
APN (Sense)	ACATCACTCTCATCCACCCT	Pig
APN (Antisense)	GCAATCACAGTGACAACTCG	Pig
SGLT1 (Sense)	GTGCTGATTGGCATCAGCAT	Pig
SGLT1 (Antisense)	TGGGACAGTTGCTGGGCTCCA	Pig
GAPDH (Sense)	ATGCCTCCTGTACCACCAAC	Pig
GAPDH (Antisense)	CACAACCTGGTGCTCAGTGT	Pig

Table 3.3 PCR primer sequences for Real-Time PCR

Gene name	Primer sequence
Bear PepT1 (Sense)	CGGCCATCTACCACACGTTT
Bear PepT1 (Antisense)	TCAGCGATTAAAGCACCAAGAA
Bear SGLT1 (Sense)	GCTGTCTTCCTGCTTGCTGTT
Bear SGLT1 (Antisense)	CCGATGATCAGTCCCCAAAA
Bear NBAT (Sense)	GCCACCATAGCCATCATTGC
Bear NBAT (Antisense)	GAAGGATCTCGGGTAGATCTGGTA
Bear $b^{o,+}AT$ (Sense)	CGGCACCACCTCCATGA
Bear $b^{o,+}AT$ (Antisense)	GGATGGGCTCAGAAAATCTCAA
Bear ASCT1 (Sense)	TGGTTGTGTTGGAGACTTGTTTTT
Bear ASCT1 (Antisense)	GCAGTTGCTAACAGCAAGTCTGA
Bear GAPDH (Sense)	TCGTCATATTTGGCAGCTTTCTC
Bear GAPDH (Antisense)	TCCCCACCCCCAACGT
Bear APN (Sense)	CCCACCGTCCCTGTTACG
Bear APN (Antisense)	GTCGACATTATAGGTGTGGTTTCAA

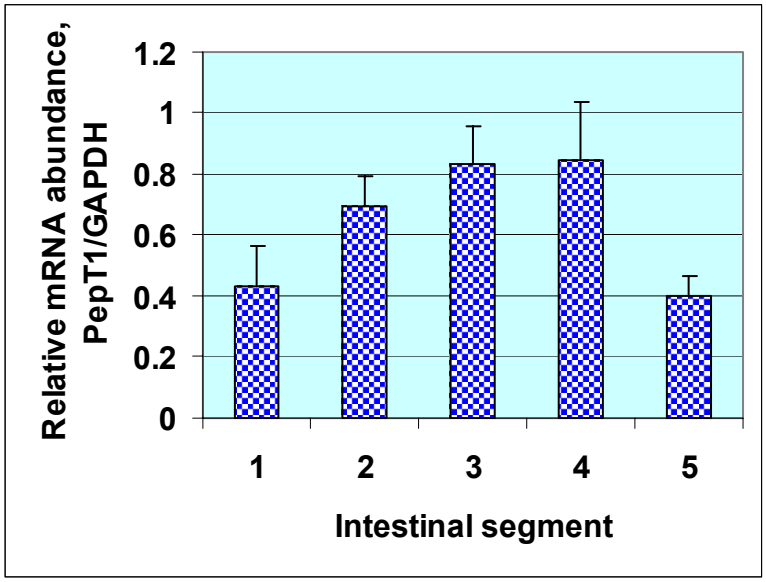

Figure 3.1 Relative mRNA abundance of PepT1. Quadratic ($P < 0.05$). Values are expressed as the mean plus standard error. Abundances are expressed as a ratio of PepT1 mRNA to GAPDH mRNA.

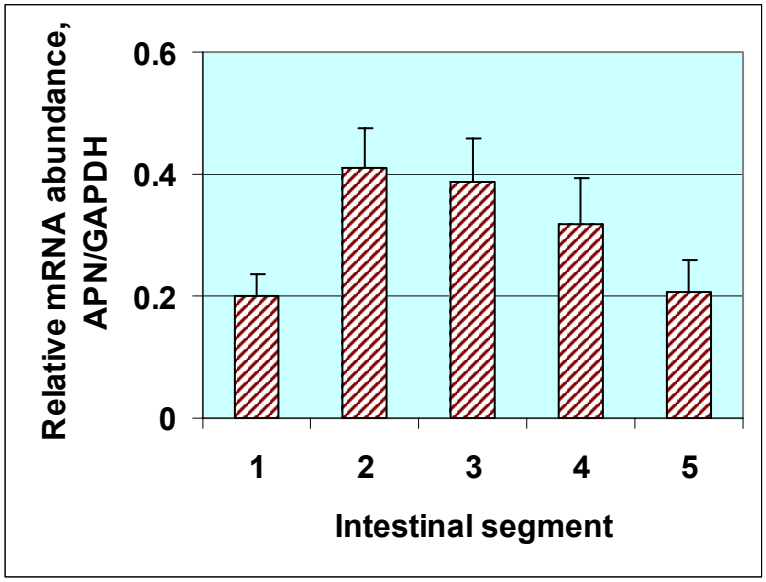

Figure 3.2 Relative mRNA abundance of APN. Quadratic ($P < 0.05$). Values are expressed as the mean plus standard error. Abundances are expressed as a ratio of APN mRNA to GAPDH mRNA.

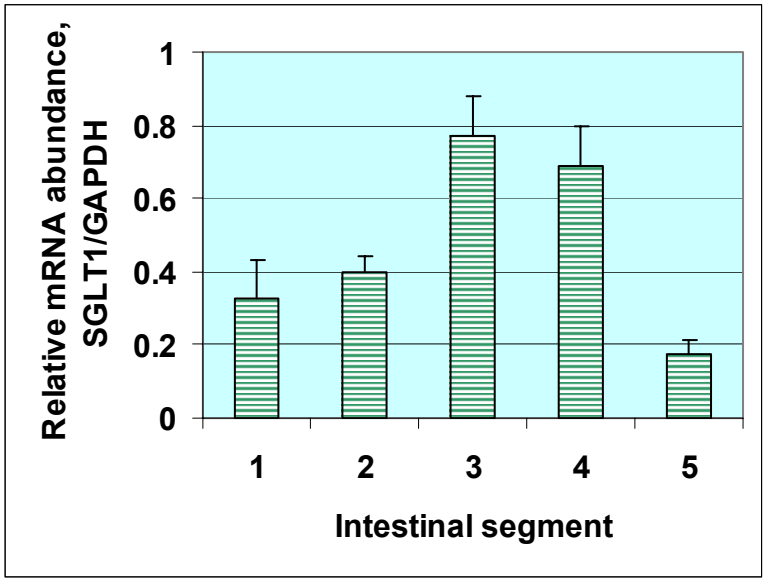

Figure 3.3 Relative mRNA abundance of SGLT1. Quadratic ($P < 0.0001$). Values are expressed as the mean plus standard error. Abundances are expressed as a ratio of SGLT1 mRNA to GAPDH mRNA.

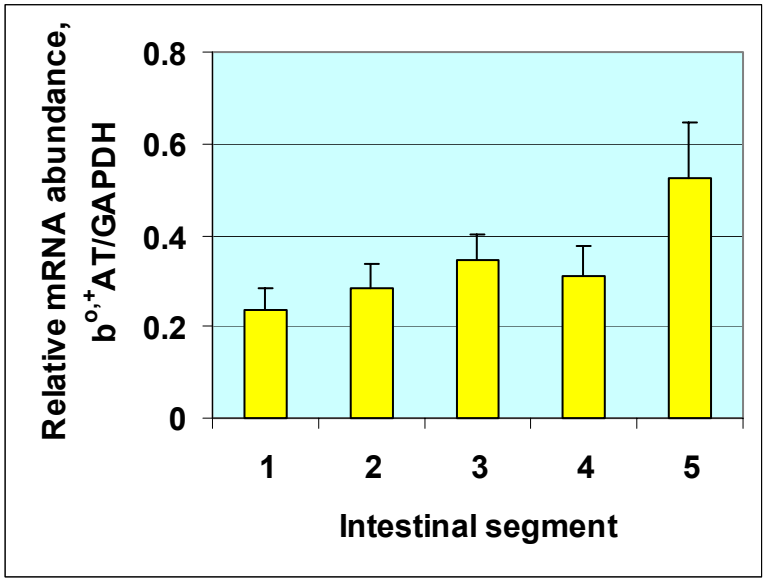

Figure 3.4 Relative mRNA abundance of $b^{o,+}AT$. Linear ($P < 0.05$). Values are expressed as the mean plus standard error. Abundances are expressed as a ratio of $b^{o,+}AT$ mRNA to GAPDH mRNA.

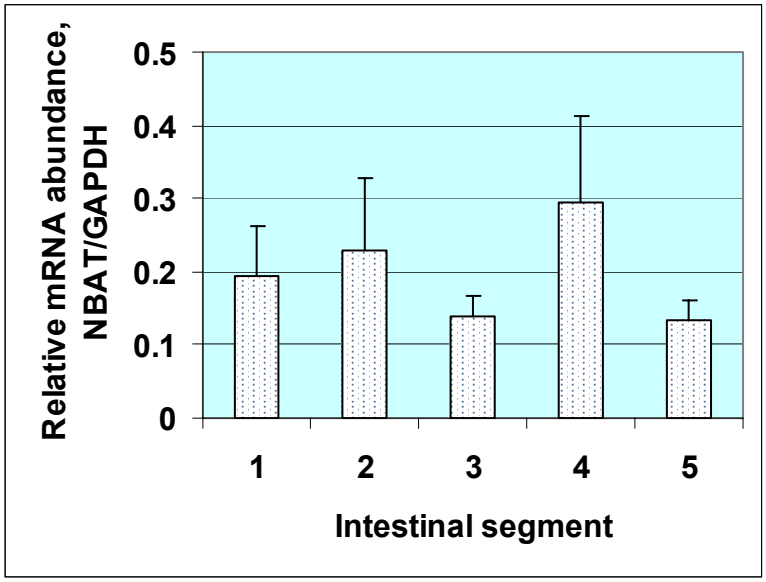

Figure 3.5 Relative mRNA abundance of NBAT. No difference among segments. Values are expressed as the mean plus standard error. Abundances are expressed as a ratio of NBAT mRNA to GAPDH mRNA.

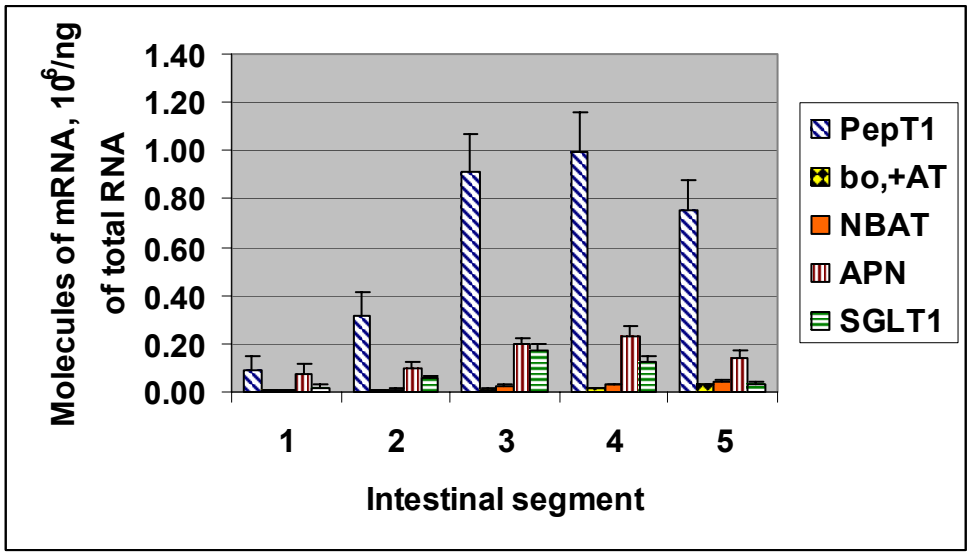

Figure 3.6 Molecules of PepT1, $b^{o,+}AT$, NBAT, APN, and SGLT1 mRNA (10^6)/ng of total RNA starting template. Values are expressed as means plus the standard error. PepT1 ($P < 0.0003$), SGLT1 ($P < 0.0003$), and APN ($P < 0.02$) changed quadratically from the proximal to distal intestine with abundance being greatest in the mid-region, and $b^{o,+}AT$ and NBAT mRNA increased linearly ($P < 0.0001$) from the proximal to distal intestine.

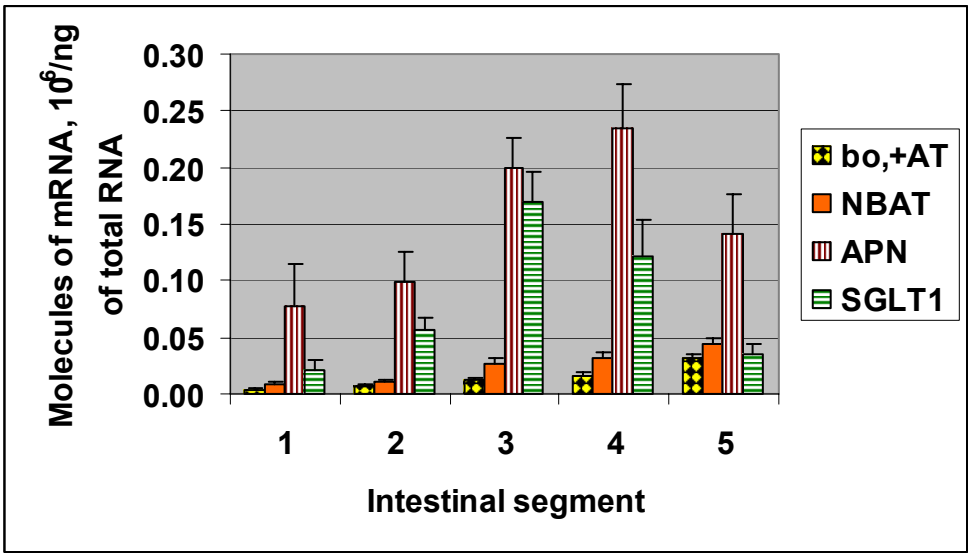

Figure 3.7 Molecules of $b^{o,+}AT$, NBAT, APN, and SGLT1 mRNA (10^6)/ng of total RNA starting template. Values are expressed as means plus the standard error. SGLT1 ($P < 0.0003$), and APN ($P < 0.02$) changed quadratically from the proximal to distal intestine with abundance being greatest in the mid-region, and $b^{o,+}AT$ and NBAT mRNA increased linearly ($P < 0.0001$) from the proximal to distal intestine.

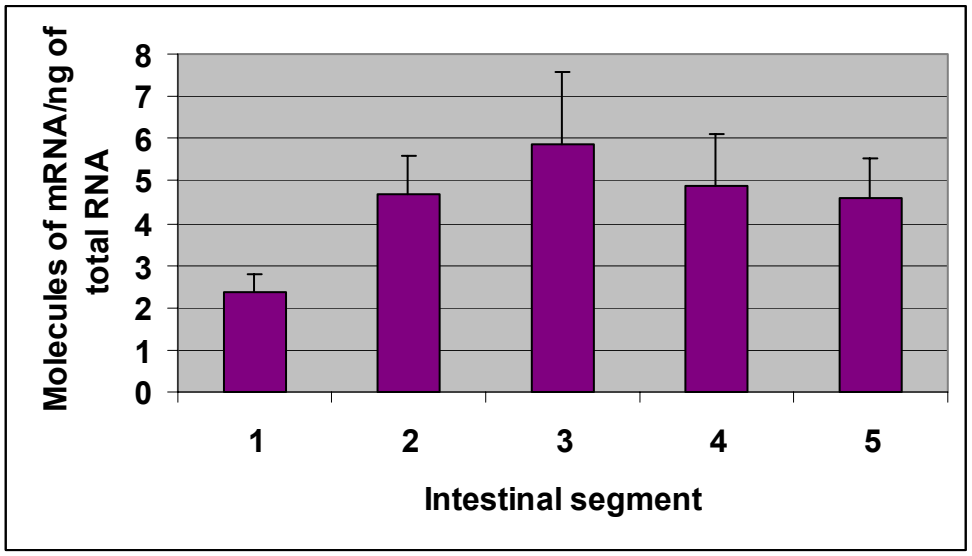

Figure 3.8 Molecules of ASCT1 mRNA per ng of total RNA starting template. Values are expressed as means plus the standard error.

Chapter IV

Epilogue

A greater understanding of the relative contribution of peptide transport vs. free AA transport will potentially be valuable to formulating diets for livestock species. Given the present data, it might be advantageous to provide AA supplements in a peptide form, which are absorbed faster and more efficiently by the animal, saving the producer money in the long run. In addition, previous research has demonstrated that some AA are labile or insoluble in the free form, emphasizing the usefulness of providing the AA to the animal in a peptide form. Absolute mRNA expression levels of these transporter genes should be investigated in other species to confirm the significance of these findings.

A greater understanding of the physiological processes associated with nutrient assimilation by the black bear will be valuable to understanding the nutritional status of animals. Cloning and functional characterization of PepT1 and other transporters in the intestinal tract of black bears, and a comparison to other species, will facilitate a greater understanding of these processes. Information about nutrient absorption in the black bear adds to our knowledge base, and if we find similar mechanisms occurring among species, it can strengthen our arguments. Major differences among species stimulate us to try and understand the physiological significance of those differences. For example, if lower numbers of molecules of PepT1 mRNA relative to AA transporter mRNA are observed in other species, this may represent an important adaptation of the bear to maximize AA assimilation during uncertain environmental conditions. As comparative nutritionists, it is of interest to understand the structural and functional difference among species, and to learn how different species have evolved to cope with different lifestyles.

It would be of interest to explore seasonal changes in nutrient transporter expression in the bear and to examine the question of whether there is maintenance of intestinal function during hibernation. Carey and Martin (1996) found that SGLT1 expression was maintained during hibernation conditions in the ground squirrel. They suggested that most genes were maintained at similar expression levels during hibernation to ready the animal for arousal. It would be of interest to look at transporter expression levels in the spring after bears emerge from hibernation and begin to consume a high protein diet. In our laboratory, the focus of much research involves investigating how livestock species, such as the pig or chicken, are able to adapt to differing levels of protein in the diet, and the capacity to adapt and exhibit higher feed efficiencies. Therefore, it is relevant to attempt to understand how bears are able to so quickly adapt to a high protein diet, especially after not having consumed feed for such a long period of time. It is also of interest to look at how transport expression shifts when the bears begin to consume a high carbohydrate diet in the summer, and if SGLT1 is upregulated during this time. Interestingly, during hibernation the total surface area of the intestinal epithelium decreases in squirrels, but the microvilli are preserved, and nutrient transporter expression is preserved, most likely in anticipation of food intake during infrequent periods of arousal from the den (Carey and Sills, 1996). In humans, no physical activity and little or no food intake would lead to imminent starvation, muscle atropy, and osteoperosis, but bears exhibit an amazing abililty to adapt to the physiological conditions required during hibernation, and do not exhibit any of these clinical symptoms.

It would also be of interest to look at urea cycle regulation in bears, and expression of transporters and enzymes in the liver, and how they may be seasonally regulated. Obviously, during the winter, with no excretion of urea, the process of recycling the urea back to the gut, where bacteria take up and hydrolyze the urea with urease, is tightly controlled. Recycling probably occurs to a minimal extent, because of the minimal substrate available for the bacteria in the gut, due to a lack of feed intake for months at a time. It appears that this tight control of nitrogen metabolism ensuring that no net increase in urea synthesis occurs is the mechanism by which the hibernating state is maintained in bears (Nelson, 1980).

Future studies of transporter expression in bears should also include intestinal histology. In spite of the fact that bears consume a large amount of vegetative material, they have a relatively short, undifferentiated gut similar to carnivores. It has been suggested that retention of a gut similar to their carnivorous ancestors is an adaptation to digest food quickly like carnivores, sacrificing the ability to efficiently digest low quality food, allowing them to take advantage of increased availability of foods and exhibit hyperphagia prior to denning (Bunnell and Hamilton, 1983). It would be of interest to compare villous height and crypt depth in the different intestinal segments to gain some insight about differences in intestinal function among the segments. Perhaps villous height and crypt depth are enhanced to increase the absorptive surface area in order to better accommodate the nutritional needs of the animal, in spite of the short length of the tract. This is of great interest in the colon, where we would not expect to see a substantial amount of nutrient absorption, but in the bear, the distal segment of the intestine appears to exhibit a higher capacity for absorption than the proximal region of the intestine. In

other species, villi are usually not present in the colon; however, polarized epithelial cells are present with the capability to absorb nutrients, and in other species, low levels of PepT1 mRNA have been observed in the proximal colon (Freeman et al., 1995).

Digestive enzyme and nutrient transporter research could have huge nutritional implications in a zoo-type atmosphere where, in many cases, the primary goal is reproduction, which is influenced directly by the nutritional status of the animal. A better understanding of the absorptive capacities for different nutrients enables enhanced diet formulation that takes advantage of the performance capabilities of the animal. For example, amino acid supplements could be provided in a peptide-form, allowing for a more rapid and energetically efficient assimilation of the constituent amino acids. A huge challenge in supplying the right nutrition to captive wildlife is the lack of known requirements, especially when natural ingredients are unavailable. In many situations domestic animals may serve as a model for formulating diets for captive exotic species, but a different physiology, feeding behavior and metabolism can limit the usefulness of these comparisons. Nutritional information gathered from the black bear could have practical applications to other species that exhibit similar physiologies and behavior.

Also, there are many potential human health advances to be gained from a greater understanding of bear physiology. Patients who experience damage to the intestine or intestinal resection exhibit a drastically reduced capacity for absorption. Therefore, providing AA in the feeding solutions in the form of peptides maximizes the capacity for absorption. The metabolic adaptations to survive hibernation are incredible. Bears have adapted to undergo a period of hibernation lasting approximately six months, during which time the animal subsists off of fat stores, with very modest lean muscle mass loss,

and during which time they do not eat, drink, urinate, or defecate. During this time parturition and lactation occurs, and the cubs nurse for several months before the bears emerge from hibernation, which involves a tremendous expenditure of metabolic energy. In other species, including humans, an extended period of starvation would not support gestation or lactation. Also, humans who are bed-ridden for months at a time experience osteoperosis (Leblanc et al., 1990). Black bears are amazing in that they do not lose any bone integrity, appearing to maintain levels of bone formation during hibernation that are similar to the active period (Donahue et al., 2003). Also, bears exhibit high circulating levels of cholesterol, but do not develop arterial plaques (Bagget, 1984). During hibernation bears subsist off of fat stores, with low circulating levels of ketone bodies in the blood (Nelson et al., 1983). In humans, ketone levels reach 5.0 m*M* during fasting, but in the bear, during the same period of fasting, ketones only reach approximately 0.2 m*M* (Castellini and Rea, 1992). Perhaps many of these amazing metabolic adaptations occurring during hibernation are related to the function of the gut, which supports the daily metabolic needs of the remainder of the body. Understanding the functions and adaptations of the bear gut has the potential to enhance our understanding of human obesity and diabetes, and how a hibernating species is able to so narrowly regulate body weight gain and weight loss, without any of the adverse effects observed in humans.

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Vita

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