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Expression Cloning and Functional Characterisation of cDNAs Encoding Nucleoside Transporter and Amino Acid/Nucleoside Transport Regulator Proteins from Rat Jejunum

BY

Sylvia Y.M. Yao

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for a degree of Doctor Of Philosophy.

Department of Physiology

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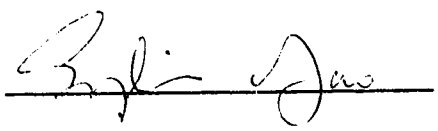

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Expression Cloning and Functional Characterisation of cDNAs Encoding Nucleoside Transporter and Amino Acid/Nucleoside Transport Regulator Proteins from Rat Jejunum** submitted by **Sylvia Yuet Mui Yao** in partial fulfillment of the requirements for the degree of Doctor Of Philosophy.

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ABSTRACT

Transmembrane and transcellular movements of nucleosides and amino acids are mediated and controlled by specific concentrative (Na^+-dependent) and equilibrative (Na^+-independent) transport mechanisms. This Thesis describes the expression cloning and functional characterisation in *Xenopus laevis* oocytes of two rat jejunal cDNAs, one encoding a Na^+-dependent nucleoside transporter protein (cNT1), the other encoding an amino acid/nucleoside transport regulator protein (4F2hc).

cNT1 is a protein of 648 amino acids with 14 putative transmembrane domains. Data base searches indicate significant sequence similarity to the NupC H^+/nucleoside symporter of *Escherichia coli* (27% amino acid identity), but there was no sequence similarity between cNT1 and proteins of mammalian origin. Functionally, cNT1 exhibited the transport characteristics of the concentrative nucleoside transporter N2 (selective for pyrimidine nucleosides and adenosine) and accepted two antiviral nucleoside analogs, 3'-azido-3'-deoxythymidine (AZT) and 2',3'-dideoxycytidine (ddC) as permeants. Transcripts for cNT1 were detected in kidney as well as jejunum. Expression of NupC in *Xenopus* oocytes confirmed that it is a H^+-dependent nucleoside transporter with a substrate specificity similar to that of cNT1.

Rat jejunal 4F2hc is a 527 amino acid type II membrane glycoprotein. It is 28% identical in amino acid sequence to the D2/rBAT glycoprotein which is defective in cystinuria, a human inherited disorder of intestinal and renal cystine and cationic amino acid transport. Expression of rat jejunal 4F2hc and rat renal D2/rBAT in *Xenopus* oocytes induced Na^+-dependent and Na^+-independent L-leucine transport, respectively,

corresponding to amino acid transport systems y^+L and $b^{o,+}$. In addition, 4F2hc and D2/rBAT stimulated Na^+-dependent uptake of uridine and, to a lesser extent, pyruvate.

In summary, cNT1 from rat jejunum is the first mammalian representative of a new gene family of polytopic nucleoside transporter proteins. cNT1 transports AZT and ddC and its human homolog may be involved in the intestinal absorption and renal handling of drugs used to treat acquired immunodeficiency syndrome (AIDS). Rat jejunal 4F2hc, in contrast, has a single transmembrane domain and is structurally and functionally related to the cystinuria protein D2/rBAT. 4F2hc and D2/rBAT mediate multiple transport activities when expressed in *Xenopus* oocytes and are likely to function as transport activators.

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ABBREVIATIONS

4F2	4F2 cell surface antigen
4F2hc	4F2 heavy chain
4F2lc	4F2 light chain
AIDS	Acquired immunodeficiency syndrome
AZT	3'-azido-3'-deoxythymidine
AZTDP	AZT 5'-diphosphate
AZTMP	AZT 5'-monophosphate
AZTTP	AZT 5'-triphosphate
BCH	2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid
bp(s)	Nucleotide base pair(s)
BSA	Bovine serum albumin
cDNA	Complememtary DNA
CNS	Central nervous system
cNT1	Concentrative nucleoside transporter
cRNA	Complememtary RNA
ddC	2',3'-dideoxycytidine
ddCTP	ddC 5'-triphosphate
ddI	2',3'-dideoxyinosine
ddITP	ddI 5'-triphosphate
DEAE	Diethylaminoethyl
DNA	Deoxynucleic acid
EDTA	Ethylenediaminetetraacetic acid

EGTA	Ethylene glycol-bis(ß-aminoethyl ether)-tetraacetic acid
FTIR	Fourier-transform infrared
GABA	γ-amino-butyric acid
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immuno-deficiency virus
HPLC	High performance liquid chromatography
LB	Lauria-Bertani medium
LMP	Low melting point
kDa	kilodaltons
K_m	Permeant concentration at half-maximal unidirectional flux
MBM	Modified Barth's medium
MDCK	Madin-Darby canine kidney
MeAIB	2-methylaminoisobutyric acid
MFS	Major facilitator superfamily
min	Minute
Mr	Molecular weight
NBMPR	Nitrobenzylthioinosine; 6-[(4-nitrobenzyl)-thio]-9-ß-D-ribofuranosylpurine
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
ORF	Open reading frame
PBS	Phosphate buffered saline

PCMBS	p-chloromercuriphenylsulphonate
PCR	Polymerase chain reaction
PKC	Protein kinase C
PMS	Phenazine methosulphate
PMSF	Phenylethylsulphonyl fluoride
RNA	Ribonucleic acid
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
SSCP	Single strand conformational polymorphism
SSSS	Sodium solute symporter superfamily
TB	Transport buffer
TM	Transmembrane domain
Tris	Tris-(hydroxymethyl) aminomethane
UDP	Uridine-5'-diphosphate
UMP	Uridine-5'-monophosphate
UTP	Uridine-5'-triphosphate
UV	Ultraviolet light
V_{max}	Maximum transport rate
v/v	Volume per unit volume
w/v	Weight per unit volume
w/w	Weight per unit weight

CHAPTER 1

General Introduction

1.1. PREAMBLE

Transmembrane and transcellular movements of nutrient molecules such as nucleosides, amino acids and sugars are mediated and controlled by specific concentrative (Na^+ and H^+-dependent) and equilibrative transport mechanisms. At the time of commencement of my graduate studies in Biochemistry at the Chinese University of Hong Kong in 1989 and subsequently in Physiology at the University of Alberta in 1990, very little was known about the molecular properties of the proteins that mediate nucleoside and amino acid transport in mammalian cells and no complementary DNAs (cDNAs) encoding mammalian nucleoside and amino acid transporter proteins or their regulators had been isolated. In contrast, the first mammalian cDNA encoding an equilibrative glucose transporter (GLUT1) was cloned in 1985 (1) and the first cDNA encoding a mammalian Na^+-dependent glucose transporter (SGLT1) was isolated in 1987 (2).

My supervisor, Dr. James D. Young, relocated his laboratory to Edmonton from Hong Kong in April, 1990. The experiments described in Chapters 1-6 of this Thesis were carried out at the University of Alberta between 1990 and 1994 with the objective of employing the technique of functional expression in *Xenopus* oocytes to isolate cDNAs encoding mammalian nucleoside and amino acid transporter (and transporter-associated) proteins from rat jejunum. Jejunal enterocytes were selected as the tissue source of mRNA for cDNA cloning because of their rapid turnover and physiological importance in nutrient absorption. My Thesis describes the isolation and functional characterisation in *Xenopus* oocytes of two rat jejunal cDNAs, the first encoding a Na^+-linked nucleoside transporter protein (cNT1) and the second encoding an amino acid/nucleoside transport regulator protein (4F2hc). cNT1 transports pyrimidine nucleosides, antiviral pyrimidine nucleoside analogs and adenosine (3) and is the first mammalian representative of a new gene family of membrane transporter proteins that includes the *Escherichia coli* nucleoside/proton symporter, NupC whose functional expression in *Xenopus* oocytes is also described. 4F2hc is structurally related to a type II membrane glycoprotein (D2/rBAT) that has recently been shown to be defective in cystinuria, a human inherited disorder of intestinal and renal cystine and cationic amino acid transport (4).

This Thesis also contains two Appendices. Appendix 1 describes a pilot research project from my first year of graduate studies in Hong Kong that resulted in the first reported reconstitution of an erythrocyte amino acid transporter into proteoliposomes (5). Subsequent attempts to purify this leucine transporter (rat erythrocyte system L) were not successful, but characterisation of one of the DEAE-cellulose membrane fractions from this study led to the isolation and N-terminal amino acid sequence analysis of a 73 kDa rat erythrocyte CD36 antigen (6, Appendix 1). CD36 is a membrane glycoprotein with no defined physiological function but has been implicated in attachment of malaria-infected erythrocytes to endothelial cells (7). Appendix 2 describes expression of amino acid transport activity in *Xenopus* oocytes injected with rat jejunal mRNA (8). These experiments were performed in Edmonton as a preliminary to the 4F2hc studies presented in Chapter 5.

The remainder of this Chapter presents literature reviews of nucleoside and amino

acid transport (Sections 1.2 and 1.3) and an introduction to the *Xenopus* oocyte expression system (Section 1.4). Five recently characterised nutrient transporter families are described in Section 1.5. My research objectives are presented in Section 1.6 and Section 1.7 outlines the organisation of the remainder of the Thesis.

1.2. NUCLEOSIDE TRANSPORT

Natural and synthetic nucleosides have important physiologic and pharmacologic activities in humans. For example, inosine serves as energy source for erythrocytes from adult pigs which lack a functional glucose transporter (9,10). Adenosine acts as a local hormone and is involved in many physiological processes such as regulation of blood flow, platelet function, lipolysis, cardiac contractility, renal vasoconstriction and coronary vasodilation (11-13). Nucleoside antimetabolites have therapeutic applications in human neoplastic and viral diseases, including leukemias and acquired immunodeficiency syndrome (AIDS) (14-17). In excess, some physiologic nucleosides are cytotoxic. For example, in patients with adenosine deaminase or purine nucleoside phosphorylase deficiencies, the accumulation of deoxyadenosine and adenosine causes severe immunodeficiency disease (18).

Nucleoside transport systems are classified functionally on the basis of cation dependence, sensitivity to inhibition by nitrobenzylthioinosine (NBMPR) and permeant selectivity. Both equilibrative (Na^+-independent) and concentrative (Na^+-dependent) nucleoside transport mechanisms are present in mammalian cells (Table 1.1). In contrast, only concentrative nucleoside transport systems are present in bacteria (19). Bacterial and mammalian nucleoside transport systems have similar substrate specificities but the former are coupled to H^+ transport.

1.2a. Equilibrative Nucleoside Transport in Mammalian Cells

Equilibrative nucleoside transport processes are widely distributed in mammalian cells (Table 1.1) and exhibit the classic features of facilitated diffusion (20-26). Their permeant selectivities are broad, transporting both purine and pyrimidine endogenous nucleosides as well as many structural analogs with various substituents in the base and/or sugar moieties (19-26). One notable exception is the antiviral pyrimidine nucleoside analog 3'-azido-3'-deoxythymidine (AZT)(27,28). Two functionally distinct groups of equilibrative nucleoside transporters can be distinguished on the basis of sensitivity to NBMPR, a specific, tight-binding inhibitor of nucleoside transport in human erythrocytes and many other cell types (29-37). The *es* nucleoside transporters are inhibited by low concentrations ($\leq$ 1 nM) of NBMPR as a result of a noncovalent interaction of NBMPR with high affinity (K_d ~ 0.1 nM) binding sites (29) located on the extracellular face of the plasma membrane (38,39). In contrast, the *ei* equilibrative nucleoside transporters are unaffected by NBMPR, or are inhibited by only high NBMPR concentrations (> 10 μM)(33-37). Both *es* and *ei* nucleoside transporters are inhibited by the vasodilators dipyridamole and dilazep (0.1 - 100 nM)(20-26), although there are substantial differences between species and cell types (36,40-44).

The specificity of NBMPR for interaction with *es* nucleoside transporters (29-31),

together with its high affinity (29) and photoreactivity (45,46) has permitted identification and purification of *es* nucleoside transporter proteins from both human and pig erythrocytes (47-50). Reconstitution studies have demonstrated that a single polypeptide species (45-65 kDa) possesses both transport and NBMPR-binding activities (50). Nucleoside transport (*es*) polypeptides from a variety of cell types have been photolabelled with [^{3}H]-NBMPR (43,51-58). M_r differences have been reported for some, reflecting, in the case of human and pig erythrocytes, intrinsic differences in protein structure (59-61) and, in other cases, differences in glycosylation (56,57,59-61). No one has succeeded in purifying *ei* nucleoside transport proteins.

In its size and glycosylation, the equilibrative nucleoside transporter of human erythrocytes more closely resembles the more abundant GLUT1 glucose transporter (10^4 *vs* 10^5 copies per cell, respectively)(62). Although the two transporter proteins co-purify during DEAE-cellulose chromatography of detergent-solubilised membranes, it was possible to separate them and purify the nucleoside transporter polypeptide by passage through an affinity column of polyclonal antibodies specific for GLUT1 (50). Gas-phase sequence analysis of the purified transporter gave a single N-terminal sequence of which the first eleven residues were hydrophilic and the next eight hydrophobic, as might be expected for a transport protein (60). The sequence is unlike that of any other currently in the protein sequence data bases but is highly homologous to the corresponding N-terminal sequence of the purified pig erythrocyte nucleoside transporter (60). The site of *N*-linked glycosylation has been localised to close to the N-terminus of the human erythrocyte transporter and the site of NBMPR labelling to within 16 kDa of that site (61). Studies with a human *es*-specific antibody have established the presence of multiple *es* transporter isoforms within a single species and tissue (63,64).

Transport studies on a human choriocarcinoma (BeWo) cell line derived from a placental tumor have shown that these cells exhibit extraordinarily large numbers of NBMPR binding sites (> 10^7/cell) and a correspondingly high NBMPR-sensitive thymidine transport activity (58). Expression of BeWo mRNA in *Xenopus* oocytes induced a 3- to 4-fold stimulation of [^{3}H]uridine uptake compared to water-injected oocytes and this uptake was abolished by NBMPR (65). Several potential *es* nucleoside transporter cDNAs have recently been isolated from a BeWo lambda gt11 cDNA library by immunologic screening with a polyclonal antibody raised against the human erythrocyte *es* nucleoside transporter and are presently undergoing characterisation (66,67). In other recent developments, a putative intracellular nucleoside transporter cDNA has been isolated from murine L1210 cells (67,68). This cDNA was obtained initially in truncated form by functional complementation of thymidine transport deficiency in *Saccharomyces cerevisiae* and encodes a 219 amino acid Golgi-associated four transmembrane protein (MTP)(67,68). An MTP deletion construct lacking the putative Golgi retention signal (localised to the C-terminal 21 amino acid residues) mediated large thymidine fluxes when expressed in *Xenopus* oocytes (68). MTP exhibits no sequence homology to known procaryote and eucaryote transporter proteins.

1.2b. Concentrative Nucleoside Transport in Mammalian Cells

Na^+-dependent nucleoside transport systems have been demonstrated in a variety

of cell types and tissues. Five subclasses (N1/*cif*, N2/*cit*, N3/*cib*, N4/*cit* and N5/*cs*) are presently recognised on the basis of permeant selectivity and sensitivity to inhibition by NBMPR and dipyridamole (Table 1.1). System N1 is generally purine-specific and guanosine serves as a model substrate, whereas system N2 is generally pyrimidine specific and thymidine serves as a model substrate. Uridine and adenosine are transported by both systems. System N3 has a broad specificity for both purine and pyrimidine nucleosides while the permeant selectivity of system N4 is similar to that of system N2, *i.e.* selective for pyrimidine nucleosides and adenosine, except that guanosine is also transported. The substrate selectivity of system N5 is not known. In contrast, to the other Na^+-linked systems however, N5 is inhibited by nanomolar concentrations of NBMPR and by dipyridamole.

N1-type nucleoside transport activity has been demonstrated in rat macrophages (69,70), rat hepatocytes (71), Walker 256 rat carcinosarcoma cells (72), murine L1210 leukemia cells (73-76), cultured murine S49 cells (77), murine splenocytes (78,79), murine enterocytes (80) and brush border membrane vesicles from bovine, rat and rabbit kidney (81-84) and rabbit intestine (85-87). N2-type transport activity has been found in murine enterocytes (80) and brush border membrane vesicles from rat (82,83,88), bovine (81) and rabbit kidney (84) and rabbit intestine (87). System N3 has been demonstrated in cultured human colorectal carcinoma CaCo cells (89), cultured human promyelocytic leukemia HL-60 cells (90) and rabbit choroid plexus (91,92). Systems N4 and N5 have been identified recently in human kidney brush border membrane vesicles and freshly isolated human leukemia cells, respectively (93-95). With the exception of system N5, no specific, high-affinity inhibitors of Na^+-dependent nucleoside fluxes have been identified. Systems N1 and N2 have a Na^+:nucleoside coupling stoichiometry of 1:1 (69-71, 75,76,79,81,88) whereas that for system N3 is 2:1 (91).

Several recent studies have described expression of Na^+-dependent nucleoside transport activity in *Xenopus* oocytes (96-100). Two laboratories have reported expression of system N2 activity in oocytes that had been injected with rabbit intestinal mRNA (96,97). Another study demonstrated the expression of low levels of Na^+-dependent uridine transport activity in oocytes injected with complementary RNA (cRNA) encoding a rabbit kidney protein (SNST1) belonging to the Na^+-dependent glucose transporter (SGLT) family (98)(Section 1.5). SNST1-mediated nucleoside transport activity has N3-type characteristics. N3-type transport activity has also observed in oocytes injected with rabbit choroid plexus mRNA (99). This laboratory has demonstrated expression of N1, N2 and N3 transport activities in oocytes injected with rat jejunal mRNA (100).

1.2c. Nucleoside Transport in Bacteria

The inner membrane of the bacterial cell envelop contains specific transport proteins that allow selective uptake of nucleosides by H^+-linked mechanisms (19, 101-106). *E. coli*, for example, obtain nucleotides for DNA and RNA synthesis by nucleoside salvage pathways as well as by *de novo* biosynthesis (19,107). Two major high-affinity nucleoside transport systems, designated NupC and NupG, have been identified in *E. coli*. NupG transports a broad range of purine and pyrimidine

nucleosides, but is resistant to inhibition by the uridine analog showdomycin (106). NupC, in contrast, is strongly inhibited by showdomycin and has a low affinity for guanosine and inosine (18,108-110).

The NupC and NupG genes have been mapped at 51.8 and 63.4 min, respectively, on the *E. coli.* chromosome (111,112). The NupG gene encodes a 418 amino acid polytopic membrane protein with a relative molecular mass of 45,000 (111) while that for NupC encodes a structurally unrelated 401 amino acid protein with a relative molecular mass of 43,000 (113). Other nucleoside transport systems may be present in *E. coli* (Dr. M.P. Gallagher, University of Edinburgh, personal communication), but have not yet been characterised.

1.3. TRANSPORT SYSTEMS FOR AMINO ACIDS

Amino acids have important metabolic functions, act as neurotransmitters and participate in cell volume regulation. Nine of the twenty amino acids commonly found in proteins are essential to the human diet. Amino acids are either neutral (zwitterionic), cationic or anionic, and mediated transport systems are required for their transport into or out of cells (114-118). Amino acid transporters are subject to substrate, hormonal and developmental control and have been identified as potential targets for oncogene action, as retrovirus receptors and as regulators of cell growth (119-124). Defective amino acid transport is responsible for several human inherited disorders, including cystinuria and lysine malabsorption syndrome (125,126). Generally, amino acid transport processes fall into four categories: Na^+-independent (equilibrative), Na^+-dependent, Na^+/Cl^--dependent and Na^+/K^+-dependent. Permeant selectivity can be relatively broad, but is usually restricted to neutral, cationic or anionic amino acids. Cells typically express multiple systems with overlapping substrate selectivities. Table 1.2 summarises the properties of the major mammalian amino acid transport systems that have been defined by functional analyses of amino acid fluxes in intact cells and plasma membrane vesicles.

Large neutral amino acids are mainly transported by Na^+-independent system L and Na^+-dependent system A (Table 1.2). System L prefers neutral amino acids with hydrophobic side chains, such as leucine and phenylalanine, and the amino acid analog 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) serves as a model substrate (127). System A has a broader selectivity for neutral amino acids than system L, is tolerant of N-methylation and is regulated by hormones and by amino acid availability (128,129). 2-methylaminoisobutyric acid (MeAIB) serves as a model system A substrate. Na^+-dependent System N mediates transport of glutamine, asparagine and histidine (130,131). Na^+/Cl^--dependent systems include transporters selective for glycine (system Gly), taurine (system beta) and proline (IMINO system)(132-137). The anionic amino acids aspartate and glutamate are transported by Na^+/K^+-dependent system X^-_{AG} (138,139).

Several functionally (and potentially structurally) related Na^+-interactive and Na^+-independent amino acid transport systems have apparently similar permeant recognition sites that bind the halucinogenic alkaloid harmaline (a Na^+-site inhibitor) and interact with both neutral and cationic amino acids (114-120,140-151). This group of

transporters includes systems ASC, asc, $b^{0,+}$, y^+ and y^+L (Table 1.2). The presence of both Na^+-dependent and Na^+-independent systems within the same family is a novel occurrence in transport biology and is a consequence of the way in which these transport systems structurally equate (Na^+ + neutral amino acid) with a cationic amino acid (114,140,144-147,152). System ASC mediates Na^+-dependent uptake of neutral amino acids of intermediate size such as serine, but is subjected to Na^+-independent inhibition by arginine (141,142,153). System asc has the same permeant selectivity as system ASC and is Na^+-independent (141,149). System $b^{0,+}$ mediates Na^+-independent transport of a wide range of neutral and cationic amino acids (150,154). Na^+-independent system y^+ is specific for cationic amino acids, but interacts with small neutral amino acids in the presence of Na^+ (148,149). System y^+L mediates Na^+-independent transport of cationic amino acids and Na^+-dependent transport of large neutral amino acids such as leucine (155-157). System $B^{0,+}$ catalyses Na^+-dependent transport of both neutral and cationic amino acids and may not be related to the above neutral/cationic amino acid transporters (158-160).

In the small intestine, absorption of essential amino acids such as leucine and lysine is a consequence of net uptake from the lumen into epithelial cells and, subsequently, efflux across the basolateral membrane towards the blood. Most mammalian cells transport leucine and lysine by separate mechanisms (systems A and L for leucine and system y^+ for lysine, Table 1.2). In the small intestine, in contrast, leucine transport across the brush border membrane is inhibited by lysine and lysine transport is inhibited by leucine (114,114,161-166). The presence of a $b^{0,+}$-type (Table 1.2) transport system in enterocytes has been invoked to account for this interaction (150), but the extent to which intestinal leucine and lysine transport are Na^+-dependent or Na^+-independent has not been determined unequivocally, and this is complicated by dietary and species variation as well as by differences down the length of the intestine (163,164,166). There is a consensus that the basolateral membrane of the intestine contains amino acid transport systems which are similar to those described in other mammalian cells, whereas in the brush border membrane additional, novel transport systems seem to be present (167-170).

Prior to 1991, almost nothing was known about the molecular properties of mammalian amino acid transporters. Recent progress in this area is reviewed in Section 1.5.

1.4. CLONING OF cDNAs ENCODING MEMBRANE TRANSPORTER PROTEINS BY FUNCTIONAL EXPRESSION IN *XENOPUS* OOCYTES

Isolated oocytes of *Xenopus laevis* accurately translate heterologous mRNA and have been used for the expression cloning of cDNAs of a number of membrane transporter and channel proteins for which antibody or oligonucleotide probes are not available (171). Expression of foreign proteins in *Xenopus* oocytes following injection with heterologous mRNA was pioneered by Gordon and Lane (172) using a procedure originally developed to inject nuclei into oocytes (173) and was first applied to membrane proteins by Sumikawa *et al* (174) who expressed specific ^{125}I-α-bungarotoxin binding sites

in the oocyte plasma membrane following injection of cells with mRNA from the electric organ of *Torpedo marmorata*. Fully mature oocytes from *Xenopus laevis* are extraordinarily large with a diameter of 1-1.2mm (0.5-0.9 μl) and can be injected with volumes with ranging from 10 to 50nl (175). Oocytes efficiently translate injected mRNA, process the nascent polypeptides, and target them to the appropriate subcellular compartment. Expression of membrane transporter proteins in oocytes can be readily determined by incubating the oocytes in a medium containing the appropriate radiolabelled substrate. Technically, oocytes are ideal for transport studies. Their large size means that initial rates of uptake (*i.e.*, transport) are sustained for much longer periods of time than can be achieved with alternative expression systems such as cultured mammalian cells, bacteria and yeast. Expression cloning is ideally suited to low membrane abundance transporter proteins because no prior purification of the protein of interest is required. The technique also avoids the danger of isolating and sequencing "false positive" clones, such as occurs in conventional antibody or oligonucleotide screening of cDNA libraries.

The strategy of using *Xenopus* oocytes for functional expression cloning of cDNAs encoding heterologous membrane transporter proteins requires, in the first instance, expression of the specific function of that protein in oocytes injected with total mRNA from an appropriate tissue/cell source. The mRNA can then be size-fractionated, the active fraction identified and, after reverse transcription into cDNA, used for the construction of a directional cDNA library in an appropriate expression plasmid. The total cDNA library or pools of clones derived from that library can then be transcribed into cRNA, capped and injected into oocytes. Subsequent assays for expression of transport function can be used to confirm the presence of potential full length clones in the library. A cDNA encoding that function may then be isolated by progressive subdivision of the library. Cloning by functional expression in *Xenopus* oocytes was first applied to membrane transporter proteins by Hediger and coworkers who isolated a cDNA encoding the rabbit intestinal Na^+-glucose cotransporter (SGLT1) (2). Other membrane transporter cDNAs to be isolated by this strategy include the rat hepatocyte Na^+/bile cotransporter (176), the Madin-Darby canine kidney (MDCK) cell Na^+/myoinositol cotransporter (177), the MDCK cell Na^+/betaine cotransporter (178), the rat renal Na^+/SO_4^{2-}cotransporter (179), the rabbit intestinal glutamate transporter (180), the rabbit renal facilitated urea transporter (181) and, most recently, the rabbit intestinal H^+/oligopeptide cotransporter (182).

In addition to mammalian membrane transporter proteins, cRNAs encoding bacterial channels and plant transporters have also been expressed in *Xenopus* oocytes. The glycerol facilitator GlpF is a channel protein in the *E. coli* inner membrane (183). Uptake of glycerol mediated by this protein was demonstrated in *Xenopus* oocytes following injection of GlpF cRNA and showed functional properties consistent with previous studies of GlpF in intact bacteria (184). Two plant transporters, the *Chlorella* H^+/monosaccharide symporter (HUP1) (185) and the *Arabidopsis* H^+/hexose symporter (STP1) (186) have also been expressed in *Xenopus* oocytes.

1.5. FAMILIES OF MEMBRANE TRANSPORTER PROTEINS

Five distinct gene families of mammalian organic solute/nutrient transporters and their procaryotic counterparts are presently recognised on the basis of amino acid sequence homology (Table 1.3). These are the equilibrative glucose transporter (GLUT) family (Section 1.5a), the Na^+-dependent glucose transporter (SGLT) family (Section 1.5b), the Na^+/Cl^--dependent neurotransmitter transporter (GAT) family (Section 1.5c), the Na^+/K^+-dependent glutamate transporter family (Section 1.5d), and the cationic amino acid transporter (MCAT) family (Section 1.5e). Transporter proteins belonging to these gene families have multiple potential transmembrane domains (6-14), with the most common number being 12. Distant structural similarities between divergent transporter gene families have led to the suggestion that most eucaryotic and procaryotic transporter proteins belong to two transporter superfamilies (187-193). Section 1.5f reviews the D2(rBAT)/4F2hc family. Members of this family of glycoproteins, in contrast to those described in Sections 1.5a-1.5e, have single transmembrane domains, but mediate amino acid transport activity when expressed in oocytes.

1.5a. Equilibrative Glucose Transporter (GLUT) Family

Equilibrative glucose transport in mammalian cells is mediated by a family of homologous membrane proteins. The cDNA encoding the first of these glucose transporter proteins (GLUT1) was isolated by immunologic screening of a human hepatoma HepG2 cDNA library with a polyclonal antibody raised against the purified human erythrocyte glucose transporter protein (1). GLUT1 is expressed in erythrocytes, placenta, blood-brain barrier, fetal tissues and transformed cells. GLUT2 is a high capacity, high K_m glucose transporter which is expressed at highest levels in the liver, pancreatic ß cells, and basolateral membranes of kidney and small intestine epithelia (194-196). GLUT3 is a brain-specific glucose transporter isoform in adults whose cDNA was isolated from a human fetal muscle cDNA library (197). GLUT4, the insulin-responsive glucose transporter protein, is found in muscle and adipose tissue (198-202). In the presence of insulin, GLUT4 is translocated to the plasma membrane from intracellular vesicles, increasing the V_{max} of glucose influx (203). GLUT5 appears to be localised in the brush border membrane of intestinal epithelial cells where it may participate fructose absorption (204,205). GLUT6 is a pseudogene with multiple stop codons and frame shifts and has a ubiquitous tissue distribution (204). GLUT7 is the hepatic microsomal glucose transporter (206). A consensus motif of six amino acid residues (KKMKND) at the C-terminus of GLUT7 has been identified as the region of sequence responsible for retention of the protein in the endoplasmic reticulum (206).

Analyses of the predicted amino acid sequences of GLUT1-7 show that they are highly homologous to each other (40%-68% amino acid identity). They also show sequence homology to a wide variety of sugar and other H^+-linked transporters in cyanobacteria, *E. coli*, yeast, algae, protozoa and plants (207). Common features of these transporters include a predicted 12 transmembrane domain topology with the N- and C-termini located intracellularly, a large extracellular loop between TM1 and TM2 and an even larger cytoplasmic loop between TM6 and TM7. Repetition of conserved motifs between the N- and C-terminal halves of the proteins suggest that the 12 transmembrane domain topology arose by gene duplication of an ancestral six

transmembrane domain protein. *Xenopus* oocytes injected with a mixture of two separate cRNAs corresponding to the N- and C-terminal halves of GLUT1 express glucose transport activity, while oocytes injected with either cRNA alone do not (208). Both halves of GLUT1 are therefore required for transport function, each representing a stable and separate domain which can noncovalently associate with the other in the plasma membrane to form a functional glucose transporter.

1.5b. Sodium-dependent Glucose Transporter (SGLT) Family

A cDNA encoding the Na^+-linked glucose cotransporter (SGLT1) from rabbit intestine was isolated by functional expression cloning in *Xenopus* oocytes (2). The cDNA of SGLT1 encodes a 664 amino acid integral membrane protein with 12 putative transmembrane domains. The predicted N-terminus does not contain a signal sequence and is suggested to reside on the cytoplasmic side of the cell membrane. SGLT1 exhibits no discernible sequence homology with the equilibrative glucose transporter (GLUT) family or the other transporter families described in Sections 1.5c-1.5e. Members of the Na^+-glucose cotransporter (SGLT) family includes a low affinity human renal Na^+/glucose cotransporter (SGLT2)(209), Na^+/nucleoside cotransporter (SNST1) from rabbit kidney (98), a Na^+/amino acid cotransporter (SAAT1) from porcine LLC-PK_1 cells (210), a Na^+/myoinositol cotransporter from dog kidney (SMIT1) (177), and two transporter proteins from *E. coli.*, the PutP Na^+/proline cotransporter (211), and the PanF Na^+/pantothenate cotransporter (212).

The SNST1 cDNA was isolated by screening a rabbit renal cDNA library with the rabbit renal SGLT1 cDNA (98). Expression of SNST1 in *Xenopus* oocytes resulted in a small increase (2-fold) of Na^+-dependent uridine uptake which was inhibited by both purine (adenosine, guanosine) and pyrimidine nucleosides (uridine, cytidine) and by 2',3'-dideoxycytidine. Message for SNST1 was absent from rabbit intestine but present in heart, a tissue that has not previously been shown to express Na^+-dependent nucleoside transport activity (98). Expression of SNST1 in Sf9 cells induced Na^+-dependent uptake of thymidine and formycin B (an analog of inosine) suggesting that SNST1 is a broad specificity N3-type nucleoside transporter (Table 1.1) (213).

The cDNA of SAAT1 was isolated by low stringency hybridisation screening of a LLC-PK_1 cDNA library (210). Expression of SAAT1 in COS-7 cells induced low-level Na^+-dependent neutral amino acid transport activity with transport characteristics corresponding to amino acid transport system A (Table 1.2). Message for SAAT1 was present in spleen, liver, muscle but not intestine. In contrast, the cDNA of SMIT1 was independently isolated by functional expression screening of a dog kidney library in *Xenopus* oocytes and subsequently shown to be homologous to SGLT1 (177).

The predicted amino acid sequences of SNST1, SAAT1 and SMIT1 are closely related to SGLT1 (61%, 74% and 46% identity, respectively). Hydropathy analyses indicate that each of these proteins have 12 potential transmembrane domains. Regions of particularly high homology include putative transmembrane domains 2,3,7 and 8. A structural motif (GL...AxxxxLxxxGR) is conserved in all members of the SGLT family and has been suggested to be related to cation recognition and/or binding (214). This sodium-binding (SOB) motif is also present in the glutamate transporter family (Section

1.5e), but is absent from the neurotransmitter (GAT) family (Section 1.5c). A conserved aspartate residue at position 28 in the human homolog of rabbit intestinal SGLT1 is mutated to an asparagine in human glucose/galactose malabsorption (215).

1.5c. Sodium/Chloride-dependent Neurotransmitter Transporter (GAT) Family

A number of amino acids and related metabolites serve as neurotransmitters to mediate chemical transmission across synapses. Specific high-affinity Na^+/Cl^--dependent transporters on the postsynaptic membrane mediate active reuptake of neurotransmitters present in the synaptic cleft to ensure rapid termination of synaptic transmission. The recent isolation of a large number of cDNAs encoding such neurotransmitter transporters have defined the gene family designated GAT (216,217).

In 1986, Radian and co-workers purified a Na^+-dependent γ-aminobutyric acid (GABA) transporter from rat brain (218). The sequence of the longest proteolytic fragment of the purified protein was used to design a oligonucleotide probe and subsequent hybridisation screening of a rat brain cDNA library with this probe led to the cloning of the first neurotransmitter transporter cDNA, GAT1 (219). Expression of this clone in *Xenopus* oocytes induced high-affinity Na^+/Cl^--dependent GABA transport. cDNAs encoding a diverse array of homologous neurotransmitter transporters have been isolated from neuronal tissue of various mammalian species and include other GABA transporters (GAT2, GAT3 and GAT4) (220), system Gly-type glycine transporters (GLYT1 and GLYT2) (221-223) (Table 1.2), dopamine transporters (DAT) (224-226), serotonin transporters (SERT) (227,228), a noradrenaline transporter (NET) (229), a system beta-type taurine transporter (TAUT) (230) (Table 1.2), an IMINO-type proline transporter (PROT) (231) (Table 2) and a choline transporter (CHOT) (232). A related hypertonicity-regulated betaine transporter (BGT) has also been isolated from dog kidney (178). Members of GAT family of transporters have deduced amino acid sequences of between 618 and 627 amino acid residues with sequence identity in the range of 30-65%. They share a common 12 putative transmembrane spanning domain topology in which the first 8 transmembrane sequences are highly conserved. There is a large extracellular hydrophilic loop between helices 3 and 4 which contains 1-4 potential N-linked glycosylation sites. Two tryptophan residues in helice 4 of the GAT protein are essential for transport activity (233,234).

1.5d. Sodium/Potassium-dependent Glutamate Transporter Family

Excessive synaptic concentrations of the neurotransmitter glutamate may cause cell death by over-activation of N-methyl-D-aspartate (NMDA) receptors and subsequent calcium entry. Neuronal and glial glutamate transporters (system X^-_{AG}, Table 1.2) function to ensure rapid reuptake of glutamate from the synaptic cleft. Recently, three high-affinity glutamate transporter cDNAs were isolated independently from rat brain (GLT-1 and GLAST)(235,236) and rabbit intestine EAAC1 (237). EAAC1 cDNA was isolated by expression cloning in Xenopus oocytes, while the GLT-1 and GLAST cDNAs were cloned by immunologic hybridisation screening using antibodies raised against a purified rat brain glutamate transport protein and a purified rat brain protein of unknown function, respectively.

Proteins encoded by these cDNAs have sizes ranging from 524 to 573 amino acid residues and are highly homologous (51-55% amino acid identity). These glutamate transporters are structurally and functionally unrelated to members of the GAT family of neurotransmitter transporters (238,239). Members of GAT family have 12 putative transmembrane domains and transport is driven by Na^+ and Cl^- cotransport. In contrast, the glutamate transporters have 6 to 10 putative transmembrane domains (235-237) and substrate uptake is driven by Na^+ and K^+/OH^- countertransport (240).

Data base searches show significant amino acid sequence homology between mammalian glutamate transporters and several bacterial transporters including the H^+-coupled glutamate transporter of *E. coli* (GltP) (241), the Na^+/H^+-coupled glutamate transporters (GltT) of *Bacillus stearothermophilus* and *Bacillus caldotenax* (242), and the C4-dicarboxylate transporter (DctA) of *Rhizobium meliloti* (243).

Two cDNAs (ASCT1 and SATT) were isolated from human brain by screening cDNA libraries for sequences related to rat glutamate transporters (242,245). The two cDNAs encode the same 532-amino acid protein which has 34%, 39% and 39% amino acid sequence identity to GLT-1, GLAST and EAAC1, respectively (242,243, Dr. B.K. Tamarappoo, personal communication). Expression of ASCT1/SATT cRNA in *Xenopus* oocytes elicited Na^+-dependent uptake of alanine, serine and cysteine (apparent K_m values 71, 88 and 29 μM, respectively) but not glutamate or MeAIB, and Northern blots revealed a ubiquitous tissue distribution of transporter mRNA (244,245). It was concluded that ASCT1/SATT was a Na^+-dependent ASC-type transporter (but please see Section 1.5e). Sequence homology between ASCT1/SATT and the rabbit/rat glutamate transporters is most pronounced in the C-terminal halves of the transporters and the motif AA(I,L,V)FIQA (residues 377-383 of EAAC1) is conserved in all transporters.

1.5e. Cationic Amino Acid Transporter (MCAT) Family

MCAT1 was obtained from mouse fibroblasts as an ecotropic murine retrovirus receptor (ecoR) of unknown function (122). Functional expression of MCAT1 in *Xenopus* oocytes identified its role as a Na^+-independent cationic amino acid transporter with y^+-type characteristics (123,124). The cDNA of MCAT1 encodes an integral membrane protein with 14 putative transmembrane domains. MCAT1 is widely expressed in mouse tissue and has a high apparent affinity for cationic amino acids (arginine apparent K_m value 70 μM). In contrast to expectations from functional studies (Section 1.3) MCAT1 is not related to the ASC-type human brain transporter (ASC1/SATT) described in Section 1.5d. Expression studies in this laboratory suggest that the latter transporter may not conform to "classic" system ASC as defined by functional studies in erythrocytes and other mammalian cells (A.W.I. Lo, University of Alberta, personal communication).

A cDNA encoding the human homolog of MCAT1 (HCAT) (87% amino acid sequence identity with MCAT1) was obtained by hybridisation screening of a human T-cell cDNA library (247) and cDNAs encoding two other MCAT isoforms (MCAT2A and MCAT2B, 61% identity to MCAT1) have also been cloned. MCAT2A and MCAT2B were obtained from murine hepatocytes and macrophages, respectively, and are identical in amino acid sequence except for a region of 41 amino acids between putative

transmembrane domains 8 and 9 (248,249). MCAT2A has an order of magnitude lower affinity for cationic amino acids (arginine apparent K_m 2.5 mM) than MCAT1 or MCAT2B and corresponds to a low-affinity hepatic y^+-type amino acid transporter defined previously in kinetic studies of cationic amino acid transport in that tissue (250). The transport properties of MCAT2B are similar to those of MCAT1 (249). Based upon sequence similarity, MCAT1, MCAT2A, MCAT2B and HCAT have been grouped with polyamine and choline transporters from yeast, fungi and eubacteria to form the APC gene family of membrane transporter proteins (191).

1.5f. D2(rBAT)/4F2hc Family

Two highly homologous cDNAs encoding putative amino acid transporters (82% amino acid sequence identity) were isolated simultaneously from rat kidney (D2 or NAA-Tr) (251,252) and rabbit kidney (rBAT) (253,254) by functional expression cloning in *Xenopus* oocytes. Expression of this transport activity, which had the characteristics of system $b^{o,+}$ (Table 1.2), was unaffected by treatment of oocytes with actinomycin-D (252). In contrast to the transporter proteins described in Sections 1.5a-1.5e, D2 and rBAT are type II membrane glycoproteins with single transmembrane domains (255). A leucine zipper motif at the C-terminus (residues 543 to 564 of rBAT) may be involved in the oligomerisation of D2/rBAT proteins with other transporter components.

An ability of D2 and its homologs to induce cystine and cationic amino acid transport in oocytes led to the consideration that this protein might be involved in the human autosomal recessive disease cystinuria, a disorder of cystine and cationic amino acid transport in epithelial cells of the renal tubule and the intestine (256). Defective kidney reabsorption of cystine and cationic amino acids results in precipitation of poorly soluble cystine in renal tubules, leading to the formation of cystine stones. Deposition of kidney stones causes obstruction of renal tubules, infection and ultimately renal insufficiency (257). Consistent with a potential physiological role of D2(rBAT) in cystine/cationic amino acid transport, transcripts for D2(rBAT) were present mainly in the kidney cortex and the small intestine, and immunocytochemical studies have localised D2(rBAT) to the microvilli of the kidney proximal tubule (S3 segment) (258-260).

Very recently, the human D2(rBAT) gene was mapped to chromosome 2p (261), the same location as the cystinuria gene (262). Six missense mutations in the human rBAT gene detected by single strand conformational polymorphism (SSCP) analysis segregated with the cystinuria phenotype and accounted for 30% of the cystinuria chromosomes studied (3). Expression of the most common D2(rBAT) mutation found in cystinuria patients (methionine 467 changed to threonine; M467T) abolished 80% of D2(rBAT)-induced amino acid transport activity in *Xenopus* oocytes (3). These findings are in accord with the hypothesis that D2(rBAT) is involved in epithelial cystine and cationic amino acid transport and that mutations in the D2(rBAT) gene causes cystinuria.

D2 and rBAT are homologous to the 4F2 heavy chain cell surface antigen (4F2hc) (29% amino acid identity, 45% amino acid similarity) which is widely expressed in proliferating cells (263-266). The 4F2 antigen is a 120-125 kDa disulfide-linked heterodimer glycoprotein which is composed of a heavy chain (85 kDa) and a light chain (40 kDa). The latter protein has not yet been characterised (267). The physiological

function of 4F2hc is not clear, but its expression is induced during the process of cell activation and proliferation (268). Expression of human 4F2hc in oocytes induces y^+L transport activity (Table 1.2) (265,266). Like D2(rBAT), 4F2hc is a type II membrane glycoprotein with a single transmembrane domain (255).

The fact that D2(rBAT) and 4F2hc have a structure which predicts a single transmembrane domain does not *a priori* exclude the possibility that they function as transporters. It is more likely, however, that they are transporter activators, perhaps analogous to the R1 modulator of SGLT (269). Evidence in this regard is presented in Chapter 5. A conserved cysteine residue in D2(rBAT) and 4F2hc (cysteine 103 of rBAT) may serve for covalent disulfide linkage with other transporter components.

1.6. RESEARCH OBJECTIVES

Transport of nucleosides and amino acids has been studied extensively in mammalian cells over the last 25 years and multiple transport systems have been defined on the basis of substrate specificity, kinetic properties, inhibitor susceptibility and ionic requirements. However, at the time I commenced my graduate studies in 1989, none had been identified unequivocally at the molecular level (except for the equilibrative nucleoside transporter of human erythrocytes) and no mammalian nucleoside and amino acid transporter cDNAs had been isolated. Experiments in my supervisor's laboratory demonstrated expression of multiple Na^+-dependent nucleoside transport activities in *Xenopus* oocytes injected with rat jejunal mRNA (100). As well, I was able to show parallel expression of Na^+-dependent and Na^+-independent leucine transport activities (8, Appendix 2). A previous study in my supervisor's laboratory to which I contributed also established that oocytes injected with rat jejunal mRNA expressed lysine transport activity, which was predominantly Na^+-independent (270). These studies established the basis for molecular cloning of cDNAs encoding these different nutrient transport activities.

The aim of my research project was to isolate and characterise cDNA(s) encoding nutrient transporter systems from rat jejunum by functional expression cloning in *Xenopus* oocytes. Specific research objectives were: (i) to isolate cDNAs encoding nucleoside and amino acid transport proteins (and/or their regulators) from a rat jejunal cDNA library by functional expression in *Xenopus* oocytes, (ii) to sequence these cDNAs and deduce the amino acid sequences, transmembrane topologies and evolution associations of the encoded proteins, (iii) to determine the transport characteristics of the cloned cDNAs by functional expression in *Xenopus* oocytes.

1.7. ORGANISATION OF THESIS

The remainder of this Thesis is divided into five Chapters. Chapter 2 describes the cloning and functional characterisation of a cDNA encoding a novel mammalian Na^+-dependent nucleoside transporter protein (cNT1) which exhibits the transport characteristics of Na^+-dependent system N2 (selective for physiological pyrimidine nucleosides and adenosine) (Table 1.1). cNT1 and its homolog NupC (a H^+/nucleoside

symporter from *E. coli*) form a new gene family of transporter proteins.

Chapter 3 describes the functional characterisation of cNT1-mediated transport of two nucleoside analogs (3'-azido-3'-deoxythymidine (AZT) and 2',3'-dideoxycytidine (ddC)) which are used in the treatment of acquired immunodeficiency syndrome (AIDS). The results presented in this Chapter demonstrate that AZT and ddC are permeants of cNT1. This is the first direct evidence that AZT enters cells by transporter-mediated processes as well as by passive diffusion and suggests that the human counterpart of cNT1 might be involved in the intestinal absorption and renal handling of AZT.

Chapter 4 describes the functional expression of NupC in a eucaryotic expression system, *Xenopus* oocytes. The experiments presented in this Chapter investigate the cation specificity of NupC and demonstrate that it has a substrate specificity similar to that of cNT1. This is the first demonstration of the functional expression of a bacterial membrane transporter protein in oocytes.

Chapter 5 describes the cloning of a cDNA encoding a rat jejunal 4F2hc glycoprotein by a combination of functional expression in *Xenopus* oocytes and diagnostic PCR (polymerase chain reaction). Rat 4F2hc and its homolog D2 were characterised functionally in *Xenopus* oocytes with respect to amino acid transport activity and were tested for their abilities to induce other nutrient fluxes. D2 cRNA induced uptake of pyruvate and uridine in oocytes, the latter flux having properties of Na^+-dependent system N3 (Table 1.1). 4F2hc cRNA also induced Na^+-dependent uridine uptake, but with a specificity different from systems N1, N2 or N3 (Table 1.1). These experiments indicate that 4F2hc and D2 may have physiological roles in addition to amino acid transport and suggest that they function not as transporters, but as transport modulators.

Chapter 6 is a general discussion of the results presented in previous Chapters. Appendix 1 describes reconstitution studies of neutral amino acid transport system L from rat erythrocytes (Table 1.2) and is the first reported reconstitution of an erythrocyte amino acid transporter. Appendix 2 describes the expression of novel Na^+-dependent and Na^+-independent leucine transport activities in *Xenopus* oocytes injected with rat jejunal mRNA.

Table 1.1 Nucleoside transport systems in mammalian cells

			Permeants	Inhibitors
I.	Equilibrative (Na^+-independent)			
	i)	*es*	Purine and pyrimidine nucleosides	NBMPR, dipyridamole and dilazep
	ii)	*ei*	Purine and pyrimidine nucleosides	Dipyridamole and dilazep
II.	Concentrative (Na^+-dependent)			
	i)	N1 (*cif*)	Purine nucleosides, uridine and formycin B	None
	ii)	N2 (*cit*)	Pyrimidine nucleosides and adenosine	None
	iii)	N3 (*cib*)	Purine and pyrimidine nucleosides	None
	iv)	N4 (*cit*)	Pyrimidine nucleosides, adenosine and guanosine	None
	v)	N5 (*cs*)	Not determined	NBMPR, dipyridamole and dilazep

Table 1.2. Amino acid transport systems

System	Substrate	Ion-dependence	Distribution
A	Most neutral amino acids, MeAIB	Na^+	Ubiquitous
ASC	Ala, Cys, Ser, Thr	Na^+	Ubiquitous
N	Gln, Asn, His	Na^+	Hepatocytes and muscle
Gly	Gly, sarcosine	Na^+,Cl^-	Ubiquitous
IMINO	Pro, pipecolic acid	Na^+,Cl^-	Small intestine brush-border
ß (beta)	ß-Ala, taurine	Na^+,Cl^-	Ubiquitous
X^-_{AG}	Glu, Asp	Na^+,Cl^- (OH^-)	Ubiquitous
L	Amino acids with branched and apolar side chains	-	Ubiquitous
T	Trp, Tyr, Phe	-	Human red blood cells and hepatocytes
asc	Ala, Ser, Cys, Thr	-	Ubiquitous
y^+	Arg, Lys, Ornithine	-	Ubiquitous
$b^{o,+}$	Neutral and cationic amino acids	-	Ubiquitous
y^+L	Neutral and cationic amino acids	Na^+ (for neutral amino acids)	Human red blood cells
$B^{o,+}$	Branched chain, bicyclic, and cationic amino acids	Na^+	Mouse blastocysts, sea urchin eggs

Table 1.3. Glossary of membrane transporter proteins

1) Equilibrative glucose transporter (GLUT) family

GLUT1	: Erythrocyte-type equilibrative glucose transporter
GLUT2	: Liver-type equilibrative glucose transporter
GLUT3	: Brain-type equilibrative glucose transporter
GLUT4	: Insulin-responsive equilibrative glucose transporter
GLUT5	: Small-intestine equilibrative glucose/fructose transporter
GLUT6	: Pseudogene sequence
GLUT7	: Hepatic microsomal equilibrative glucose transporter

2) Sodium-dependent glucose transporter (SGLT) family

SGLT1	: Rabbit intestinal Na^+-dependent glucose transporter
SGLT2	: Human renal Na^+-dependent glucose transporter
SNST1	: Rabbit renal Na^+-dependent nucleoside transporter
SAAT1	: Porcine LLC-PK_1 Na^+-dependent system A-type amino acid transporter
SMIT1	: Dog renal Na^+-dependent myoinositol transporter
PutP	: *E. coli* Na^+-dependent proline transporter
PanF	: *E. coli* Na^+-dependent pantothenate transporter

3) Sodium/chloride-dependent neurotransmitter transporter (GAT) family

GAT1	: Rat brain Na^+/Cl^--dependent GABA transporter
GAT2	: Mouse brain Na^+/Cl^--dependent GABA transporter
GAT3	: Mouse brain Na^+/Cl^--dependent GABA transporter
GAT4	: Mouse brain Na^+/Cl^--dependent GABA transporter
GLYT1	: Rat brain Na^+/Cl^--dependent glycine transporter
GLYT2	: Mouse brain Na^+/Cl^--dependent glycine transporter
SERT	: Rat brain Na^+/Cl^--dependent serotonin transporter
NET	: Human brain Na^+/Cl^--dependent noradrenaline transporter
TAUT	: Mouse brain Na^+/Cl^--dependent taurine transporter
PROT	: Rat brain Na^+-dependent proline transporter
CHOT	: Rat brain Na^+-dependent choline transporter
BGT	: Dog renal Na^+/Cl^--dependent betaine transporter

/...

Table 1.3. Glossary of membrane transporter proteins (continued)

4) Sodium/potassium-dependent glutamate transporter family

GLT-1	: Rat brain Na^+/K^+-dependent glutamate transporter
GLAST	: Rat brain Na^+/K^+-dependent glutamate/aspartate transporter
EAAC1	: Rabbit intestinal Na^+/K^+-dependent glutamate transporter
GltP	: *E. coli* H^+-dependent glutamate transporter
GltT	: *B. stearothermophilus* and *B. caldotenax* Na^+/H^+-dependent glutamate transporters
DctA	: *R. meliloti* C4-dicarboxylate transporter
ASCT/SATT	: human brain Na^+-dependent ASC-type amino acid transporter

5) Cationic amino acid transporter (MCAT) family

MCAT1	: Mouse fibroblast y^+-type transporter
HCAT	: Human T-cell y^+-type transporter
MCAT2A	: Mouse low-affinity hepatocyte y^+-type transporter
MCAT2B	: Mouse macrophage y^+-type transporter

6) D2(rBAT)/4F2hc family (putative transport regulatory family)

D2/NAA-Tr	: Rat renal system $b^{o,+}$-type amino acid transport activator
rBAT	: Rabbit renal system $b^{o,+}$-type transport activator
4F2hc	: Human/mouse system y^+L amino acid transport activator

1.7. BIBLIOGRAPHY

1. Mueckler, M., Caruso, C., Baldwin, S., Panico, M., Blench, I., Morris, H., Allard, W.J., Lunhard, G., and Lodish, H. (1985). Sequence and structure of a human glucose transporter. *Science* **229**, 941-945.

2. Hediger, M.A., Coady, M.J., Ikeda, T.S., and Wright, E.M. (1987). Expression cloning and cDNA sequencing of the Na^+/glucose cotransporter. *Nature* **330**, 379-381.

3. Huang, Q.Q., Yao, S.Y.M., Ritzel, M.W.L., Paterson, A.R.P., Cass, C.E., and Young, J.D. (1994). Cloning and functional expression of a complementary DNA encoding a mammalian nucleoside transport protein. *J. Biol. Chem.* **269**, 17757-17760.

4. Calonge, M.J., Gasparini, P., Chillaron, J., Chillon, M., allucci, M., Rousaud, F., Zelante, L., Testar, X., Dallapoccola, B., Silverio, F.D., Barcelo, P., Estivill, X., Zorzano, A., Nunes, V., and Palacin, M. (1994). Cystinuria caused by mutations in rBAT, a gene involved in the transport of cystine. *Nature Genet* **6**, 420-425.

5. Yao, S.Y.M., George, R., and Young, J.D. (1993). Reconstitution studies of amino acid transport system L in rat erythrocytes. *Biochem. J.* **292**, 655-660.

6. Yao, S.Y.M., George, R., and Young, J.D. (1994). Purification and N-terminal amino acid sequence analysis of a CD36-like membrane glycoprotein from rat erythrocytes. (*J. Biol. Chem.*, in preparation).

7. Greenwalt, D.E., Lipsky, R.H., Ockenhouse, C.F., Ikeda, H., Tandon, N.N., and Jamieson, G.A. (1992). Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction, and transfusion medicine. *Blood* **80**, 1105-1115.

8. Yao, S.Y.M., Muzyka, W.R., Elliott, J.F., Cheeseman, C.I., and Young, J.D. (1994). Poly(A^+)RNA from the mucosa of rat jejunum induces novel Na^+-dependent and Na^+-independent leucine transport activities in oocytes of *Xenopus laevis*. *Mol. Membr. Biol.* **11**, 109-118.

9. Young, J.D., Paterson, A.R.P., and Henderson, J.F. (1985). Nucleoside transport and metabolism in erythrocytes from the Yucatan minature pig. Evidence that inosine functions as *in vivo* energy substrate. *Biochim. Biophys. Acta* **842**, 214-224.

10. Young, J.D., Jarvis, S.M., Clanachan, A.S., Henderson, J.F. and Paterson, A.R.P. (1986). Nitrobenzylthioinosine-an *in vivo* inhibitor of pig erythrocyte energy metabolism. *Am. J. Physiol.* **251**, C90-C94.

11. Jacobson, K.A., Daly, J.W. and Mangeniello, V. (1990). Purines in Cellular Signalling: targets for new drugs. Springer Verlag, New York.

12. Belardinelli, L., Linden, J., and Berne, R.M. (1989). The cardiac effects of adenosine. *Prog. Cardiovasc. Dis.* **32**, 73-97.

13. Dacal, J.-L., Nehlig, A., and Nicolas, F. (1991). Physiological and pharmacological properties of adenosine: therapeutic implications. *Life Sci.* **49**, 1435-1453.

14. Bonnet, P.A., and Robins, R.K. (1993). Modulation of leukocyte genetic expression by novel purine nucleoside analogues. A new approach to antitumor and antiviral agents. *J. Med. Chem.* **36**, 635-653.

15. Langtry, H.D. and Campoli-Richards, D.M. (1989). Zidovudine, a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy. *Drugs* **37**, 408-450.

16. Clumeck, N. (1993). Current use of anti-HIV drugs in AIDS. *J. Antimicrob. Chemother* **32**, Supp.A 133-138.

17. Perigaud, C., Gosselin, G., and Imbach, J.-L. (1992). Nucleoside analogues as chemotherapeutic agents: a review. *Nucleosides and Nucleotides* **11**, 903-945.

18. Markert, M.L., Hershfield, M.S., Schiff, R.I.,and Buckley, R. (1987). Adenosine deaminase and purine nucleoside phosphorylase deficiencies: evalution of therapeutic intervention in eight patients. *J. Clin. Immunol.* **7**, 389-399.

19. Munch-Petersen, A., and Mygind, B. (1983). Transport of nucleic acid precursor. In *Metabolism of nucleotides, nucleosides and nucleobases in microorganisms* (ed. Munch-Petersen, A.), Academic Press, London, pp258-305.

20. Cass, C.E., Belt, J.A., and Paterson, A.R.P. (1987). Adenosine transport in cultured cells. *Prog. Clin. Biol. Res.* **230**, 13-40.

21. Paterson, A.R.P., and Cass, C.E. (1986). Transport of nucleoside drugs in animal cells. In *Membrane transport of antineoplasmic agents.* (ed. Goldman, I.D.), Pergamon Press, Oxford, pp309-329.

22. Young, J.D., and Jarvis, S.M. (1983). Nucleoside transport in animal cells. *Biosci. reports* **3**, 309-322.

23. Plagemann, P.G.W., Wohlhueter, R.M., and Woffendin, C. (1988). Nucleoside and nucleobase transport in animal cells. *Biochim. Biophys. Acta* **947**, 405-443.

24. Gati, W.P. and Paterson, A.R.P. (1989). Nucleoside transport. In *Red blood cell membranes.* (eds. Agre, P. and Parker, J.C.), Marcel Dekker, New York, pp635-661.

25. Cabantchik, Z.I. (1989). Nucleoside transport across red cell membranes. *Methods Enzymol.* **230**, 13-40.

26. Paterson, A.R.P., Clanachan, A.S., Craik, J.D., Gati, W.P., Jakobs, J.S., and Cass, C.E. Plasma membrane transport of nucleosides, nucleobases and nucleotides: an overview. In *Role of adenosine and adenine nucleotides in biological systems.* (eds. Imai, S., and Nakazawa, M.), Elsevier Science Publisher BV, pp133-148.

27. Zimmerman, T.P., Mahony, W.B., and Prus, K.L. (1987). 3'-Azido-3'-deoxythymidine, an unusual nucleoside analogue that permeates the membrane of human erythrocytes and lymphocytes by non-facilitated diffusion. *J. Biol. Chem.* **262**, 5748-5754.

28. Domin, B.A., Mahony, W.B., and Zimmerman, T.P. (1993). Membrane permeation mechanisms of 2',3'-dideoxynucleosides. *Biochem. Pharmacol.* **46**, 725-729.

29. Cass, C.E., Gaudette, L.A., and Paterson, A.R.P. (1974). Mediated transport of nucleosides in human erythrocytes. Specific binding of the inhibitor nitrobenzylthioinosine to nucleoside transport sites in the erythrocyte membrane. *Biochim. Biophys. Acta* **345**, 1-10.

30. Cass, C.E., and Paterson, A.R.P. (1976). Nitrobenzylthioinosine binding sites in the erythrocyte membrane. *Biochim. Biophys. Acta* **419**, 285-294.

31. Jarvis, S.M., and Young, J.D. (1980). Nucleoside transport in human and sheep erythrocytes: evidence that nitrobenzylthioinosine binds specifically to functional nucleoside transport sites. *Biochem. J.* **190**, 377-383.

32. Dahlig-Harvey, E., Eilam, Y., Paterson, A.R.P., and Cass, C.E. (1981). Binding of nitrobenzylthioinosine to high-affinity sites on the nucleoside transport mechanism of HeLa cells. *Biochem. J.* **200**, 295-305.

33. Belt, J.A. (1983). Nitrobenzylthioinosine-insensitive uridine transport in human lymphoblastoid and murine leukemia cells. *Biochem. Biophys. Res. Commun.* **110**, 417-423.

34. Belt, J.A. (1983). Heterogeneity of nucleoside transport in mammalian cells. Two types of transport activity in L1210 and other cultured neoplastic cells. *Mol. Pharmacol.* **24**, 479-484.

35. Belt, J.A., and Noel, L.D. (1985). Nucleoside transport in Walker 256 rat carcinosarcoma and S49 mouse lymphoma cells. *Biochem. J.* **232**, 681-687.

36. Plagemann, P.G.W. and Wohlhueter, R.M. (1985). Nitrobenzylthioinosine-sensitive and -resistant nucleoside transport in normal and transformed rat cells. *Biochim. Biophys. Acta* **816**, 387-395.

37. Jarvis, S.M., and Young, J.D. (1986). Nucleoside transport in rat erythrocytes: two components with differences in sensitivity to inhibition by nitrobenzylthioinosine and p-chloromercuriphenyl sulphonate. *J. Membr. Biol.* **43**, 1-10.

38. Jarvis, S.M., McBride, D., and Young, J.D. (1982). Erythrocyte nucleoside transport: asymmetrical binding of nitrobenzylthioinosine to nucleoside permeation sites. *J. Physiol.* **324**, 31-46.

39. Agbanyo, F.R., Paterson, A.R.P., and Cass, C.E. (1988). External location of sites on pig erythrocytes that bind nitrobenzylthioinosine. *Mol. Pharmacol.* **33**, 332-337.

40. Plagemann, P.G., Wohlhueter, R.M. (1984). Nucleoside transport in cultured mammalian cells. Multiple forms with different sensitivity to inhibition by nitrobenzylthioinosine or hypoxanthine. *Biochim. Biophys. Acta* **773**, 39-52.

41. Plagemann, P.G.W., and Woffendin, C. (1988). Species differences in sensitivity of nucleoside transport in erythrocytes and cultured cells to inhibition by nitrobenzylthioinosine, dipyridamole, dilazep and lidoflazine. *Biochim. Biophys. Acta* **969**, 1-8.

42. Hammond, J.R. (1991). Comparative pharmacology of the nitrobenzylthioguanosine-sensitive and -resistant nucleoside transport mechanisms of Ehrlich ascites tumor cells. *J. Pharmacol. Exp. Ther.* **259**, 799-807.

43. Jones, K.W., And Hammond, J.R. (1992). Heterogeneity of [^{3}H]dipyridamole binding to CNS membranes: correlation with [^{3}H]nitrobenzylthioinosine binding and [^{3}H]uridine influx studies. *Neurochem.* **59**, 1363-1371.

44. Boleti, H., and Cass, C.E. (1992). Nitrobenzylthioinosine-insensitive nucleoside transport processes of K562 cells. *Proc. Amer. Assoc. Cancer. Res.* **33**, 18.

45. Young, J.D., Jarvis, S.M., Robins, M.J., and Paterson, A.R.P. (1983). Photoaffinity labelling of the human erythrocyte nucleoside transporter by N^6-(p-azidobenzyl)adenosine and nitrobenzylthioinosine. Evidence that the transporter is a band 4.5 polypeptide. *J. Biol. Chem.* **258**, 2202-2208.

46. Jarvis, S.M., and Young, J.D. (1987) Photoaffinity labelling of nucleoside transporter polypeptides. *Pharmac. Therapeut.* **32**, 339-359.

47. Jarvis, S.M., and Young, J.D. (1981). Extraction and partial purification of the nucleoside-transport system from human erythrocytes based on the assay of nitrobenzylthioinosine-binding activity. *Biochem. J.* **194**, 331-339.

48. Wu, J.S.R., Kwong, F.Y.P., Jarvis, S.M., and Young, J.D. (1983). Identification of the erythrocyte nucleoside transporter as a band 4.5 polypeptide. Photoaffinity labelling studies using nitrobenzylthioinosine. *J. Biol. Chem.* **258**, 13745-13751.

49. Kwong, F.Y.P., Tse, C.M., Jarvis, S.M., Choy, M.Y., and Young, J.D. (1987). Purification and reconstitution studies of the nucleoside transporter from pig erythrocytes. *Biochim. Biophys. Acta* **904**, 105-116.

50. Kwong, F.Y.P., Davies, A., Tse, C.M., Young, J.D., Henderson, P.J.F., and Baldwin, S.A. (1988). Purification of the human erythrocyte nucleoside transporter by immunoaffinity chromatography. *Biochem. J.* **255**, 243-249.

51. Shi, M.M., Wu, J.S.R., Lee, C.M., and Young, J.D. (1984). Nucleoside transport. Photoaffinity labelling of high-affinity nitrobenzylthioinosine binding site in rat and guinea pig lung. *Biochem. Biophys. Res. Commun.* **118**, 584-600.

52. Wu, J.S.R., and Young, J.D. (1984). Photoaffinity labelling of nucleoside-transport proteins in plasma membranes isolated from rat and guinea-pig liver. *Biochem. J.* **220**, 499-506.

53. Young, J.D., Jarvis, S.M., Belt, J.A., Gati, W.P., and Paterson, A.R.P. (1984). Identification of the nucleoside transporter in cultured mouse lymphoma cells. Photoaffinity labelling of plasma enriched fractions from nucleoside transport competent (S49) and nucleoside transport-deficient (AE1) cells with [^{3}H]nitrobenzylthioinosine. *J. Biol. Chem.* **259**, 8363-8365.

54. Kwan, K.J., and Jarvis, S.M. (1984). Photoaffinity labelling of adenosine transporter in cardiac membranes with nitrobenzylthioinosine. *Am. J. Physiol.* **246**, H710-H715.

55. Jarvis, S.M., and Ng, A.S. (1985). Identification of the adenosine uptake sites in guinea pig brain. *J. Neurochem.* **44**, 183-188.

56. Crawford, C.R., Ng, C.Y.C., Ullman, B., and Belt, J.A. (1990). Identification and reconstitution of the nucleoside transporter of CEM human leukemia cells. *Biochim. Biophys. Acta* **1024**, 289-297.

57. Hogue, D.L., Hodgson, K.C. and Cass, C.E. (1990). Effects of inhibition of N-linked glycosylation by tunicamycin on nucleoside transport polypeptides of L1210 leukemia cells. *Biochem. Cell Biol.* **68**, 199-209.

58. Boumah, C.E., Hogue, D.L., and Cass, C.E. (1992). Expression of high levels of nitrobenzylthioinosine-sensitive nucleoside transport in cultured human choriocarcinoma (BeWo) cells. *Biochem. J.* **288**, 987-996.

59. Kwong, F.Y.P., Fincham, H.E., Davies, A., Beaumont, N., Henderson, P.J.F., Young, J.D., and Baldwin, S.A. (1992). Mammalian nitrobenzylthioinosine-sensitive nucleoside transport proteins. *J. Biol. Chem.* **267**, 21954-21960.

60. Beaumont, N., Baldwin, S.A., Cass, C.E., and Young, J.D. (1994). In *Adenosine and adenine nucleotides: from molecular biology to integrative physiology*. (eds. Belardinelli, L., and Pelleg, A.), Kluwer Academic Publishers, Norwell, M.A. (in press).

61. Kwong, F.Y.P., Wu, J.S.R., Shi, M.M., Fincham, H.E., Davies, A., Henderson, P.J.F., Baldwin, S.A., and Young, J.D. (1993). Enzymatic cleavage as a probe of the molecular structures of mammalian equilibrative nucleoside transporters. *J. Biol. Chem.* **268**, 22127-22134.

62. Wu, J.S.R., Jarvis, S.M., and Young, J.D. (1983). The human erythrocyte nucleoside and glucose transporters are both band 4.5 membrane polypeptides. *Biochem. J.* **214**, 995-997.

63. Barros, L.F., Beaumont, N., Jarvis, S.M., Young, J.D., Henderson, P.J.F., Yudilevich, D.L., Thrasivoulou, C., and Baldwin, S.A. (1992). Immunological detection of nucleoside transporters in human placental trophoblast brush-border plasma membranes and placental capillary endothelial cells. *J. Physiol. London* **452**, 348P.

64. Barros, L.F., Beaumont, N., Jarvis, S.M., Young, J.D., Yudelivich, D.L., Thrasivoulou, C., and Baldwin, S.A. (1994). Immunological detection of nucleoside transporters in human placenta. *Eur. J. Physiol.* (in press).

65. Boumah, C.E., Harvey, C.M., Paterson, A.R.P., Baldwin, S.A., Young, J.D., and Cass, C.E. (1994). Functional expression of the nitrobenzylthioinosine-sensitive nucleoside transporter of human choriocarcinoma (BeWo) cells in isolated oocytes of *Xenopus laevis*. *Biochem. J.* **299**, 769-773.

66. Boumah, C.E., Harvey, C.M., Paterson, A.R.P., Baldwin, S.A., Young, J.D., and Cass, C.E. (1993). Candidate cDNA clones encoding the nitrobenzylthioinosine-sensitive transporter of nucleosides in cultured human choriocarcinoma (BeWo) cells. *Biochem. Cell. Biol.* **71**, AXVI.

67. Cass, C.E., Boumah, C.E., Hogue, D.L., Baldwin, S.A., and Young, J.D. (1994). Molecular cloning of nucleoside transport proteins. *Drug Development Research* **31**, 255.

68. Hogue, D.L., Ellison, M.J., Young, J.D., and Cass, C.E. (1994). Molecular cloning of a cDNA encoding a novel protein associated with golgi membranes of mammalian cells by complementation of the thymidine transport deficiency of *Saccharomyces cerevisiae*. *EMBO J.* (submitted).

69. Plagemann, P.G.W., and Aran, J.M. (1990). Characterization of Na^+-dependent, active nucleoside transport in rat and mouse peritoneal macrophages, a mouse macrophage cell line and normal rat kidney cells. *Biochim. Biophys. Acta* **1028**, 289-298.

70. Plagemann, P.G.W. (1991). Na^+-dependent, concentrative nucleoside transport in rat macrophages. Specificity for natural nucleosides and nucleoside analogs, including dideoxynucleosides, and comparison of nucleoside transport in rat, mouse and human macrophages. *Biochem. Pharmacol.* **42**, 247-252.

71. Che, M., Nishida, T., Gatmaitan, Z., and Arias, I.M. (1992). A nucleoside transporter is functionally linked to ectonucleotidases in rat liver canalicular membrane. *J. Biol. Chem.* **267**, 9684-9688.

72. Crawford, C.R., and Belt, J.A. (1991). Sodium-dependent, concentrative nucleoside transport in Walker 256 rat carcinosarcoma cells. *Biochem. Biophys. Res. Commun.* **715**, 846-851.

73. Crawford, C.R., Ng, C.Y.C., Noel, D., Belt, J.A. (1990). Nucleoside transport in L1210 murine leukemia cells. *J. Biol. Chem.* **265**, 9732-9736.

74. Dagnino, L., and Paterson, A.R.P. (1990). Sodium-dependent and equilibrative nucleoside transport systems in L1210 mouse leukemia cell: effect of inhibitors of equilibrative systems on the content and retention of nucleosides. *Cancer Res.* **50**, 6549-6553.

75. Dagnino, L., Bennett, L.L., and Paterson, A.R.P. (1991). Sodium-dependent nucleoside transport in mouse leukemia L1210 cells. *J. Biol. Chem.* **266**, 6308-6311.

76. Dagnino, L., Bennett, L.J., and Paterson, A.R.P. (1991). Substrate specificity, kinetics, and stoichiometry of sodium-dependent adenosine transport in L1210/AM mouse leukemia cells. *J. Biol. Chem.* **266**, 6312-6317.

77. Plagemann, P.G.W. (1991). Na^+-dependent, active nucleoside transport in S49 mouse lymphoma cells and loss in AE_1 mutant deficient in facilitated nucleoside transport. *J. Cell. Biochem.* **46**, 54-59.

78. Darnowski, J.W., Holdridge, C., and Handschumacher, R.E. (1987). Concentrative uridine transport by murine splenocytes: kinetics, substrate specificity, and sodium dependency. *Cancer Res.* **47**, 2614-2619.

79. Plagemann, P.G.W., Aran, J.M., and Woffendin, C. (1990). Na^+-dependent, active and Na^+-independent, facilitated transport of formycin B in mouse spleen lymphocytes. *Biochim. Biophys. acta.* **1022**, 93-102.

80. Vijayalakshimi, D. and Belt, J.A. (1988). Sodium-dependent nucleoside transport in mouse intestinal epithelial cells. Two transport systems with differing substrate specificities. *J.Biol. Chem.* **263**, 19419-19423.

81. Williams, T.C., and Jarvis, S.M. (1991). Multiple sodium-dependent nucleoside transport systems in bovine renal brush-border membrane vesicles. *Biochem. J.* **274**, 27-33.

82. Le Hir, M., and Dubach, U.C. (1984). Sodium gradient-energized concentrative transport of adenosine in renal brush border vesicles. *Pflugs Arch.* **401**, 58-63.

83. Le Hir, M. (1990). Evidence for separate carriers for purine nucleosides and for pyrimidine nucleosides in the renal brush border membrane. *Renal Physiol. Biochem.* **13**, 154-161.

84. Williams, T.C., Doherty, A.J., Griffith, D.A., and Jarvis, S.M. (1989). Characterization of sodium-dependent and sodium-independent nucleoside transport systems in rabbit renal brush-border and basolateral plasma-membrane vesicles from the renal outer cortex. *Biochem. J.* **264**, 223-231.

85. Betcher, S.L., Forrest, J.N.Jr., Knickelbein, R.G., and Dobbins, J.W. (1990). Sodium-adenosine cotransport in brush-border membranes from rabbit ileum. *Am. J. Physiol.* **259**, G504-G510.

86. Roden, M., Paterson, A.R.P., and Turnheim, K. (1991). Sodium-dependent nucleoside transport in rabbit intestinal epithelium. *Gastroenterology* **100**, 1553-1562.

87. Jarvis, S.M. (1989). Characterization of sodium-dependent nucleoside transport in rabbit intestinal brush-border membrane vesicles. *Biochim. Biophys. Acta* **979**, 132-138.

88. Lee, C.W., Cheeseman, C.I., and Jarvis, S.M. (1990). Transport characteristics of renal brush-border Na^+- and K^+-dependent uridine carriers. *Am. J. Physiol.* **258**, F1203-F1210.

89. Belt, J.A., Marina, N.M., Phelps, D.A., and Crawford, C.R. (1993). Nucleoside transport in normal and neoplastic cells. *Adv. Enzyme Regul.* **33**, 235-252.

90. Belt, J.A., and Harper, E., Byl, J. (1992). Na^+-dependent nucleoside transport in human myeloid leukemia cell lines and freshly isolated myeloblasts. *Cancer Res.* **33**, 20.

91. Wu, X., Yuan, G., Brett, C.M., Hui, A.C., and Giacomini, K.M. (1992). Sodium-dependent nucleoside transport in choroid plexus from rabbit. Evidence for a single transporter for purine and pyrimidine nucleosides. *J. Biol. Chem.* **267**, 8813-8818.

92. Wu, X., Gutierrez, M.M., and Giacomini, K.M. (1994). Further characterization of the sodium-dependent nucleoside transporter (N3) in choroid plexus from rabbit. *Biochim. Biophys. Acta* **1191**, 190-196.

93. Gutierrez, M.M., Brett, C.M., Ott, R.J., Hui, A.C., and Giacomini, K.M. (1992). Nucleoside transport in brush border membrane vesicles from human kidney. *Biochim. Biophys. Acta* **1105**, 1-9.

94. Gutierrez, M.M., and Giacomini, K.M. (1993). Substrate selectivity, potential sensitivity and stoichiometry of Na^+-nucleoside transport in brush-border membrane vesicles from human kidney. *Biochim. Biophys. Acta* **1149**, 202-208.

95. Paterson, A.R.P., Gati, W.P., Vijayalakshmi, D. (1993). Inhibitor-sensitive, Na^+-linked transport of nucleoside analogs in leukemia cells from patients. *Proc. Amer. Assoc. Cancer Res.* **34**, 14.

96. Jarvis, S.M., and Griffith, D.A. (1991). Expression of the rabbit intestinal N2 Na^+/nucleoside transporter in *Xenopus leavis* oocytes. *Biochem. J.* **278**, 605-607.

97. Terasaki, T., Kadowaki, A., Higashida, S., Nakayama, K., Tamai, I., and Tsuji, A. (1993). Expression of the Na^+-dependent transport system of rabbit small intestine: studies with mRNA-injected *Xenopus laevis* oocytes. *Biol. Pharm. Bull.* **16**, 493-496.

98. Pajor, A.M., and Wright, E.M. (1992). Cloning and functional expression of a mammalian Na^+/nucleoside cotransporter. A member of the SGLT family. *J. Biol. Chem.* **267**, 3557-3560.

99. Wu, X., and Giacomini, K.M. (1994). Expression of the choroid plexus sodium-nucleoside cotransporter (N3) in *Xenopus laevis* oocytes. *Biochem. Pharmacol.* **48**, 432-434.

100. Huang, Q.-Q., Harvey, C.M., Paterson, A.R.P., Cass, C.E. and Young, J.D. (1993). Functional expression of Na^+-dependent nucleoside transport systems of rat intestine in isolated oocytes of *Xenopus laevis*. Demonstration that rat jejunum expresses the purine-selective system N1 (*cif*) and a second novel system N3 having board specificity for purine and pyrimidine nucleosides. *J. Biol. Chem.* **268**, 20613-20619.

101. Peterson, R.N., and Koch, A.L. (1966). The relationship of adenosine and inosine transport in *Escherichia coli*. *Biochim. Biophys. Acta* **126**, 129-145.

102. Peterson, R.N., Boniface, J., and Koch, A.L. (1967). Energy requirements, interactions and distinctions in the mechanisms for transport of various nucleosides in *Escherichia coli*. *Biochim. Biophys. Acta* **135**, 771-783.

103. Roy-Burman, S., and Visser, D.W. (1975). Transport of purines and deoxyadenosine in *Escherichia coli*. *J. Biol. Chem.* **250**, 9270-9275.

104. Roy-Burman, S., von Dippe, P., and Visser, D.W. (1978). Mechanism of energy coupling for transport of deoxycytidine, uridine, uracil, adenine and hypoxanthine in *Escherichia coli*. *Biochim. Biophys. Acta* **511**, 285-296.

105. Leung, K.-K., and Visser, D.W. (1977). Uridine and cytidine transport in *Escherichia coli* B and transport-deficient mutants. *J. Biol. Chem.* **252**, 2492-2497.

106. Munch-Petersen, A., Mygind, B., Nicolaisen, A., and Pihl, N.J. (1979). Nucleoside transport in cells and membrane vesicles from *Escherichia coli* K12. *J. Biol. Chem.* **254**, 3730-3737.

107. Hammer-Jespersen, K. (1983). Nucleoside catabolism. In *Metabolism of nucleosides and nucleobases in microorganisms.* (ed. Munch-Petersen, A.), Academic Press, London, pp203-238.

108. Komatsu, Y., and Tanaka, K. (1971). Mechanism of action of showdomycin. IV. Interactions between the mechanisms for transport of showdomycin and various nucleosides in *Escherichia coli. Agric. Biol. Chem.* **35**, 1328-1339.

109. Komatsu, Y., and Tanaka, K. (1972). A showdomycin resistant mutant of *Escherichia coli* K12 with altered nucleoside transport character. *Biochim. Biophys. Acta* **288**, 390-403.

110. Munch-Petersen, A., and Mygind, B. (1976). Nucleoside transport systems in *Escherichia coli* K12: specificity and regulation. *J. Cell. Physiol.* **89**, 551-560.

111. Westh Hansen, S.E., Jensen, N., and Munch-Petersen, A. (1987). Studies on the sequence and structure of the *Escherichia coli* K-12 *nupG* gene, encoding a nucleoside-transport system. *Eur. J. Biochem.* **168**, 385-391.

112. Zhang, Y., Craig, J.E., and Gallagher, M.P. (1992). Location of the *nup*C gene on the physical map of *Escherchia coli* K-12. *J. Bacteriol.* **174**, 5758-5759.

113. Craig, J.E., Zhang, Y., and Gallagher, M.P. (1994). Cloning of the *nup*C gene of *Escherichia coli* encoding a nucleoside transport system, and identification of an adjacent insertion element, IS186. *Mol. Microbiol.* **11**, 1159-1168.

114. Christensen, H.N. (1979). Exploiting amino acid structure to learn about membrane transport. *Adv. Enzymol.* **49**, 41-101.

115. Christensen H.N. (1984). Organic ion transport during seven decades. The amino acids. *Biochim. Biophys. Acta* **779**, 255-269.

116. Kilberg, M.S., Stevens, B.R., and Novak, D.A. (1993). Recent advances in mammalian amino acid transport. *Annu. Rev. Nutr.* **13**, 137-165.

117. Souba, W.W., and Pacitti, A.J. (1992). How amino acids get into cells: mechanisms, models, menus, and mediators. *J.P.E.N.* **16**, 569-578.

118. Guidotti, G.G. (1993). Amino acid transport systems. *Ital. J. Gastroenterol.* **25**, 37-41.

119. Amino acid transport in eucaryotic cells and tissues (ed. Kilberg, M.S.)(1986). *Fed. Proc.* **45**, 2438-2454.

120. Shotwell, M.A., Kilberg, M.S., and Oxdender, D.L. (1983). The regulation of neutral amino acid transport in mammalian cells. *Biochim. Biophys. Acta* **737**, 267-284.

121. Saier, M.H.Jr., Daniels, G.A., Boerner, P., Lin, J. (1988). Neutral amino acid transport in animal cells: potential targets of oncogene action and regulators of cellular growth. *J. membr. Biol.* **104**, 1-20.

122. Albritton, L.M., Tseng, L., Scadden, D., and Cunningham, J.M. (1989). A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. *Cell* **57**, 659-666.

123. Kim, J.W., Closs, E.I., Albritton, L.M., and Cunningham, J.M. (1991). Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature* **352**, 725-728.

124. Wang, H., Kavanaugh, M.P., North, R.A., and Kabat, D. (1991). Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter. *Nature* **352**, 729-731.

125. Wellner, D., and Meister, A. (1981). A survey of inborn errors of amino acid metabolism and transport in man. *Ann. Rev. Biochem.* **50**, 911-968.

126. Christensen, H.N. (1988). Amino acid transport systems of lysosomes: possible substitute utility of a surviving transport system for one congenitally defeative or absent. *Biosci. Rep.* **8**, 121-129.

127. Christensen, H.N., Handlogten, M., E., Lam, I., Tager, H.S., and Zand, R. (1969). A bicyclic amino acid to improve discriminations among transport systems. *J. Biol. Chem.* **244**, 1510-1520.

128. Palacio, A.R.T. (1992). Regulation, characterization and factors affecting the amino acid transport systems. *Rev. Inv. Clin.* **44**, 427-439.

129. Kilberg, M.S., Stevens, B.R., and Novak, D.A. (1993). Recent advances in mammalian amino acid transport. *Annu. Rev. Nutr.* **13**, 137-165.

130. Joseph, S.K., Bradford, N.M., and MiGivan, J.D. (1978). Characteristics of the transport of alanine, serine and glutamine across the plasma membrane of isolated rat liver cells. *Biochem. J.* **176**, 827-864.

131. Kilberg, M.S., Handlogten, M.E., and Christensen, H.N. (1980). Characteristics of an amino acid transport system in rat liver for glutamine, asparagine, histidine, and closely related analogs. *J. Biol. Chem.* **255**, 4011-4019.

132. Christensen, H.N., and Handlogten, M.E. (1981). Role of system Gly in glycine transport in monolayer cultures of liver cells. *Biochim. Biophys. Acta* **98**, 102-107.

133. Ellory, J.C., Jones, S.E.M., and Young, J.D. (1981). Glycine transport in human erythrocytes. *J. Physiol.* **320**, 403-422.

134. Scalera, V., Corcelli, A., Frassanito, A., and Storelli, C. (1987). Chloride dependence of the sodium-dependent glycine transport in pig kidney cortex brush-border membrane vesicles. *Biochim. Biophys. Acta* **903**, 1-10.

135. Collarini, E.J., Oxender, D.L. (1987). Mechanisms of transport of amino acids across membranes. *Annu. Rev. Nutr.* **7**, 75-90.

136. Barnard, J.A., Thaxter, S., Kikuchi, K., and Ghishan, F.K. (1988). Taurine transport by rat intestine. *Am. J. Physiol.* **254**, G334-G338.

137. Munck, L.K., and Munck, B.G. (1994). Chloride-dependent intestinal transport of imino- and ß-amino acids in guinea pig and rat. *Am. J. Physiol.* **266**, R997-R1007.

138. Corcelli, A., and Storelli, C. (1983). The role of potassium and chloride ions on the Na^+/acidic amino acid cotransport system in rat intestinal brush-border membrane vesicles. *Biochim. Biophys. Acta* **732**, 24-31.

139. Romano, P.M., Ahearn, G.A., Adams, M.B., and Ramaswamy, K. (1987). Na^+-dependent L-glutamate transport by eel intestinal BBMV: role of K^+ and Cl^-. *Am. J. Physiol.* **257**, R180-R188.

140. Thomas, E.L., and Christensen, H.N. (1971). Nature of cosubstrate action of Na^+ and neutral amino acid in a transport system. *J. Biol. Chem.* **246**, 1682-1688.

141. Young, J.D., Mason, D.K., Fincham, D.A. (1988). Topological similarities between harmaline inhibition sites on Na^+-dependent amino acid transport system ASC in human erythrocytes and Na^+-independent system asc in horse erythrocytes. *J. Biol. Chem.* **263**, 140-143.

142. Fincham, D.A., Mason, D.K., and Young, J.D. (1988). Dibasic amino acid interactions with Na^+-independent transport system asc in horse erythrocytes. Kinetic evidence of functional and structural homology with Na^+-dependent system ASC. *Biochim. Biophys. Acta* **937**, 184-194.

143. Fincham, D.A., Ellory, J.C., and Young, J.D. (1992). Characterization of a novel variant of amino acid transport system asc in erythrocytes from Przewalski's horse (*Equus przewalskii*). *Can. J. Physiol. Pharmacol.* **70**, 1117-1127.

144. Christensen, H.N., and Antonioli, J.A. (1969). Cationic amino acid transport in the rabbit reticulocyte: Na^+-dependent inhibition of Na^+-independent transport. *J. Biol. Chem.* **244**, 1497-1504.

145. Christensen, H.N., and Handlogten, M.E. (1969). Reactions of neutral amino acids plus Na^+ with a cationic amino acid transport system. *Fed. Eur. Biochem. Soc. Lett.* **3**, 14-17.

146. Christensen, H.N., Handlogten, M.E., and Thomas, E.L. (1969). Na^+-facilitated reactions of neutral amino acid with a cationic amino acid transport system. *Proc. Natl. Acad. Sci. U.S.A.* **63**, 948-955.

147. Thomas, E.L., Shao, T.C. and Christensen, H.N. (1971). Structural selectivity in interaction of neutral amino acid and alkali metal ions with a cationic amino acid transport system. *J. Biol. Chhem.* **246**, 1677-1681.

148. White, [illegible] (1985). The transport of cationic amino acid across the plasma membrane of mammalian cells. *Biochim. Biophys. Acta* **822**, 355-374.

149. Young, J.D., Fincham, D.A., and Harvey, C.M. (1991). Cation and harmaline interactions with Na^+-independent dibasic amino acid transport system y^+ in human erythrocytes and in erythrocytes from a primitive vertebrate the pacific hagfish (*Eptatretus stouti*). *Biochim. Biophys. Acta* **1070**, 111-118.

150. Van Winkle, L.J., Campione, A.L., and Gorman, J.M. (1988). Na^+-independent transport of basic and zwitterionic amino acids in mouse blastocysts by a shared system and by processes which distinguish between these substrates. *J. Biol. Chem.* **263**, 3150-3163.

151. Van Winkle, L.J., Campione, A.L., and Gorman, J.M. (1990). Inhibition of transport system $b^{o,+}$ in blastocysts by inorganic cations yields insight into the structure of its amino acid receptor site. *Biochim. Biophys. Acta* **1025**, 215-224.

152. Thomas, E.L., and Christenseen, H.N. (1970). Indications of spatial relations among structures recognizing amino acids and Na^+ at a transport receptor site. *Biochem. Biophys. Res. Commum.* **40**, 277-283.

153. Young, J.D., Wolowyk, M.W., Jones, S.E.M., and Ellory, J.C. (1983). Red cell amino acid transport. Evidence for the presence of system ASC in mature red blood cells. *Biochem. J.* **216**, 349-357.

154. Van Winkle, L.J. (1988). Amino acid transport in developing animal oocytes and early conceptuses. *Biochim. Biophys. Acta* **947**, 173-208.

155. Deves, R., Chavez, P., and Boyd, C.A.R. (1992). Identification of a new transport system (y^+L) in human erythrocytes that recognizes lysine and leucine with high affinity. *J. Physiol.* **454**, 491-501.

156. Deves, R., Angelo, S., and Chavez, P. (1993). *N*-ethylmaleimide discriminates between two lysine transport systems in human erythrocytes. *J. Physiol.* **468**, 753-766.

157. Lo, A.W.I., Qureshy, S.A., Harvey, C.M., and Young, J.D. (1993). Cation and neutral amino acid interactions with a novel lysine transporter (system y^+L) in human erythrocytes. *Biochem. Cell Biol.* **71**, Axiv.

158. Van Winkle, L.J., Christensen, H.N., and Campione, A.L. (1985). Na^+-dependent transport of basic, zwitterionic, and bicyclic amino acids by a broad-scope system in mouse blastocysts. *J. Biol. Chem.* **260**, 12118-12123.

159. Christensen, H.N. (1990). Role of amino acid transport and countertransport in nutrition and metabolism. *Physiol. Rev.* **70**, 43-77.

160. Van Winkle, L.J. (1993). Endogenous amino acid transport systems and expression of mammalian amino acid transport proteins in *Xenopus* oocytes. *Biochim. Biophys. Acta* **1154**, 157-172.

161. Munck, B.G. (1980). Lysine transport across the small intestine. *J. Membr. Biol.* **53**, 45-53.

162. Mircheff, A.K., Kippen, I., Hirayama, B., and Wright, E.M. (1982). Delineation of sodium-stimulated amino acid transport pathways in rabbit kidney brush border vesicles. *J. Membr. Biol.* **64**, 113-122.

163. Cassano, G., Leszczynska, B., and Murer, H. (1983). Transport of L-lysine by rat intestinal brush border membrane vesicles. *Pfluggers Arch.* **397**, 114-120.

164. Munck, B.G. (1983). Comparative aspects of amino acid in guinea pig, rabbit and rat small intestine. In *Intestinal transport* (eds. Gilles-Ballien, M., and Gilles, R.), Springer, Berlin, pp260-263.

165. Lawless, K., Maenz, D., and Cheeseman, C. (1987). Is leucine an allosteric modulator of lysine transporter in the intestinal basolateral membrane? *Am. J. Physiol.* **253**, G637-G642.

166. Munck, L.K., and Munck, B.G. (1992). Variation in amino acid transport along the rabbit small intestine. Mutual jejunal carriers of leucine and lysine. *Biochim. Biophys. Acta* **1116**, 83-90.

167. Stevens, B.R., Kaunitz, J.D., and Wright, E.M. (1984). Intestinal transport of amino acids and sugars: advances using membrane vesicles. *Ann. Rev. Physiol.* **46**, 417-433.

168. Silbernagl, S. (1988). The renal handling of amino acids and oligopeptides. *Physiol. Rev.* **68**, 911-948.

169. Malo, C. (1991). Multiple pathways for amino acid transport in brush border membrane vesicles isolated from the human fetal small intestine. *Gastroenterology* **100**, 1644-1652.

170. Del Castillo, J.S., and Muniz, R. (1991). Neutral amino acid transport by isolated small intestinal cells from guinea pigs. *Am. J. Physiol.* **261**, G1030-G1036.

171. Sigel E. (1990). Use of *Xenopus* oocytes for the functional expression of plasma membrane proteins. *J. Memb. Biol.* **117**, 201-221.

172. Gurdon, J.B., Lane, C.D., Woodland, H.R. and Marbaix, G. (1971). Use of frog eggs and oocytes for the study of messenger RNA and its translation in living cells. *Nature* **80**, 539-551.

173. Gurdon, J.B. (1968). Changes in somatic cell nuclei inserted into growing and maturing amphibian oocytes. *J. Embryol. exp. Morph.* **20**, 401-414.

174. Sumikawa, K., Houghton, M., Emtage, J.S., Richards, B.M., and Barnard, E.A. (1981). Active multi-subunit Ach receptor assembled by translation of heterologous mRNA in *Xenopus* oocytes. *Nature* **292**, 862-864.

175. Gurdon, J.B. (1974). Translation control and message-injection into living cells. *In The control of gene expression in animal development.* (ed. Gurdon, J.B.), Harvard University Press, Cambridge, pp37-76.

176. Hagenbuch, B., Stieger, B., Foguet, M., Lubbert, H., and Meier, P.J. (1991). Functional expression cloning and characterization of the hepatocyte Na^+/bile acid cotransport system. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10629-10633.

177. Kwon, H.M., Yamauchi, A., Uchida, S., Preston, A.S., Garcia-Perez, A., Burg, M.B., and Handler, J.S. (1992). Cloning of the cDNA for a Na^+/myo-inositol cotransporter, a hypertonicity stress protein. *J. Biol. Chem.* **267**, 6297-6301.

178. Yamauchi, A., Uchida, S., Kwon, H.M., Preston, A.S., Brooks-Robey, R., Garcia-Perez, A., Burg, M.B., and Handler, B.L. (1992). Cloning of a Na^+-and Cl^- dependent betaine transporter that is regulated by hypertonicity. *J. Biol. Chem.* **267**, 649-652.

179. Markovich, D., Forgo, J., Stange, G., Biber, J., and Murer, H. (1993). Expression cloning of rat renal Na^+/SO_4^{2-} cotransport. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8071-8077.

180. Kanai, Y., and Hediger, M.A. (1993). Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature* **360**, 467-471.

181. You, G., Smith, C.P., Kanai, Lee, W.-S., Stelzner, M., and Hediger, M.A. (1993). Expression cloning and characterization of the vasopressin-regulated urea transporter. *Nature* **365**, 844-847.

182. Fei, Y.-J., Kanai, S., Nussberger, S., Ganapathy, V., Romero, M.F., Singh, S.K., Boron, W.F., and Hediger, M.A. (1994). Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature* **368**, 563-566.

183. Berman-Kurtz, M., Lin, E.C.C., and Richey, D.P. (1971). Promoter-like mutant with increased expression of the glycerol kinase operon of *Escherichia coli*. *J. Bacteriol.* **106**, 724-731.

184. Maurel, C., Reizer, J., Schroeder, J.I., Chrispeels, M.J., and Saier, M.H.Jr. (1994). Fuctional characterization of the *Escherichia coli* glycerol facilitator, GlpF, in *Xenopus* oocytes. *J. Biol. Chem.* **269**, 1-4.

185. Aoshima, H., Yamada, M., Sauer, N., Komor, E., and Schobert, C. (1993). Heterologous expression of the H^+/hexose cotransporter from *Chlorella* in *Xenopus* oocytes and its characterization with respect to sugar specificity, pH and membrane potential. *J. Plant. Physiol.* **141**, 293-297.

186. Boorer, K.J., Forde, B.G., Leigh, R.A., and Miller, A.J. (1992). Functional expression of a plant plasma membrane transporter in *Xenopus* oocytes. *FEBS Lett.* **302**, 166-168.

187. Henderson, P.J.F. (1990). Proton-linked sugar transport systems in bacteria. *J. Bioenerg. Biomembr.* **22**, 525-569.

188. Henderson, P.J.F. (1993). The 12-transmembrane helix transporters. *Curr. Opinion Cell. Biol.* **5**, 708-721.

189. Saier, M.H., Jr. and Reizer, J. (1991). Families and superfamilies of transport proteins common to prokaryotes and eukaryotes. *Curr. Opin. Struct. Biol.* **1**, 362-368.

190. Griffith, J.K., Baker, M.E., Rouch, D.A., Page, M.G.P., Skurray, R.A., Paulsen, I.T., Chater, K.F., Baldwin, S.A., and Henderson, P.J.F. (1992). Membrane transport proteins: implications of sequence comparisons. *Curr. Opin. Cell. Biol.* **4**, 684-695.

191. Reizer, J., Finley, K., Kakuda, D., MacLeod, C.L., Reizer, A., and Saier, M.H. (1993). Mammalian integral membrane receptors are homologous to facilitators and antiporters of yeast, fungi, and eubacteria. *Protein Sci.* **2**, 20-30.

192. Maloney, P.C. (1994). Bacterial transporters. *Curr. Opin. Cell Biol.* **6**, 571-582.

193. Reizer, J., Reizer, A., and Saier, M.H.Jr. (1994). A functional superfamily of sodium/solute symporters. *Biochim. Biophys. Acta* **1197**, 133-166.

194. Orci, L., Thorens, B., Ravazzola, M., and Lodish, H.F. (1989). Localization of the pancreatic beta cell glucose transporter to specific plasma membrane domains. *Science* **245**, 295-297.

195. Thorens, B., Sarkar, H.K., Kaback, H.R., and Lodish, H.F. (1988). Cloning and functional expression in bacteria of a novel glucose transporter present in liver, intestine, kidney, and ß-pancreatic islet cells. *Cell* **55**, 281-290.

196. Thorens, B., Cheng, Z.-Q., Brown, D., and Lodish, H.F. (1990). Liver glucose transporter: a basolateral protein in hepatocytes and intestine and kidney cells. *Am. J. Physiol.* **259**, C279-C285.

197. Nagamatsu, S., Kornhauster, J.M., Burant, C.F., Seino, S., Mayo, K.E., and Bell, G.I. (1992). Glucose transporter expression in brain. cDNA sequence of mouse GLUT3, the brain facilitative glucose transporter isoform, and identification of sites of expression by *in situ* hybridization. *J. Biol. Chem.* **267**, 467-472.

198. James, D.E., Strube, M.I., and Meuckler, M. (1989). Molecular cloning and characterization of an insulin-regulatable glucose transporter. *Nature* **338**, 83-87.

199. Birnbaum, M.J. (1989). Identification of a novel gene encoding insulin-responsive glucose transporter protein. *Cell* **57**, 305-315.

200. Charron, M.J., Brosius, F.C., Alper, S.L., and Lodish, H.F. (1989). A glucose transport protein expressed predominately in insulin-responsive tissues. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2535-2539.

201. Kaestner, K.H., Christry, R.J., McLenithan, J.C., Briterman, L.T., Cornelius, P., Pekala, P.H., and Lane, M.D. (1989). Sequence, tissue distribution, and differential expression of mRNA ... a putative insulin-responsive glucose transporter in mouse 3T3-L1 adi... *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3150-3154.

202. Fukumoto, H., Kayano, T., Buse, J.B., Edwards, Y., Pitch, P.F., Bell, G.I., and Seino, S. (1989). Cloning and characterization of the major insulin-responsive glucose transporter expressed in human skeletal muscle and other insulin-responsive tissues. *J. Biol. Chem.* **264**, 7776-7779.

203. Haney, P.M., Slot, J.W., Piper, R.C., James, D.E., and Mueckler, M. (1991). Intracellular targeting of the insulin-regulatable glucose transporter (GLUT4) is isoform specific and independent of cell type. *J. Cell. Biol.* **114**, 689-699.

204. Kayano, T., Burrant, C.F., Fukumoto, H., Gould, G.W., Fan, Y., Eddy, R.L., Byers, M.G., Shows, T.B., Seino, S., and Bell, G.I. (1990). Human facilitative glucose transporters. Isolation, functional characterization, and gene localization of cDNAs encoding an isoform (GLUT5) expressed in small intestine, kidney, muscle, and adipose tissue and an unusual glucose transporter pseudogene-like sequence (GLUT6). *J. Biol. Chem.* **265**, 13276-13282.

205. Davidson, N.O., Hausman, A.M.L., Ifkovits, C.A., Buse, J.B., Gould, G.W., Burant, C.F., and Bell, G.I. (1992). Human intestinal glucose transporter expression and localization of GLUT5. *Am. J. Physiol.* **262**, C795-C800.

206. Waddell, I.D., Zomerschoe, A.G., Voice, M.W., and Burchell, A. (1992). Cloning and expression of a hepatic microsomal glucose transprter protein. Comparison with liver plasma-membrane glucose transporter protein GLUT2. *J. Biol. Chem.* **256**, 7090-7093.

207. Baldwin, S.A. (1993). Mammalian passive glucose transporters:members of an ubiquitous family of active and passive transport proteins. *Biochim. Biophys. Acta* **1154**, 17-49.

208. Preston, R.A.J., Sami, J.A., Charalambous, B.M., and Baldwin, S.A. (1994). Production of functional GLUT1 by co-expression of *N*-and *C*-terminal half molecules in *Xenopus* oocytes. *Biochem. Soc. Tranc.* **22**, 276S.

209. Wells, R.G., Pajor, A.M., Kanai, Y., Turk, E., Wright, E.M., and Hediger, M.A. (1992). Cloning of a human cDNA with similarity to the sodium-glucose cotransporter. *Am. J. Physiol.* **263**, F459-F465.

210. Kong, C.-T., Yet, S.-F., and Lever, J.E. (1993). Cloning and expression of a mammalian Na^+/amino acid cotransporter with sequence similarity to Na^+/glucose cotransporters. *J. Biol. Chem.* **268**, 1509-1512.

211. Nakao, T., Yamato, I., and Anraku, Y. (1987). Nucleotide sequence of Put P, the proline carrier gene of *Escherichia coli* K_{12}. *Mol. Gen. Genet.* **208**, 70-75.

212. Jackowski, S., and Alix, J.-H. (1990). Cloning, sequence and expression of the pantothenate permease (*pan F*) gene of *Escherichia coli* B. *J. Bacteriol.* **172**, 3842-3848.

213. Pajor, A.M. (1994). Molecular cloning and expression of SNST1, a renal sodium/nucleoside cotransporter. *Drug Development Res.* **31**, 305.

214. Deguichi, Y., Yamato, I., and Anraku, Y. (1990). Nucleotide sequence of *gltS*, the Na^+/glutamate symport carrier gene of *Escherichia coli* B. *J. Biol. Chem.* **256**, 21704-21708.

215. Turk, E., Zabel, B., Mundlos, S., Dyer, J., and Wright, E.M. (1991). Glucose/galactose malabsorption caused by a defeat in the Na^+/glucose cotransporter. *Nature* **350**, 354-356.

216. Uhl, G.R. (1992). Neurotransmitter transporters (plus): a promising new gene family. *TINS* **15**, 265-268.

217. Schloss, P., Mayser, W., Betz, H. (1992). Neurotransmitter transporters, a novel family of integral plasma membrane proteins. *FEBS* **307**, 76-80.

218. Radian, R., Bendahan, A., and Kanner, B.I. (1986). Purification and identification of the functional sodium- and chloride-coupled gamma-aminobutyric acid transport glycoprotein from rat brain. *J. Biol. Chem.* **261**, 15437-15441.

219. Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Miedel, M.C., Davidson, N., Lester, H.A., and Kanner, B.L. (1990). Cloning and expression of a rat brain GABA transporter. *Science* **249**, 1303-1306.

220. Liu, Q.-R., Lopez-Coruera, B., Mandiyan, S., Nelson, H., and Nelson, N. (1993). Molecular characterization of four pharmacologically distinct α-aminobutyric acid transporters in mouse brain. *J. Biol. Chem.* **268**, 2106-2112.

221. Guastella, J., Brecha, N., Weigmann, C., Lester, H.A., and Davidson, N. (1992). Cloning, expression, and localization of a rat brain high-affinity glycine transporter. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7189-7193.

222. Smith, K.E., Borden, L.A., Hartig, P.R., Branchek, T., and Weinshank, R.L. (1992). Cloning and expression of a glycine transporter reveal colocalization with NMDA receptors. *Neuron* **8**, 927-935.

223. Liu, Q.-R., Nelson, H., Mandiyan, S., Lopez-Corcuera, B., and Nelson, N. (1992). Cloning and expression of a glycine transporter from mouse brain. *FEBS* **305**, 110-114.

224. Giros, B., Mestikawy, S.E., Bertrand, L., and Caron, M.G. (1991). Cloning and functional characterization of a cocaine-sensitive dopamine transporter. *FEBS* **295**, 149-154.

225. Kilty, J.E., Lorang, D., and Amara, S.G. (1991). Cloning and expression of a cacaine sensitive rat dopamine transporter. *Science* **254**, 578-579.

226. Shimada, S., Kitayama, S., Lin., C.-L., Patel, A., Nanthankumar, E., Gregor, P., Kuhar, M., and Uhl, G. (1991). Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. *Science* **254**, 576-578.

227. Blakely, R.D., Berson, H.E., Fremeau, R.T., Caron, M.G., Peek, M.M., Prince, H.K., and Bradley, C.C. (1991). Cloning and expression of a functional serotonin transporter from rat brain. *Nature* **354**, 66-70.

228. Hoffman, B.J., Mezey, E., and Brownstein, M.J. (1991). Cloning of a serotonin transporter affected by antidepressants. *Science* **254**, 579-580.

229. Pacholczyk, T., Blakely, R.D., and Amara, S.G. (1991). Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. *Nature* **350**, 2563-2568.

230. Liu, Q.-R., Lopez-Corcurea, B., Nelson, H., Mandiyan, S., and Nelson, N. (1992). Cloning and expression of a cDNA encoding the transporter of taurine and ß-alanine in mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 12145-12149.

231. Fremeau, R.T., Caron, M.G., and Blakely, R.D. (1992). Molecular cloning and expression of a high-affinity L-proline transporter expressed in putative glutamatergic pathways of rat brain. *Neuron* **8**, 915-926.

232. Mayser, W., Schloss, P., and Betz, H. (1992). Primary structure and functional expression of a choline transporter expressed in the rat nervous system. *FEBS* **305**, 31-36.

233. Pantanowitz, S., Bendahan, A., and Kanner, B.I. (1993). Only one of the charged amino acids located in the transmembrane α-helices of the γ-aminobutyric acid transporters (subtype A) is essential for its activity. *J. Biol. Chem.* **268**, 3222-3225.

234. Kleinberger-Doron, N., and Kanner, B.I. (1994). Identification of the tryptophan residues critical for the function and targeting of the γ-aminobutyric acid transporter (subtype A). *J. Biol. Chem.* **269**, 3063-3067.

235. Pines, G., Danbolt, N.C., Bijoras, M., Zhang, Y., Bandahan, A., Eide, L., Koepsell, H., Storm-Mathisen, J., Seeberg, E., and Kanner, B.I. (1992). Cloning and expression of a rat brain L-glutamate transporter. *Nature* **360**, 464-467.

236. Storck, T., Schulte, S., Hofmann, K., and Stoffel, W. (1992). Structure, expression and functional analysis of a Na^+-dependent glutamate/aspartate transporter from rat brain. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10955-10959.

237. Kanai, Y., and Hediger, M.A. (1992). Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature* **360**, 467-471.

238. Kanner, B.I. (1993). Glutamate transporters from brain: a novel neurotransmitter transporter family. *FEBS* **325**, 95-99.

239. Kanai, Y., Smith, C.P., and Hediger, M.A. (1994). A new family of neurotransimitter transporters: the high affinity glutamate transporters. *FASEB J.* **8**, 1450-1459.

240. Bouvier, M., Szatkowski, M., Amato, A., and Attwell, D. (1992). The glial cell glutamate uptake carrier countertransports pH-changing anions. *Nature* **360**, 471-474.

241. Tolner, B., Poolman, B., Wallace, B., and Konings, W.N. (1992). Revised nucleotide sequence of the gltP gene, which encodes the proton glutamate-aspartate transport protein of *Escherichia coli* K-12. *J. Bacteriol.* **174**, 2391-2393.

242. Tolner, B., Poolman, B., and Konings, W.N. (1992). Characterization and functional expression in *Escherichia coli* of the sodium/proton/glutamate symport

proteins of *Bacillus stearothermophilus* and *Bacillus caldotenax*. *Mol. Microbiol.* **6**, 2845-2856.

243. Engelke, T., Jording, D., Kapp, D., and Puhlerhler, A. (1989). Identification and sequence analysis of the *Rhizobium meliloti* dctA gene encoding the C4-dicarboxylate carrier. *J. Bacteriol.* **171**, 5551-5560.

244. Arriza, J.L., Kavanaugh, M.P., Fairman, W.A., Wu, Y.-N., Murdoch, G.H., North, R.A., and Amara, S.G. (1993). Cloning and expression of a human neutral amino acid transporter with structural similarity to the glutamate transporter gene family. *J. Biol. Chem.* **268**, 15329-15332.

245. Shafqat, S., Tamarappoo, B.K., Kilberg, M.S., Puranam, S., McNamara, J.O., Guadano-Ferraz, A., and Fremeau, R.T.Jr. (1993). Cloning and expression of a novel Na^+-dpendent neutral amino acid transporter structurally related to mammalian Na^+/glutamate cotransporters. *J. Biol. Chem.* **268**, 15351-15355.

247. Yoshimoto, A.R., Yoshimoto, E., and Meruelo, D. (1991). Molecular cloning and characterization of a novel human gene homologous to the murine ecotropic retroviral receptor. *Virology* **185**, 10-17.

248. Closs, E.I., britton, L.M., Kim, J.W., and Cunningham, J.M. (1993). Identification of a low affinity, high capacity transporter of cationic amino acids in mouse liver. *J. Biol. Chem.* **268**, 7538-7544.

249. Closs, E.I., Lyons, C.R., Kelly, C., and Cunningham, J.M. (1993). Characterization of the third member of the MCAT family of cationic amino acid transporters. *J. Biol. Chem.* **268**, 20796-20800.

250. Closs, E.I., Rinkes, I.H.M.B., Bader, A., Yarmush, M.L., and Cunningham, J.M. (1993). Retroviral infection and expression of cationic amino acid transporters in rodent hepatocytes. *J. Virol.* **67**, 2097-2102.

251. Wells, R.G., and Hediger, M.A. (1992). Cloning of a rat kidney cDNA that stimulates dibasic and neutral amino acid transport and has sequence similarity to glucosidases. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5596-5600.

252. Tate, S.S., Yan, N., and Udenfriend, S. (1992). Expression cloning of a Na^+-independent neutral amino acid transporter from rat kidney. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1-5.

253. Bertran, J., Werner, A., Moore, M.L., Stange, G., Markovich, D., Biber, J., Testar, X., Zorzano, A., Palacin, M., and Murer, H. (1992). Expression cloning of a cDNA from rabbit kidney cortex that induces a single transport system for

cystine and dibasic and neutral amino acids. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5606-5610.

254. Markovich, D., Stange, G., Bertran, J., Palacin, M., Werner, A., Biber, J., and Murer, H. (1993). Two mRNA transcripts (rBAT-1 and rBAT-2) are involved in system $b^{o,+}$-related amino acid transport. *J. Biol. Chem.* **268**, 1362-1367.

255. Holland, E.C., and Drickamer, K. (1985). Signal recognition particle mediates the insertion of a transmembrane protein which has a cytoplasmic NH_2 termi... *J. Biol. Chem.* **261**, 1286-1292.

256. Segal, S., and Thier, S.O. (1989). Cystinuria. In *The Metabolic basis of inherited diseases* (eds. Scriver, C.H., Beaudet, A.L., Sly, W.S., and Valle, D.), McGraw-Hill, New York, pp2479-2496.

257. Rosenberg, L.E., Durant, J.L., and Holland, I.M. (1965). Intestinal absorption and renal extraction of cystine and cysteine in cystinuria. *New Engl. J. Med.* **273**, 1239-1345.

258. Kanai, Y., Stelzner, M.G., Lee, W.-S., Wells, R.G., Brown, D., and Hediger, M.A. (1992). Expression of mRNA (D2) encoding a protein involved in amino acid transport in S3 proximal tubule. *Am. J. Physiol.* **263**, F1087-F1093.

259. Pickel, V.M., Nirenberg, M.J., Chan, J., Mosckovitz, R., Udenfriend, S., and Tate, S.S. (1993). Ultrastructural localization of a neutral and basic amino acid transporter in rat kidney and intestine. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7779-7783.

260. Furriols, M., Chillaron, J., Mora, C., Castello, A., Bertran, J., Camps, M., Testar, X., Vilaro, S., Zorzano, A., and Palacin, M. (1993). rBAT, related to L-cystine transport, is localized to the microvilli of proximal straight tubules, and its expression is regulated in kidney by development. *J. Biol. Chem.* **268**, 27060-27068.

261. Lee, W.-S., Wells, R.G., Sabbag, R.V., Mohandas, T.K., and Hediger, M.A. (1993). Cloning and chromosomal localization of a human kidney cDNA involved in cystine, dibasic, and neutral amino acid transport. *J. Clin. Invest.* **91**, 1959-1963.

262. Pras, E., Arber, N., Aksentijevich, I., Katz, G., Schapiro, J.M., Prosen, L., Gruberg, L., Harel, D., Liberman, U., Weissenbach, J., Pras, M., and Kastner, D. (1994). Localization of a gene causing cystinuria to chromosome 2p. *Nature Genet.* **6**, 415-419.

263. Quackenbush, E., Clabby, M., Gottesdiener, K.M., Barbosa, J., Jones, N.H., Strominger, J.L., Speck, S., and Leiden, J.M. (1987). Molecular cloning of complementary DNAs encoding the heavy chain of the human 4F2 cell-surface antigen: a type II membrane glycoprotein involved in normal and neoplastic cell growth. *Prod. Natl. Acad. Sci. U.S.A.* **84**, 6526-6530.

264. Lumadue, J.A., Glick, A.B., and Ruddle, F.H. (1987). Cloning, sequence analysis, and expression of the large subunit of the human lymphocyte activation antigen 4F2. *Proc Natl. Acad. Sci. U.S.A.* **84**, 9204-9208.

265. Wells, R.G., Lee, W.-S., Kanai, Y., Leiden, J.M., and Hediger, M.A. (1992). The 4F2 antigen heavy chain induces uptake of neutral and dibasic amino acids in *Xenopus* oocytes. *J. Biol. Chem.* **267**, 15285-15288.

266. Bertran, J., Magagnin, S., Werner, A., Markovich, D., Biber, J., Testar, X., Zorzano, A., Kühn, L.C., Palacin, M., and Murer, H. (1992). Stimulation of system y^+-like amino acid transport by the heavy chain of human 4F2 surface antigen in *Xenopus laevis* oocytes. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5606-5610.

267. Hemler, M.E., and Strominger, J.L. (1982). Characterization of the antigen recognized by the monoclonal antibody (4F2): different molecular forms on human T and B lymphoblastoid cell lines. *J. Immunol.* **129**, 623-628.

268. Parmacek, M.S., Karpinski, B.A., Gottasdiener, K.M., Thompson, C.B., and Leiden, J.M. (1989). Structure, expression and regulation of murine 4F2 heavy chain. *Nucleic Acid Res.* **17**, 1915-1931.

269. Veyhl, M., Spangenberg, J., Püschel, B., Poppe, R., Dekel, C., Fritzsch, G., Haase, W., and Koepsell, H. (1993). Cloning of a membrane-associated protein which modifies activity and propertites of the Na^+-D-glucose cotransporter. *J. Biol. Chem.* **268**, 25041-25053.

270. Harvey, C.M., Muzyka, W.R., Yao, S.Y.M., Cheeseman, C.I., and Young, J.D. (1993). Expression of rat intestinal L-lysine transport systems in isolated oocytes of *Xenopus laevis*. *Am. J. Physiol.* **265**, G99-G106.

CHAPTER 2

Cloning and Functional Expression of a cDNA Encoding a Rat Jejunal Na^+-dependent Nucleoside Transporter Protein (cNT1)[1]

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2.1. INTRODUCTION

Natural nucleosides are important precursors of nucleic acids and energy-rich cellular metabolites in mammalian cells. Adenosine, for example, functions as a local hormone in regulation of lipolysis, neurotransmitter release, platelet aggregation, coronary vasodilation and cardiac contractility (1-3). In addition, synthetic nucleoside analogs have been used extensively in the treatment of human neoplastic and viral diseases, including leukemias and acquired immunodeficiency syndrome (AIDS) (4,5).

Mammalian cells transport nucleosides by both active and equilibrative mechanisms. Equilibrative nucleoside transporters are widely distributed, and can be divided into two classes (*es* and *ei*) on the basis of sensitivity or resistance to inhibition by nitrobenzylthioinosine (NBMPR)(Table 1.1)(6-14). Active, Na^+-linked nucleoside transporters are also present in many different mammalian cells and tissues, including choroid plexus, liver, kidney, intestine, lymphocytes, splenocytes, macrophages and both murine and human leukemia cells (15-37). Five Na^+-dependent nucleoside transporter subclasses (N1-N5) are recognised (Table 1.1)(38). System N1 is generally purine specific and guanosine serves as a model substrate (17-20,23-36). System N2 is generally pyrimidine specific and thymidine serves as a model substrate (18,19,22,23,25,26). Uridine and adenosine are transported by both systems. System N3 has broad specificity for both purine and pyrimidine nucleosides (15,16,23,24). System N4 is selective for pyrimidine nucleosides, adenosine and guanosine (22). The substrate selectivity of system N5 has not yet been determined (37). In contrast to systems N1-N4, this latter transporter is sensitive to inhibition by NBMPR, dipyridamole and dilazep (37).

In bacteria, nucleoside transport is best characterised in *E. coli* where transport is predominantly mediated by two H^+-coupled systems, NupC and NupG (39). NupG mediates transport of a wide range of purine and pyrimidine nucleosides while NupC has a low affinity for guanosine and inosine and is strongly inhibited by the nucleoside analog, showdomycin (40-43).

Studies from three different laboratories have demonstrated expression of Na^+-dependent nucleoside transport activity in *Xenopus* oocytes injected with intestinal mRNA (44-46). Rabbit intestinal mRNA expressed N2-type transport activity (44,45). Experiments in this laboratory with rat intestinal mRNA demonstrated induction of system N1, N2 and N3 transport activities (46). In this Chapter, I describe the isolation and functional characterisation of a cDNA encoding a high-affinity, Na^+-dependent nucleoside transporter protein (named cNT1) from rat jejunal epithelium by functional expression cloning in *Xenopus* oocytes. The cDNA sequence of cNT1 predicts a protein of 648 amino acids (relative molecular mass 71,000) with 14 potential transmembrane domains. Functionally, cNT1 exhibited the transport characteristics of system N2 (selective for pyrimidine nucleosides and adenosine). Adenosine was transported with a similar apparent affinity as uridine, but with a substantially lower maximum velocity. Consistent with the tissue distribution of system N2 transport activity, transcripts for cNT1 were detected in kidney as well as jejunum. Data base searches indicated significant sequence similarity to the NupC H^+/nucleoside symporter of *E. coli* and to

two other *E. coli* proteins (ECOHU4748 and ECOHU4751) of unknown function. In contrast, there was no sequence similarity between cNT1 and proteins of mammalian origin, including a putative Na^+-linked nucleoside transporter (SNST1) from rabbit kidney (47). Therefore, cNT1 and its bacterial homologs belong to a new gene family of transporter proteins. Some of the data presented in this Chapter have been published (48).

2.2. MATERIALS AND METHODS

Rat jejunal mRNA induces both nucleoside (46) and amino acid (49,50,Appendix 2) transport activities when expressed in *Xenopus* oocytes. A directional size-selected cDNA library was therefore prepared from rat jejunal epithelium in the plasmid expression vector pGEM-3Z with the objective of isolating cDNAs encoding these transport activities by functional expression cloning in *Xenopus* oocytes.

Glassware for RNA work was baked at 180°C for 8 h or more. Solutions were prepared using baked glassware and autoclaved water. Chemicals were handled with baked spatulas. Solutions were treated with 0.1% (w/v) diethyl pyrocarbonate (DEPC) for at least 12 h at 37°C and then autoclaved for 15 min on a liquid cycle to prevent RNase contamination. Siliconized tubes were used for the storage of small quantity of RNA. Plastic microcentrifuge tubes were immersed in siliconizing solution (5% (v/v) dimethyl-dichlorosilicane in chloroform) for 10 min in a fume hood prior to rinsing thoroughly with M.Q. (Millipore filtered) water and autoclaved.

2.2a. Isolation of Poly(A)$^+$RNA (mRNA)

Sprague-Dawley rats (200-250-g males), fed on Wayne Rodent Blox 8604 (PMI Feeds), were anaesthetized with 0.3 ml sodium pentobarbital (65 mg/ml in propylene glycol; MTC Pharmaceuticals). The small intestine was cut at the ligament of Trietz, and the first 30 cm of jejunum was removed and flushed immediately with ice-cold phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4). Luminal scrapings were frozen immediately in liquid nitrogen and ground into a fine powder before being homogenized in 4 M guanidinium thiocyanate. The homogenate was centrifuged (5000 x*g*, 10 min, 4°C), and the resulting supernatant was layered onto a cushion of 5.7 M caesium chloride (*d* 1.7 g/ml) in 0.01 M EDTA (pH 7.5) and centrifuged (111,000 x*g*, 24h, 20°C) in a Beckman SW28 rotor to yield RNA pellets. The RNA pellets were washed twice in 70% (v/v) ethanol and resuspend in RNase-free water.

Poly(A)$^+$RNA (mRNA) was purified from total RNA either using the polyAtract™ mRNA isolation system (Promega) or oligo d(T)-cellulose affinity chromatography. Two cycles of oligo(dT)-cellulose (Boehringer Mannheim) affinity chromatography (0.15 g dry oligo d(T)-cellulose for 5 mg of total RNA for the first column and 0.05 g for the second column) were used for mRNA purification. Dry oligo d(T)-cellulose was equilibrated in 5 ml of the elution buffer (10 mM Tris-HCl, pH7.5, 2 mM EDTA) for several min before being placed into an autoclaved Dispocolumn (BioRad). The column was washed with 5 column volumes of the binding buffer (10 mM Tris-HCl, pH7.5, 0.5 M LiCl, 1 mM EDTA, 0.1% (w/v) SDS) before use. The total RNA sample (~5 mg) was adjusted to 0.5 M LiCl with 10 M LiCl after being heat-denatured at 70°C for 10 min and chilled on ice for 10 min. The denatured RNA sample was passed through the oligo d(T) column by gravity and the eluate was collected and passed through the column again after being heat-denatured at 70°C for 10 min and chilled on ice. The column was then washed with 5 column volumes of the binding buffer followed by 5 column volumes of the wash buffer (10 mM tris-HCl, pH7.5, 0.1

M LiCl, 1 mM EDTA). Poly(A)$^+$RNA was eluted with 2 column volumes of the elution buffer. The eluted RNA was then repurified by using the second oligo d(T) column with the same procedures described above except that the final elution of the poly(A)$^+$RNA bound to the second column was achieved with 2 X 1 column volumes of the elution buffer. Poly(A)$^+$RNA was precipitated by ethanol and stored at -70°C.

2.2b. DNA/RNA Extraction and Ethanol Precipitation

Phenol (Gibco/BRL) was equilibrated to a pH > 7.8 with 0.5 M Tris.HCl, pH 8.0 at room temperature before use. Phenol was equilibrated to a pH > 7.8 because DNA will partition into the organic phase at acid pHs. An equal volume of Tris buffer (0.5 M Tris.Cl, pH 8.0 at room temperature) was added to melted phenol and stirred for 15 min. When the two phases had separated, as much as possible of the upper (aqueous) phase was aspirated. The process was repeated until the pH of the lower phase was > 7.8 (as measured with pH paper). The upper phase was removed as much as possible and 0.1 volumes 0.1 M Tris.HCl (pH 8.0), 0.1% (w/v) hydroxyquinoline was added to give the organic phase a yellowish colour. The phenol solution was then mixed with chloroform and isoamyl alcohol at a ratio of 25:24:1 (v/v/v).

An equal volume of phenol/chloroform/isoamyl alcohol was added to the DNA or RNA solution and mixed well by shaking vigorously. The upper aqueous phase containing the DNA or RNA was separated from the lower organic phase by centrifugation at 10,000 g for 1 min. The upper layer was then transferred to a new 1.5 ml microcentrifuge tube while the lower organic phase was extracted once with an equal volume of sterile water. The pooled aqueous solutions were then extracted once with an equal volume of chloroform/isoamyl alcohol (24:1, v/v) followed by centrifugation for 1 min. The upper aqueous layer was then transferred to a new 1.5 ml microcentrifuge tube and the DNA or RNA was precipitated by addition of a 1/10 volume of the 3 M sodium acetate (pH 7.0 for DNA or pH 6.0 for RNA) and 2.5 volumes of the cold 95 % (v/v) ethanol (stored at -20°C). The tube was kept at -20°C for several hours, and the DNA or RNA was collected by centrifugation at 10,000 g for 30 min at 4°C. The pellet was washed with 500 μl 80% (v/v) ethanol (-20°C) centrifuged again for 10 min at 4°C. The DNA or RNA pellet was dried in air or by a vacuum evaporator and then resuspended in sterile water at the appropriate concentration.

2.2c. Size Fractionation of mRNA

Size fractionation of mRNA was accomplished by non-denaturing sucrose step-gradient centrifugation. mRNA (400 μg in 0.5 ml TE buffer (10 mM Tris-HCl, pH7.4, 1 mM EDTA)) was heat-denatured at 70°C for 10 min and chilled on ice before loading on a gradient composed of 11x1 ml graded concentrations of sucrose (10-21 % (w/v) in TE buffer). After centrifugation (150,000 xg, 20h, 4°C, in a Beckman SW 41 rotor), 0.6 ml fractions were collected, and the mRNA was precipitated by adding 0.1 volume of 3M sodium acetate (pH 6) and 2.5 volumes of cold 95% ethanol (v/v). The precipitates were dissolved in RNase-free water at a concentration of 1 μg/μl and stored at -70°C.

2.2d. Denaturing Agarose Gel Electrophoresis of RNA

Gel electrophoresis of mRNA was performed on a 1% (w/v) agarose gel containing 0.1 volumes of 10X MOPS buffer (0.2 M MOPS, 50 mM Na acetate, 10 mM EDTA (pH 7.0), 2% (v/v) formaldehyde) in electrophoresis buffer containing 20 mM MOPS, 5 mM Na acetate and 1 mM EDTA (pH 7.0). Five μl of the RNA sample (maximum 30 μg) was mixed with 25 μl of the loading buffer (1.5 ml 10X MOPS, 7.5 ml 100% deionised formamide, 1 ml glycerol, 0.8 ml 10% bromophenol blue, 2.5 ml 37% (v/v) formaldehyde and 1.7 ml sterile water per 15 ml stock solution). The RNA sample was then heat-denatured at 65°C for 10 min and chilled on ice for on ice for 5 min. One μg of 1 mg/ml ethidium bromide was added to the sample which was run at 40 V for 5 h. RNA was viewed and photographed on a UV box. Gel lanes were scanned by laser densitometry (Gelscan XL, Pharmacia), and the size of RNA was calculated by reference to BRL molecular weight standards (Life Technologies).

2.2e. Construction of a Rat Jejunal cDNA Library

A rat jejunal RNA size-fraction (1.6-3.0 kb, median 2.3 kb) that induced peak Na^+-dependent uridine transport activity was reverse transcribed using the Riboclone (Promega) cDNA synthesis system with an *Xba* primer-adaptor consisting of oligo(dT) adjacent to an *Xba*1 restriction site. Digestion of the resulting double-stranded cDNA with *Xba*I gave orientation-specific DNA with a 5' *EcoR*I terminus and a 3' *Xba*I terminus. cDNAs $\geq$ 2 kb were ligated into the *EcoR*I and *Xba*I restriction enzyme sites of the plasmid expression vector pGEM-3Z (Figure 2.1)(Promega) and transformed into *E. coli* (JM 109) (Promega) to give a cDNA library containing 6,800 primary recombinants.

2.2f. Library Screening

The rat jejunal cDNA library was titered by serial dilution on LB plates containing ampicillin (100 μg/ml). The library was then plated out onto twenty nylon membranes (H-Bond, 150mm diameter, Amersham) placed on LB plates (150 mm diameter) containing ampicillin (100 μg/ml) and incubated overnight at 37°C. Each plate contained 500-1000 single colonies. To prepare replica plates, the nylon membranes were transferred (bacteria side up) onto several autoclaved sheets of 3MM paper (Whatman). Another set of wetted nylon replica membranes were carefully positioned on top of the master membranes and covered with several additional sheets of 3MM paper followed by a glass plate. Colonies on the master membranes were then transferred onto the replica membranes by pressing down on the glass plates. The replica membranes (bacteria side up) were then placed on LB plates containing ampicillin (100 μg/ml) and incubated overnight at 37°C. Plasmid DNA from each replica plate was purified using the Qiagen-tip 20 mini plasmid purification kit (Qiagen) while the master plates were kept at 4°C for storage. Plasmid DNA from each pool was digested with *Xba*I restriction enzyme, and extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) prior to precipitation with 95% (v/v) ethanol (Section 2.2b). Restriction enzyme digestion of DNA was performed in a reaction mixture containing 0.1 volumes of the appropriate React Buffer (10X concentration, Gibco/BRL), 0.1 volumes of the DNA

solution, 4 units of restriction enzyme per μg of DNA (the volume of the restriction enzyme did not exceed 10% of the final volume) and sterile water. Digestion was performed at 37°C for 1-3 h. The completeness of DNA digestion was analysed by electrophoresis on a 1% agarose gel (1% agarose (w/v), 0.25 μg/ml ethidium bromide, 2 mM EDTA, 40 mM Tris-acetate pH 8.5) in Tris-acetate-EDTA (TAE) buffer (2 mM EDTA and 40mM Tris-acetate, pH 8.5). DNA loading buffer (0.16 volumes of 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 40% (w/v) sucrose) was added to the DNA solution prior to electrophoresis at 80V until the migration of bromophenol blue reached two-thirds of the way down the gel. The gel was viewed and photographed on a UV light box.

Linearised plasmid DNA was transcribed with T7 RNA polymerase *in vitro* in the presence of the m^7GpppG cap using the MEGAscript™ *in vitro* transcription system (Ambion) (Section 2.2j) and the resulting cRNA was injected into oocytes (Section 2.2k). Two pools of clones were identified that increased the uptake of 10 μM uridine 8-fold above that of oocytes injected with cRNA transcribed from the total library and 140-fold above that of control water-injected oocytes (10μM, 30min, 20°C). Colonies from the master plate of one of these pools were individually seeded into the wells of 96-well flat bottom microtitre plates to produce a grid system. Testing of rows and columns for uridine transport activity identified a single positive colony from which a plasmid (pQQH1) with a 2.4-kb insert was isolated.

2.2g. cDNA Sequencing

The pQQH1 2.4 kb insert was sequenced in both directions by overlapping deletions generated by exonuclease III and verified by sequencing with synthetic oligonucleotides. pQQH1 in the vector pGEM-3Z (Figure 2.1) was subcloned into the vectors pBluescript II KS⁻ and SK⁻ (Stratagene) (Figures 2.2 and 2.3) by gel ligation and named pQQH2 and pQQH3, respectively. The Vector and insert DNAs were prepared by double-digestion with two appropriate restriction enzymes prior to gel ligation. Digested DNAs were separated on a 1 % (w/v) low melting point (LMP) agarose (Gibco/BRL) gel containing ethidium bromide (0.25 μg/ml). The insert and the vector DNAs were excised under UV light. Gel slices in microcentrifuge tubes were centrifuge briefly and melted at 70°C for 5 min before ligation. The reaction mixture contained 30 ng of insert DNA, 10 ng vector DNA, 0.2 volumes of ligase buffer (5X concentration, Gibco/BRL), 0.1 volumes of T4 DNA ligase (Gibco/BRL) and sterile water. The ligation mixture was incubated for 24h or more at room temperature and was melted at 70°C for 5 min before transformation. For transformation, 200 μl of JM109 or DH5α competent cells were thawed on ice before use. Ten μl of the ligation mixture or 10 ng of plasmid DNA was added to the competent cells and mixed gently. They were incubated for 1 h on ice. The mixture was heated to 42°C for 1 min and then chilled on ice for 1 min. One ml Lauria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, per liter and autoclaved 25 min) was added to the mixture and shaken for 1 h in a 37°C warm room. The entire mixture was plated out on an LB plate containing 100 μg/ml ampicillin and 15 g agar per liter LB medium (pH adjusted to 7.0 with NaOH) and incubated overnight at 37°C.

Overlapping deletions of pQQH2 and pQQH3 were generated by exonuclease III digestion (Erase-a-base System, Promega) according to the manufacturer's protocol for sequencing. In brief, 5 μg plasmid DNA was digested completely with *Xho*I and *Kpn*I restriction enzymes to produce exonuclease III-susceptible and resistant overhanging ends, respectively. Double digested DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) prior to ethanol precipitation (Section 2.2b). Exonuclease III digestion of the DNA yielded inserts of decreasing size. Flushed end-DNAs were then ligated to circularise the deletion-containing vectors. Ten μl of the ligation mixtures were used directly to transform DH5α competent cells (Promega) and the remaining ligation mixtures were stored at -20°C. Plasmid DNA sizes were checked by gel electrophoresis on a 1% (w/v) agarose gel before sequencing. Sequencing by the dideoxynucleotide chain termination method was performed by *Taq* DyeDeoxy terminator cycle sequencing with an automated Model 373A (Applied Biosystems) DNA Sequencer (Department of Biochemistry, DNA Sequencing Laboratory, University of Alberta).

2.2h. Sequence Analyses

Data base searches and sequence alignments were performed using programs of the Sequence Analysis Software GCG Package, including FASTA, FETCH, BESTFIT, COMPARE and PILEUP (Genetics Computer Group Inc.) and by SWEEP searches of the University of Leeds (UK) OWL protein database in conjunction with the alignment program MALIGN and the Birkbeck matrix by Drs. D. Donnelly and S.A. Baldwin, University of Leeds (51). Potential transmembrane domains (TM) of cNT1 were identified using physiochemical (Goldman, Engelman, Steitz) (52) and statistical (von Heijne) (53,54) hydropathy scales and a 21-residue trapezoid sliding window.

2.2i. Northern Blot Analysis

Poly(A)$^+$ RNA (mRNA) from rat jejunum (10 μg) was separated on a 0.8% formaldehyde agarose gel and blotted onto a Hybond-N transfer membrane (Amersham). *Pst*I-*Pst*I (420 bp) and *Acc*I-*BamH*I (617 bp) fragments of pQQH1 that represented coding sequences for cNT1 amino acid residues 75-213 and 385-588, respectively, were labelled with ^{32}P using a ^{T7}QuickPrime kit (Pharmacia). Hybridisation was for 16 h at 42°C in 50% formamide. The membrane was washed twice in 0.1 x SSC(15 mM NaCl and 1.5 mM sodium citrate, pH 7.0)/0.1 % SDS at room temperature and twice at 65°C. A multiple rat tissue blot (ClonTech) (2 μg poly(A)$^+$ RNA per lane) was probed with the ^{32}P-labeled *Acc*I-*BamH*I fragment under identical conditions.

2.2j. *In vitro* Transcription

Plasmid DNA was transcribed with T7 polymerase in the presence of m^{7}GpppG (Ambion) using an *in vitro* transcription kit (MEGAscript™, Ambion). Double-stranded pGEM-3Z plasmid DNA was digested completely with *Xba*I restriction enzyme. The DNA template was purified by phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) extraction and ethanol precipitation, and dissolved in RNase-free water at a concentration of 1 μg/μl. The reaction mixture, containing 0.1 volumes of 10X concentrated buffer, 0.1 volumes of ATP (75 mM), 0.1 volumes of CTP (75 mM), 0.1 volumes of UTP (75

mM), 0.02 volumes of GTP (75 mM), 0.15 volumes of $m^7G(5')ppp(5')G$ (40mM), 0.05 volumes of linearised DNA template (1 $\mu g/\mu l$), 0.1 volumes of the Enzyme mixture (a combination of placental ribonuclease inhibitor and bacteriophage T7 RNA polymerase) and RNase-free water, was mixed gently and incubated at 37°C for 6 h. At the end of the incubation period, template DNA was digested with DNase I at 37°C for 15 min. The reaction was terminated by adding 30 μl of RNase-free water and 25 μ. of lithium chloride precipitation solution (7.5 M lithium chloride and 50 mM EDTA). After mixing thoroughly, the tube was chilled at -20°C for at least one hour. Transcribed RNA (cRNA) was pelleted by centrifugation at $\geq$ 100,000 rpm for 30 min at 4°C. Supernatant was carefully removed. After resuspending the pellet in 200 μl RNase-free water, RNA was recovered by phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) extraction and ethanol precipitation. RNA pellets were resuspended in RNase-free water at a concentration of 1 $\mu g/\mu l$.

2.2k. Preparation of Stage VI *Xenopus* Oocytes

Mature oocyte-positive female *Xenopus* laevis (Nasco) were anesthetised with ice and killed. Ovarian lobes were removed, opened and washed into modified Barth's medium (MBM: 88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 2.5 mM Na-pyruvate, 0.1 mg/ml penicillin, 0.1 mg/ml gentamycin sulphate, 10 mM HEPES, pH 7.5). Small clumps of ovarian tissue were dissected and incubated in 4 mg/ml collagenase (Collagenase A 103578, 0.49 U/mg, Boehringer Mannheim) or 2 mg/ml collagenase (Collagenase CLS1, 184 U/mg, Worthington Biochemicals) in MBM at 20°C for 2 h or until oocytes became separated from the connective tissue. Individual oocytes were washed and sorted prior to incubation in hypertonic phosphate buffer (100 mM K_2HPO_4, pH 6.5, 0.1 % (w/v) bovine serum albumin) for 1 h at 20°C to remove remaining follicular layers. Mature healthy stage VI oocytes were maintained at 18°C for 24 h in MBM before injection.

2.2l. Expression of cNT1 in *Xenopus* Oocytes

pQQH1 was linearised with *Xba*I and *in vitro* transcribed with T7 RNA polymerase in the presence of m^7GpppG cap using the MEGAscript™ *in vitro* transcription system (Ambion) (Section 2.2j). Stage VI oocytes (Section 2.2k) were injected (Inject+Matic System, Singer Instrument Co. Ltd.) with 10 nl of cRNA of pQQH1 (1 $\mu g/\mu l$) or 10 nl of water and incubated at 18°C in MBM for 3 days with a daily change of medium before the assay of transport activity. Nucleoside uptake was traced with the respective [^{3}H]nucleosides ([2,8-^{3}H]adenosine, [5'-^{3}H]thymidine and [5,6-^{3}H]uridine) (Moravek Biochemicals) (20 μCi/ml) which were purified by high performance liquid chromatography (HPLC) before use (>96% radiochemically pure). Purification was performed by D. Mowles in the laboratory of Dr. C.E. Cass, Department of Biochemistry, University of Alberta. Flux measurements were performed in a 48-well tissue culture plate positioned on a Gyrotary shaker (New Brunswick Scientific Co.) at 20°C on groups of 8-12 oocytes in transport buffer (0.2 ml) containing 100 mM NaCl or 100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.5. For assays performed in the absence of Na^+, oocytes were washed

in choline chloride transport buffer prior to the addition of incubation medium. For Cl^- substitution experiments, Cl^- in the transport buffer was substituted by gluconate or methylsulphate. To permit complete Cl^- replacement, the normal transport buffer was simplified to contain only NaCl or Na gluconate or Na methylsulphate (100 mM) and 10 mM HEPES, pH7.5. Omission of KCl, $CaCl_2$ and $MgCl_2$ from the medium had no significant effect on the initial rate of uridine uptake. For competition experiments, oocytes were exposed to nonradioactive nucleosides simultaneously with the ^{3}H-labeled permeant. For inhibition studies with NBMPR, oocytes were preincubated for 30 min at 20°C with the inhibitor before adding [^{3}H]nucleoside. In the case of adenosine transport experiments, 1 μM deoxycoformycin was added to the transport buffer to inhibit adenosine deaminase activity. In control experiments, this concentration of deoxycoformycin had no significant effect on cNT1-mediated uridine influx. At the end of the incubation (30 s to 60 min), extracellular label was removed by six rapid ice-cold washes in the appropriate transport buffer; all washes were completed within 1 min. Individual oocytes were dissolved in 0.5 ml of 5 % (w/v) sodium dodecyl sulphate (SDS) for quantitation of oocyte-associated [^{3}H] by liquid scintillation counting (LS 6000IC, Beckman Canada Inc.). In preliminary experiments, uptake values at time zero were determined by mixing oocytes with ice-cold incubation medium containing radiolabelled permeants, followed by immediate washing and shown not to be significantly different from background.

2.2m. Data Analysis

Results for flux studies are means ± SEM for 8-12 individual oocytes. Kinetic constants (apparent K_m and V_{max}) for uridine and adenosine influx were determined by non-linear regression analysis (ENZFITTER, Elsevier-Biosoft).

2.3. RESULTS

2.3a. Nucleotide and Deduced Amino Acid Sequences of cNT1

Previously, my supervisor's laboratory demonstrated that *Xenopus* oocytes express Na^+-dependent nucleoside transport activity after injection with mRNA from rat jejunum (46). To isolate nucleoside transporter cDNA(s), a 1.5-3.0 kb rat jejunal mRNA size-fraction that showed peak expression of [^{3}H]uridine uptake in *Xenopus* oocytes (46) was used to construct a directional cDNA library in the plasmid expression vector pGEM-3Z (Figure 2.1). The library was then screened in *Xenopus* oocytes for expression of [^{3}H]uridine transport activity. This resulted in the isolation of a 2420 base-pair (bp) nucleotide transporter cDNA (named pQQH1). The open reading frame (ORF) of pQQH1 encodes a 648 amino acid protein with a predicted molecular mass of 71,000 and was named cNT1 (concentrative nucleoside transporter 1) (Figure 2.4). The ORF of pQQH1 is flanked by ~170 bp of 5'-untranslated sequence and ~300 bp of 3'-untranslated sequence. The start of the coding sequence was defined by the first ATG downstream of four in-frame stop codons. cNT1 does not contain a signal sequence suggesting that the N-terminus is located intracellularly. The protein has a relatively high cysteine content (3.1%) and an -SSSS- motif at residues 609-612. Serine clusters also occur in mammalian Na^+/K^+-dependent glutamate transporters (55), acetylcholine and biogenic amine receptors (56) and the rat jejunal 4F2hc cell surface antigen (Chapter 5). A possible Na^+-binding (SOB) motif (GL...AxxxxLxx...iP) has been identified by Deguchi *et al* (57) on the basis of sequence comparisons between different Na^+-dependent nutrient transporter proteins belonging to the SGLT and glutamate gene families (Chapter 1, Section 1.5). Two potential SOB-like motifs were identified in cNT1, one near the N-terminus ($G^{19}L^{20}..A^{25}..S^{30}..G^{34}R^{35}$) and the other near the C-terminus ($G^{553}L^{554}..A^{583}..I^{588}..P^{592}R^{593}$). The motif at the N-terminus is predicted to be intracellular, while that at C-terminus is predicted to be extracellular (please see Figure 2.5, below).

The predicted topology of cNT1 was investigated by hydropathy/charge-bias analysis (54). The topographical model shown in Figure 2.5 has the maximum number of 14 potential transmembrane domains (TMs) and a (+)- charge difference of 26. Assignments for the termini of TMs predicted by the von Heijne hydropathy scale (54) are: TM1, residues 86-106; TM2, residues 108-128; TM3, residues 154-174; TM4, residues 177-197; TM5, residues 205-225; TM6, residues 238-258; TM7, residues 265-285; TM8, residues 298-318; TM9, residues 340-360; TM10, residues 362-382; TM11, residues 428-448; TM12, residues 459-479; TM13, residues 534-554; TM14. residues 572-592. Charged residues observed at the boundaries of many of the putative TMs may play a role in determining the correct orientation of the protein within the membrane (58). In the model, both the N-terminus (residues 1-85) and C-terminus (residues 593-648) are oriented towards the cytoplasm. A panel of other hydropathy analyses (59) predicted the presence of between 10 and 14 TMs, so that alternative secondary structures are conceivable.

The protein has three potential *N*-linked glycosylation sites (Asn 543,605 and 643) and four potential protein kinase C-dependent phosphorylation sites (Ser/Thr 5, 203, 421

and 527). One potential *N*-linked glycosylation site (Asn543) is located within TM13, while the other two (Asn 605 and Asn 643) are predicted to be intracellular (Figure 2.5). Four possible *O*-linked glycosylation sites are located in the extracellular hydrophilic loop connecting TM13 and TM14 (Figure 2.5). All four potential PKC-dependent phosphorylation sites are predicted to be intracellular. There are three potential intramembraneous charged residues in cNT1; Glu 246 in TM6, Glu 307 in TM8 and Arg 469 in TM12. It is possible that Arg 469 and one of the two Glu residues may form an interacting charge pair and function in protein folding and/or stability.

A search of DNA and protein sequence databases revealed significant sequence similarity between cNT1 and the H^+/pyrimidine nucleoside symporter of *E. coli* (NupC) (60) and between these proteins and two *E. coli* sequences of unknown function (ECOHU4748 and ECOHU4751) (61). In the University of Leeds MALIGN sequence alignments shown in Figure 2.6, cNT1 is 27% identical in amino acid sequence to NupC and 34% identical to ECOHU4748 and ECOHU4751, but is ~200 amino acid residues longer than its bacterial homologs. The additional amino acid residues present in cNT1 are located mainly at the N- and C-termini and within the predicted intracellular hydrophilic loop connecting TM12 and TM13. Sequence homology between cNT1 and the bacterial proteins was particularly evident in the C-terminal half of the cNT1 sequence, *i.e.* between TM7 and TM14, inclusive Identical amino acid residues in the four proteins (indicated with an asterisk in Figure 2.6) which may be essential for function are located principally within TM11, TM12 and TM13 of cNT1. There was also a conserved five amino acid residue sequence (NEFVA) within the putative intracellular loop connecting TM12 and TM13 (residues 496-500 of cNT1). NupC, ECOHU4748 and ECOHU4751 did not contain the cNT1 -SSSS- cluster or N- and C-terminal SOB motifs and shared only one of the relatively large number of Cys residues present in cNT1 (Cys 539). GCG PILEUP sequence alignments of the four proteins (Figure 2.7) were similar to those given by the MALIGN analysis (Figure 2.6), except in the region corresponding the first 270 amino acid residues of the cNT1 sequence (TM1 - TM6). In this aligment, cNT1 is 25% and 33% identical to NupC and ECOHU4748/51, respectively. A graphical comparison of the amino acid sequences of NupC, ECOHU4748 and ECOHU4751 with that of cNT1 using the COMPARE and DOTPLOT programs of the GCG package are presented in Figures 2.8a, 2.8b and 2.8c, respectively. This analysis depicts greater N-terminal sequence homology between cNT1 and ECOHU4748/51 than between cNT1 and NupC.

Preliminary hydropathy analyses of NupC, ECOHU4748 and ECOHU4751 suggest that these proteins have 10 potential transmembrane domains, in contrast to the 14 of cNT1 (data not presented). TMs 3-10 of the bacterial proteins align closely with TMs 7-14 of cNT1. However, alignment ambiguities in the N-terminal region of cNT1 (Figures 2.6 and 2.7) make it difficult to identify the cNT1 counterparts of bacterial protein TMs 1 and 2. It is intuitively most likely that they correspond to cNT1 TMs 5 and 6.

In contrast, no sequence similarity was found between cNT1 and proteins of mammalian origin, including the equilibrative glucose transporter (GLUT) family (Chapter 1, Section 1.5a), the Na^+/Cl^--dependent neurotransmitter transporter (GAT)

family (Chapter 1, Section 1.5c), the Na^+/K^+-dependent glutamate transporter family (Chapter 1, Section 1.5d), the cationic amino acid transporter (MCAT) family (Chapter 1, Section 1.5e) and the Na^+-dependent glucose transporter (SGLT) family (Chapter 1, Section 1.5b). As described in Chapter 1, Section 1.5b, the latter includes a putative Na^+-linked nucleoside transporter cDNA isolated from rabbit kidney (SNST1) (47).

2.3b. Expression of cNT1 in *Xenopus* Oocytes

A representative time course of [^{3}H]uridine uptake (10 μM, 20°C) by oocytes injected with cNT1 cRNA is shown in Figure 2.9. After 30 min, the cellular content of uridine, which is only slowly metabolised by oocytes (46), was 64 $\pm$ 3.6 pmol/oocyte, corresponding to an intracellular concentration approximately 6-fold higher than that present in the extracellular medium. Uridine uptake (30 min) in water-injected oocytes was 0.03 pmol/oocyte, giving an expressed:basal flux ratio in excess of 10^4. In subsequent experiments, I used a 1-min incubation period to define initial rates of uridine transport (Figure 2.9, *inset*).

Figure 2.10 shows the concentration-dependence of cNT1-mediated uridine influx. The expressed uridine transport activity was saturable and conformed to simple Michaelis-Menten kinetics with an apparent K_m value of 37 μM (Table 2.1), which is within the published range for Na^+-dependent uridine transport in intact mammalian cells and vesicle preparations (15,18,22,23,25,26). The estimated V_{max} was 21 pmol/oocyte.min^{-1} (Table 2.1). Na^+-independent uridine influx in cRNA-injected oocytes (6% of the total flux at 10 μM uridine) was 3.6-fold greater than uridine influx in water-injected oocytes in Na^+-medium; the latter may represent uncoupled uridine transport (slippage) by the transporter (46, 62).

Some cotransporters are coupled to Cl^- as well as cations. To determine whether cNT1-mediated uridine uptake was Cl^--dependent, uridine influx (10 μM) was measured in simplified transport buffer containing 100 mM NaCl, 100 mM Na gluconate or 100 mM Na methylsulphate (Figure 2.11). Initial rates of uridine influx measured in media containing gluconate (6.51 $\pm$ 0.39 pmol/oocyte.min^{-1}) or methylsulphate (6.34 $\pm$ 0.31 pmol/oocyte.min^{-1}) were not significantly different from that in Cl^- transport buffer (6.67 $\pm$ 0.40 pmol/oocyte.min^{-1}). Therefore, unlike some Na^+-dependent amino acid/neurotransmitter transporters (Chapter 1, Section 1.5c), cNT1 does not require Cl^- ions for activity. NBMPR, a potent inhibitor of the equilibrative *es* nucleoside transporter and the concentrative Na^+-dependent system N5 (Table 1.1), had no significant effect on cNT1-mediated uridine influx (10 μM) at concentrations of 1 μM and 10 μM (Figure 2.12) (4.14 $\pm$ 0.26, 4.52 $\pm$ 0.30 and 3.95 $\pm$ 0.40 pmol/oocyte.min^{-1} in the absence and in the presence of 1 μM and 10 μM NBMPR, respectively).

Three types of the Na^+-dependent nucleoside transporter (systems N1, N2, N3) have been expressed in oocytes injected with intestinal mRNA (44-46). Inhibition experiments in NaCl transport buffer identified cNT1-mediated uridine transport activity as N2-type (Figures 2.13, 2.14 and 2.15). Figure 2.13 demonstrates that uridine influx (10 μM) was almost completely inhibited by 1 mM nonradioactive thymidine (95% inhibition), cytidine (97% inhibition), adenosine (93% inhibition) and uridine (94% inhibition) whereas guanosine (31% inhibition) and inosine (12% inhibition) were much

less effective. The model system N2 permeant thymidine (10 μM) gave similar results (Figure 2.14): 1 mM nonradioactive thymidine (95% inhibition), cytidine (93% inhibition), adenosine (81% inhibition), uridine (93% inhibition), guanosine (16% inhibition) and inosine (6% inhibition).

In the experiment shown in Figure 2.15, cNT1-mediated uridine influx (10 μM) was measured in the absence or in the presence of graded concentrations (5 μM - 1 mM) of unlabeled uridine, thymidine and adenosine. As expected from the data shown in Figure 2.13, all three nucleosides caused almost complete inhibition of uridine influx. Calculated apparent K_i values (assuming competitive inhibition and a uridine apparent K_m of 37 μM, Table 2.1) were 26 μM, 23 μM and 29 μM for uridine, thymidine and adenosine, respectively. To determine whether cNT1 might also accept nucleotides and nucleobases as permeants, cNT1-mediated uridine influx (10 μM) was measured in the presence of 1 mM UMP, UDP, UTP and uracil. No significant inhibition was observed (Figure 2.16). Therefore, cNT1 encodes a Na^+-dependent high-affinity N2-type nucleoside transporter protein with an apparent selectivity for pyrimidine nucleosides and adenosine.

Figure 2.17 shows a representative time course of [^{3}H]adenosine (10 μM) uptake in oocytes injected with cNT1 cRNA or water and confirms that cNT1 mediates adenosine transport. Uptake for adenosine was slower than for uridine and thymidine, and after 10 min was 2.68 $\pm$ 0.36 pmol/oocyte which was 45-fold higher than the control flux in water-injected oocytes (0.06 $\pm$ 0.008 pmol/oocyte). This is the first direct demonstration that an N2-type nucleoside transporter also accepts adenosine as permeant (please see Discussion). In the absence of Na^+ in the incubation medium, the cNT1-mediated adenosine flux was reduced by 94% (0.15 $\pm$ 0.03 pmol/oocyte.10 min^{-1}). In subsequent experiments, I used a 10-min incubation period to define initial rates of adenosine transport. Figure 2.18 shows the concentration dependence of adenosine influx measured in cRNA-injected and water-injected oocytes. cNT1-mediated adenosine influx was saturable and conformed to simple Michaelis-Menten kinetics with apparent K_m and V_{max} values of 26 μM and 0.07 pmol/oocyte.min^{-1}, respectively. Table 2.1 summarises the kinetic parameters of cNT1-mediated uridine and adenosine transport activities in *Xenopus* oocytes derived from the data presented in Figures 2.10 and 2.18. These data confirm the high apparent affinity of cNT1 for adenosine, but demonstrate that this purine nucleoside is only poorly transported compared with the pyrimidine nucleoside uridine. Calculated V_{max}/K_m ratios for uridine and adenosine are 0.57 and 0.003, respectively, a difference of almost 200-fold. cNT1-mediated adenosine (10 μM) influx was strongly inhibited by 0.2 mM thymidine (95% inhibition), cytidine (100% inhibition), adenosine (89% inhibition) and uridine (96% inhibition) (Figure 2.19). In contrast, guanosine gave a modest 18% inhibition and inosine was without effect (0% inhibition). Adenosine is more lipophilic than uridine (63,64) and showed a greater nonsaturable uptake in water-injected oocytes: at a concentration of 1 mM, adenosine and uridine fluxes were 18.8 $\pm$ 1.67 *vs* 3.06 $\pm$ 0.27 pmol/oocyte.30min^{-1}, respectively.

2.3c. Tissue Distribution of cNT1 mRNA

Hybridisation of rat jejunal mRNA at high stringency with a radiolabelled probe

of cNT1 corresponding to cNT1 amino-acid residues 75-213 identified a single transcript at 3.4 kb (Figure 2.20 (a), lane 1). A Northern blot at the same level of stringency with a different cNT1 probe representing amino-acid residues (385-588) closer to the C-terminus of the protein revealed additional transcripts in the 1.5-3.0 kb range, including bands at 1.9 and 2.5 kb that might possibly encode other related rat intestinal Na^+-dependent nucleoside transporters (Figure 2.20 (a), lane 2) (46). When a rat multiple tissue Northern blot was screened at high stringency with the 385-588 amino-acid residue cNT1 probe, only kidney gave a positive hybridisation signal (Figure 2.20 (b)). System N2 transport activity has only been demonstrate definitively in intestinal and kidney epithelia (18,19,23,25,26).

2.4. DISCUSSION

Nucleosides are transported across the brush border and basolateral membranes of enterocytes of the small intestine by concentrative (Na^+-dependent) and equilibrative (Na^+-independent) pathways, respectively (23-26), and it has been demonstrated that *Xenopus* oocytes express Na^+-dependent nucleoside transport activity when injected with intestinal mRNA from both the rat (systems N1, N2 and N3) (46) and rabbit (system N2) (44,45). In this Chapter, I describe how expression screening in *Xenopus* oocytes was used to isolate a 2420 base-pair (bp) cDNA from rat jejunal epithelium encoding a Na^+-dependent nucleoside transporter protein (named cNT1) (48).

Expression of cNT1 cRNA in *Xenopus* oocytes induced concentrative Na^+-dependent (Cl^--independent), high-affinity [^{3}H]uridine uptake (apparent K_m 37 μM) (Figures 2.9 and 2.10) and, as well, Na^+-dependent influx of [^{3}H]thymidine and [^{3}H]adenosine (Figures 2.14, 2.17 and 2.18). cNT1-mediated transport of all three [^{3}H]nucleosides was inhibited by uridine, thymidine, cytidine and adenosine, but only weakly by guanosine or inosine (Figures 2.13, 2.14 and 2.19). Transport was unaffected by nucleotides (UMP, UDP and UTP), the nucleobase uracil, and NBMPR (Figures 2.11 and 2.12). Therefore, cNT1 is a Na^+-dependent, NBMPR-insensitive nucleoside transporter with N2-type characteristics. Consistent with the tissue distribution of system N2 transport activity (18-21,23,26), transcripts for cNT1 were detected in kidney as well as intestine.

Although primarily selective for pyrimidine nucleosides, cNT1 also accepted the purine nucleoside adenosine as a substrate. Previous assignments of adenosine as a system N2 permeant have been based on competition studies (*e.g.* the ability of adenosine to block N2-mediated uridine transport in intact cells and membrane vesicles) and not on direct measurements of N2-mediated adenosine transport. Direct measurements of [^{3}H]adenosine influx have only been reported for the N1 or N3 nucleoside transport systems of murine leukemia L1210 cells (34,35), rat renal brush-border membrane vesicles (19), rat hepatocytes (17) and rabbit intestinal brush-border membrane vesicles (24). My experiments are therefore the first direct demonstration that system N2 mediates Na^+-dependent adenosine transport activity. Kinetic parameters for uridine and adenosine influx mediated by cNT1 in *Xenopus* oocytes are listed in Table 2.1. Both nucleosides were transported with high-affinity. However, the apparent V_{max} for adenosine transport was only 0.33% of that for uridine, giving an adenosine V_{max}/K_m ratio of 0.003 compared with 0.57 for uridine. Therefore, although cNT1 transports adenosine, this purine nucleoside is handled very differently from uridine. As discussed in Chapter 6 (General Discussion), system N2 is unlikely to be a major physiological route of transmembrane adenosine movements.

The deduced amino acid sequence of cNT1 showed no similarity to the rabbit kidney SNST1 nucleoside transporter (47) or to other proteins of mammalian origin. There was, however, significant homology to an *E. coli* nucleoside transporter, NupC (60), and to two *E. coli* proteins, ECOHU4748 and ECOHU4751, of unknown function. A detailed functional study of NupC expressed in *Xenopus* oocytes is presented in Chapter 4. The results are consistent with the bacterial nucleoside transport literature

which describes NupC as a H^+/nucleoside symporter selective for pyrimidine nucleosides and adenosine (39-40). Some other bacterial transporters such as the *E. coli* melibiose transporter (MelB) can utilise either H^+ or Na^+ for the uphill movement of solute, while the *B. stearothermophilus* glutamate transporter (GltT) is coupled to both H^+ and Na^+ translocation (65). Results presented in Chapter 4 suggest that NupC may not be able to substitute Na^+ for H^+. Conversely, cNT1 shows no apparent pH-dependence (please also see Chapter 4). This topic is discussed further in Chapter 6 (General Discussion).

cNT1 is larger than its bacterial homologs (648 *vs* 401-417 amino acid residues), with the additional amino acid residues localised at the N- and C-termini and, to a lesser extent, in the predicted hydrophilic loop connecting TM12 and TM13. The two putative SOB binding motifs in cNT1 are located at the ends of the transporter, so that the additional regions of sequence in cNT1 may be structural determinants of Na^+-dependence. Alternatively, they may be involved in regulation of mammalian nucleoside transport activity. On the other hand, deletions of the N- or C-termini of the *E. coli* H^+-linked Lac permease (66,67) and the mammalian GAT1 Na^+/Cl^--dependent GABA transporter (68) have no significant effect on transport function, so that it is also possible that the extreme N- and/or C-termini of cNT1 may not be functionally important.

Amino acid residues and regions of sequence conserved between cNT1 and its bacterial homologs are likely to be important or essential for transport activity, particularly in relation to substrate specificity, which appears to be identical in cNT1 and NupC. cNT1 and NupC exhibit about 27% sequence identity overall, but show particularly strong similarities in their C-terminal regions (where the substrate binding site may therefore be located), both in terms of amino acid sequence and also in terms of the predicted locations, size and number of transmembrane helices. Conserved amino acid residues between cNT1 and its bacterial counterparts are most prevalent within potential transmembrane domains, particularly in TM12 and in TM13 where there is a stretch of eleven amino acids with the sequence -LxxFANFxSIG- (residues 538-548 of cNT1). The presumed extracellular loop linking these two potential transmembrane domains also contains a group of 5 conserved amino acid residues with the sequence -NEFVA- (residues 496-500 of cNT1). Such regions of the cNT1 sequence are potential targets for future analysis by site-directed mutagenesis.

Conserved charged and polar residues are of particular interest because of their potential interactions with H^+/Na^+ and nucleosides or, for charged residues, their possible participation in structurally important ionic bonds (65,69,70). For example, site-directed mutagenesis studies of Lac permease indicate that Arg302, His322 and Glu325 in putative TMs 9 and 10 are involved in coupling H^+ and lactose translocation (71-73). Recent studies of the Lac permease by site-directed mutagenesis and cysteine scanning mutagenesis indicate that helices VII, VIII, IX, X and XI are in close proximity and interact via ion pairs (73-80). Conserved charged and polar amino acid residues located within potential TMs of cNT1 include Glu307 (TM8), Asn443 (TM11), Asn543 (TM13), Ser352 (TM9), Ser459 (TM12), Ser546 (TM13), Tyr357 (TM9) and Tyr466 (TM12) (Figure 2.5).

cNT1 is the first mammalian representative of a new gene family of transporters. As discussed in Chapter 6 (General Discussion) expression and functional characterisation

of ECOHU4748 and ECOHU4751 in *Xenopus* oocytes will help resolve whether members of the cNT1/NupC family exclusively transport nucleosides or whether transporters selective for other classes of permeant are also represented. cNT1 is more closely similar to ECOHU4748/51 than to NupC and the sequence homology may extend further into the N-terminal regions of the proteins (Figure 2.8). Determinations of ECOHU4748/51 cation specificity will therefore also be important. The absence of amino acid sequence similarity between cNT1 and the SGLT nucleoside transporter homolog from rabbit kidney SNST1 (47) is consistent with the different cation coupling ratios of mammalian system N2 (cNT1) and system N3 (SNST1) nucleoside transporters (81) and suggests that mammalian nucleoside transporters comprise at least two gene families.

In summary, a cDNA from rat jejunal epithelium encoding a Na^+-dependent nucleoside transport protein (named cNT1) was isolated by functional expression cloning in *Xenopus* oocytes. Data base searches indicate significant sequence similarity to the NupC H^+/nucleoside symporter of *E.coli*, but there is no sequence similarity between cNT1 and proteins of mammalian origin. Functionally, cNT1 exhibits the transport characteristics of the concentrative nucleoside transport system N2 (selective for pyrimidine nucleosides and adenosine). Consistent with the tissue distribution of system N2 transport activity, transcripts of cNT1 were detected in kidney as well as intestine. cNT1 and its bacterial homologs therefore belong to a new gene family of transporter proteins.

Table 2.1. Kinetic parameters of cNT1-mediated nucleoside transport in *Xenopus* oocytes.

Substrate	K_m (μM)	V_{max} (pmol/oocyte.min^{-1})	V_{max}/K_m
Uridine	37	21	0.57
Adenosine	26	0.07	0.003

Kinetic parameters are derived from data presented in Figures 2.10 and 2.18

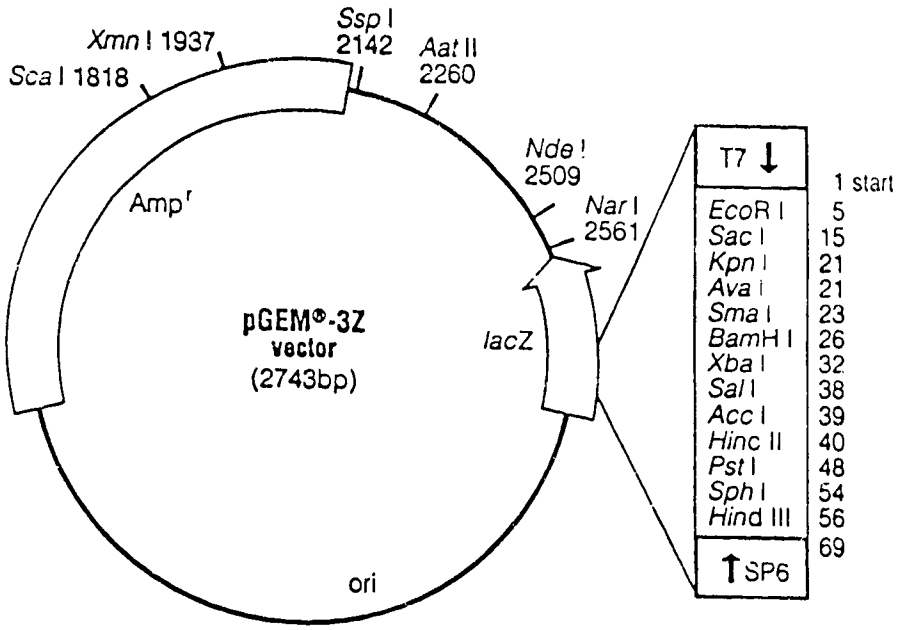
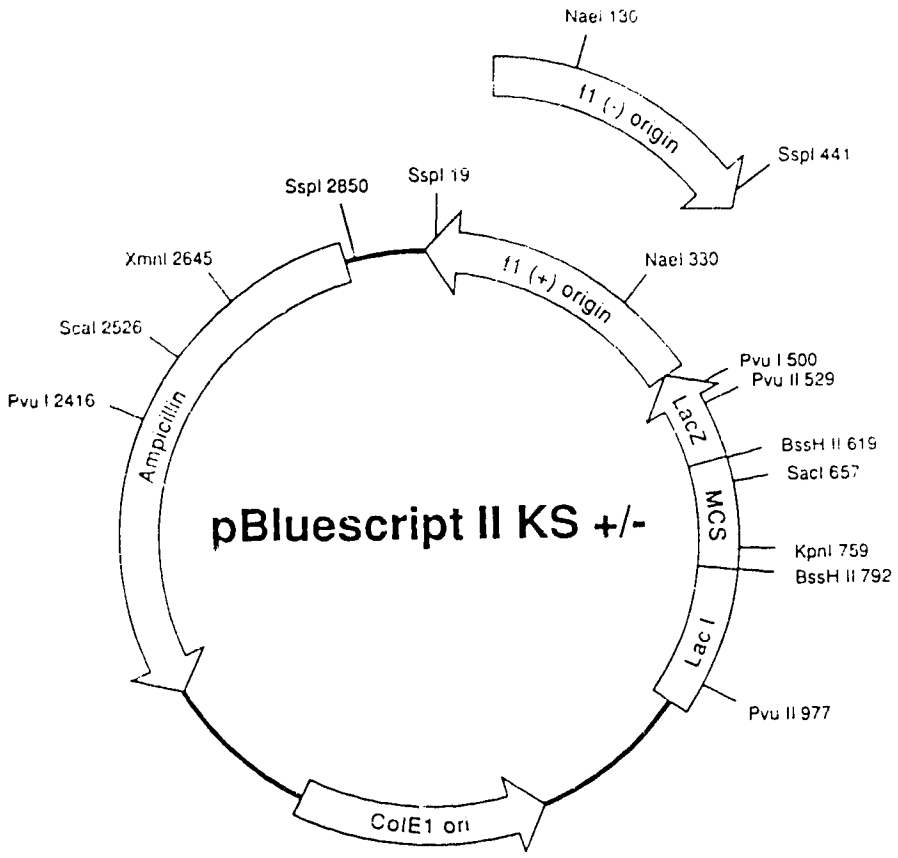


Figure 2.1. pGEM-3Z Vector Circle Map and the Multiple Cloning Site. pGEM-3Z is a 2743 bp vector that contains a multiple cloning region and allows synthesis of RNA transcripts from either strand using the SP6 and T7 RNA polymerase promotors.

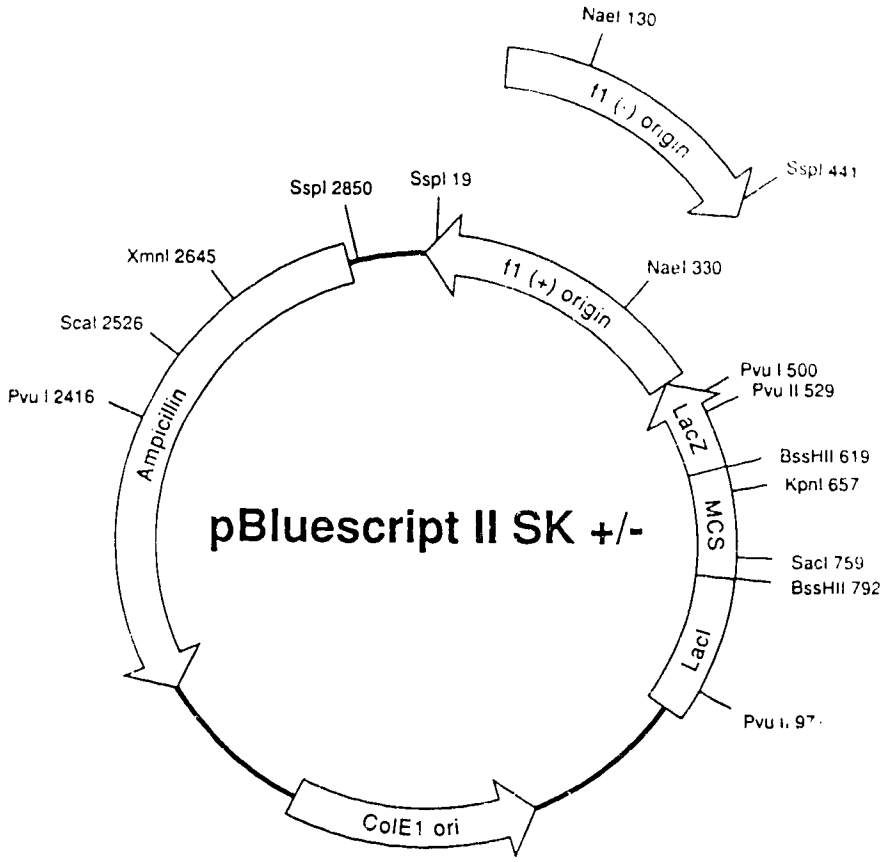


Multiple Cloning Site:

T3 promotor - KpnI ApaI DraII XhoI HincII AccI SalI ClaI HindIII EcoRV EcoRI PstI

SmaI BamHI SpeI XbaI NotI EagI BstXI SacII SacI - T7 promotor.

Figure 2.2. pBluescript II KS Vector Circle Map and the Multiple Cloning Site. pBluescript II KS is a 2961 bp vector. The multiple cloning site is flanked by T3 and T7 promotors.



Multiple Cloning Site:

T3 promotor - SacI BstXI SacII NotI EagI XbaI SpeI BamHI SmaI PstI EcoRI EcoRV

HindIII ClaI HincII AccI SalI XhoI DraII ApaI KpnI - T7 promotor.

Figure 2.3. pBluescript II SK Vector Circle Map and the Multiple Cloning Site. pBluescript II KS is a 2961 bp vector. The multiple cloning site is flanked by T3 and T7 promotors.

Figure 2.4. Nucleotide and deduced amino acid sequences of cNT1. The open reading frame encoding 648 amino acid residues is flanked by ~170bp of 5'-untranslated sequence and ~300bp of 3'-untranslated sequence. The stop codon is indicated by an asterisk.

```
                                  CCTGACGCTGCCTTCTCACTGCAGATAAGTGAGTAG
TACAGGACCCTCTCCCCTCTCTATGCAGCCCTGTGTCTGTGAGTGCCCAGGGAGCAGGCA 96
TTTACCAGGTCTGGTGGCTGCGTGTTCCACACGTCCTCATGAGGCTCAAGAGCCAAGCAC 156
ATGGCAGACAACACACAGAGGCAAAGAGAGTCCATTTCCCTCACGCCTATGGCCCACGGC 216
M  A  D  N  T  Q  R  Q  R  E  S  I  S  L  T  P  M  A  H  G      20
CTGGAGAACATGGGGGCAGAATTCCTGGAAAGCATGGAGGAAGGCCGACTCCCTCACAGT 276
L  E  N  M  G  A  E  F  L  E  S  M  E  E  G  R  L  P  H  S      40
CACTCAAGCCTGCCGGACGGTGAAGGTGGCCTGAACAAAGCAGAGCGGAAGGCCTTCTCC 336
H  S  S  L  P  E  G  E  G  G  L  N  K  A  E  R  K  A  F  S      60
CGATGGAGGAGTCTGCAGCCGACTGTGCAAGCGAGAAGCTTCTGCAGGGAGCACCGGCAG 396
R  W  R  S  L  Q  P  T  V  Q  A  R  S  F  C  R  E  H  R  Q      80
CTGTTTGGATGGATCTGCAAAGGCCTGCTCTCTACTGCATGTCTTGGCTTCTTGATGGTC 456
L  F  G  W  I  C  K  G  L  L  S  T  A  C  L  G  F  L  M  V     100
GCCTGCCTCCTGGACCTCCAGAGGGCCCTAGCACTGTTGATCATCACCTGTGTGGTTCTC 516
A  C  L  L  D  L  Q  R  A  L  A  L  L  I  I  T  C  V  V  L     120
GTCTTTCTGGCCTATGATCTGCTAAPGAGGCTTCTGGGGTCCAAGCTGAGGAGGTGTGTG 576
V  F  L  A  Y  D  L  L  K  R  L  L  G  S  K  L  R  R  C  V     140
AAGTTTCAAGGCCATTCTTGCCTGAGCCTCTGGCTGAAAAGAGGTCTAGCCCTTGCTGCT 636
K  F  Q  G  H  S  C  L  S  L  W  L  K  R  G  L  A  L  A  A     160
GGTGTGCGCCTGATCTTGTGGCTATCTCTGGACACCGCCCAGCGGCCTGAACAGCTGGTG 696
G  V  G  L  I  L  W  L  S  L  D  T  A  Q  R  P  E  Q  L  V     180
TCCTTTGCAGGGATCTGTGTGTTCCTTGTCCTTCTCTTTGCTGGCTCAAAGCATCACCGT 756
S  F  A  G  I  C  V  F  L  V  L  L  F  A  G  S  K  H  H  R     200
GCGGTGTCATGGCGAGCTGTGTCCTGGGGCCTTGGGCTGCAGTTTGTGCTTGGGCTCTTC 816
A  V  S  W  R  A  V  S  W  G  L  G  L  Q  F  V  L  G  L  F     220
GTCATCAGAACAGAACCAGGGTTCATTGCATTCCAGTGGCTAGGGGATCAGATCCAGGTC 876
V  I  R  T  E  P  G  F  I  A  F  Q  [illegible]  G  D  Q  I  Q  V     240
TTCCTGAGTTACACCGAGGCAGCTCCAGCTTCG[illegible]AGGCTCTGGTGAAGCAT 936
F  L  S  Y  T  E  A  G  S  S  F  [illegible]  A  L  V  K  D     260
GTCTTTGCCT[illegible]AGGTTTTGCCCATCATCATC[illegible]CTGCGTCATGTCTGTTCTG 996
V  F  A  F  Q  V  L  P  I  I  I  F  [illegible]  S  C  V  M  S  V  L     280
TACTATCTGGGCCTCATGCAGTGGGTGATCCTGA[illegible]TGCCTGGTTGATGCAGGTCACC 1056
Y  Y  L  G  L  M  Q  W  V  I  L  K  [illegible]  A  W  L  M  Q  V  T     300
ATGGGCACCTCAGCCACCGAGACACTGAGTGTGGCGGGAAACATCTTTGTGAGCCAGACT 1116
M  G  T  S  A  T  E  T  L  S  V  A  G  N  I  F  V  S  Q  T     320
GAAGCTCCTCTGCTGATCCGGCCCTATCTGGCAGACATGACACTCTCTGAAGTTCACGTT 1176
E  A  P  L  L  I  R  P  Y  L  A  D  M  T  L  S  E  V  H  V     340
GTCATGACTGGAGGCTATGCTACCATTGCTGGCAGCCTCCTGGGCGCCTACATCTCCTTT 1236
V  M  T  G  G  Y  A  T  I  A  G  S  L  L  G  A  Y  I  S  F     360
GGGATCGACGCTGCTTCCTTAATCGCAGCCTCTGTCATGGCCGCCCCTTGTGCGTTGGCT 1296
G  I  D  A  A  S  L  I  A  A  S  V  M  A  A  P  C  A  L  A     380
CTCTCCAAGCTGGTCTACCCAGAGGTGGAGGAGTCCAAGTTCCGGAGTGAGAATGGCGTG 1356
L  S  K  L  V  Y  P  E  V  E  E  S  K  F  R  S  E  N  G  V     400
AAGCTGACCTATGGAGACGCTCAGAACCTCTTGGAAGCAGCCAGTGCTGGGGCTGCCATC 1416
K  L  T  Y  G  D  A  Q  N  L  L  E  A  A  S  A  G  A  A  I     420
TCAGTGAAGGTCGTTGCCAACATTGC[illegible]GCCAATCTGATTGCCTTCCTGGCTGTACTAGCC 1476
S  V  K  V  V  A  N  I  A  A  N  L  I  A  F  L  A  V  L  A     440
TTCGTCAATGCTGCCCTCTCCTGGCTAGGGGACATGGTGGACATCCAGGGACTCAGCTTC 1536
F  V  N  A  A  L  S  W  L  G  D  M  V  D  I  Q  G  L  S  F     460
CAGCTCATCTGCTCCTACGTCCTGCGGCCTGTGGCCTTCTTGATGGGTGTGGCCTGCGAG 1596
Q  L  I  C  S  Y  V  L  R  P  V  A  F  L  M  G  V  A  W  E     480
GACTGTCCGGTAGTGGCTGAGTTGCTGGGC[illegible]CAAGTTCTTTCTGARTGAGTTTGTGGCC 1656
D  C  P  V  V  A  E  L  L  G  [illegible]  K  F  F  L  N  E  F  V  A     500
TATCAAGAGCTTTCCCAGTACAAGCAACGACGCCTGGCAGGGGCTGAGGAGTGGCTTGGT 1716
Y  Q  E  L  S  Q  Y  K  Q  R  R  L  A  G  A  E  E  W  L  G     520
GACAAGAAACAGTGGATCTCTGTCAGAGCAGAAATCCTGACTACATACGCCCTCTGTGGA 1776
D  K  K  Q  W  I  S  V  R  A  E  I  L  T  T  Y  A  L  C  G     540
TTTGCCAACTTCAGCTCCATCGGCATCATGTTGGGAGGCCTGACCTCCCTAGTCCCCCAG 1836
F  A  N  F  S  S  I  G  I  M  L  G  G  L  T  S  L  V  P  Q     560
CGGAGGAGCGACTTCTCCCAGATTGTACTCCGGGCACTGATCACRGGGGCTTTCGTGTCC 1896
R  R  S  D  F  S  Q  I  V  L  R  A  L  I  T  G  A  F  V  S     580
CTGCTAAACGCCCTGTGTCGCAGGGRTCCTCTATGTACCCAGGGGCGTCGAGGTGGACTGC 1956
L  L  N  A  C  V  A  G  I  L  Y  V  P  R  G  V  E  V  D  C     600
GTGTCCCTTCTGAACCAAACTGTCAGCAGCAGCAGCTTTGACGTTTACCTGTGCTGCCGC 2016
V  S  L  L  N  Q  T  V  S  S  S  S  F  E  V  Y  L  C  C  R     620
CAAGTCTTCCAGAGCACTAGCTCGGAGTTCAGCCAAGTGGCACTGGACAACTGCTGTCGA 2076
Q  V  F  Q  S  T  S  S  E  F  S  Q  V  A  L  D  N  C  C  R     640
TTTTACAACCACACAGTCTGCACATAGCTGGGACGGAGCRTCTTCGTAGCCTCAGGGCTC 2136
F  Y  N  H  T  V  C  T  *
ATCCAGCCCAGAGAGGCGTGGGACTCGTCACTACCTCGATCCCACAATTGGGAAGGGTG 2196
CAACGGTCATCGCTGCTCCCRTGTCTGCCTCTCCAAGTACGAGTTCCCAGAGTCTGGTCT 2256
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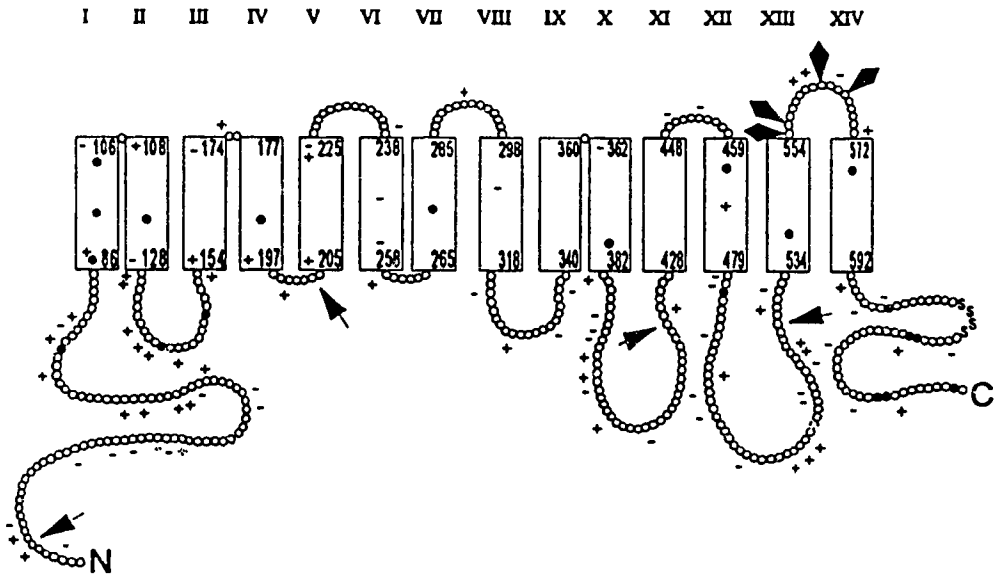


Figure 2.5 Topographical model of cNT1. Potential membrane spanning domains are numbered and shown as rectangles. The relative positions of acidic (Glu, Asp), basic (Lys, Arg) and Cys residues are indicated by (-), (+) and (●), respectively. Four potential *O*-linked glycosylation sites are located in the extracellular loop connecting transmembrane domains 13 and 14 (♦). All four potential PKC-dependent phosphorylation sites are predicted to be intracellular (↑).

Figure 2.6. Alignment of cNT1 with NupC, ECOHU4748 and ECOHU4751 (MALIGN analysis). Analysis was performed by Drs. D. Donnelly and S.A. Baldwin, University of Leeds. Predicted transmembrane domains of cNT1 are marked by bars below the sequence alignment. Amino acids identical in all four proteins are indicated by asterisks. Gaps were introduced to optimise the sequence alignment.

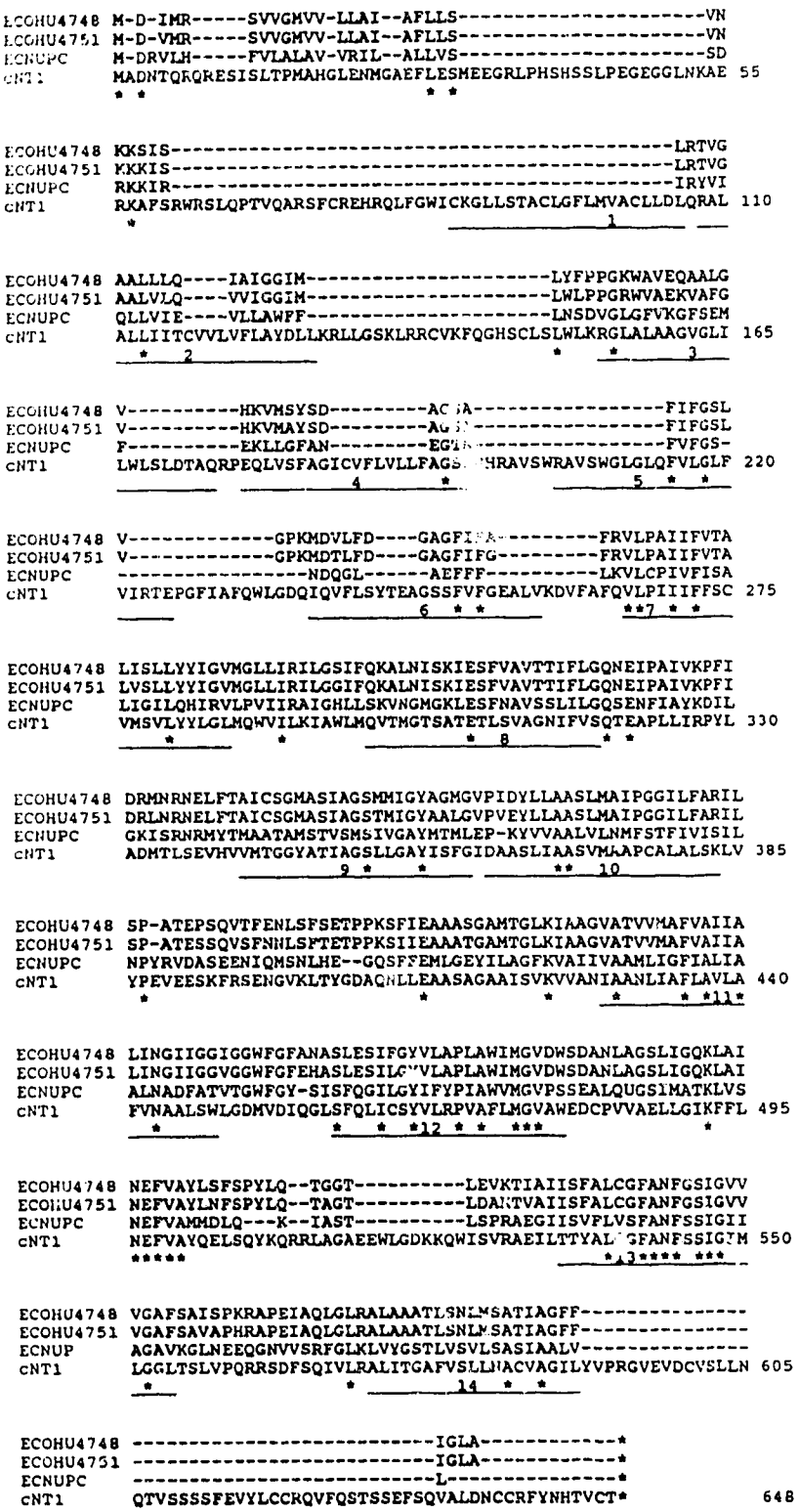

ECOHU4748 M-D-IMR-----SVVGMVV-LLAI--AFLLS----------VN
ECOHU4751 M-D-VMR-----SVVGMVV-LLAI--AFLLS----------VN
ECNUPC M-DRVLH-----FVLALAV-VRIL--ALLVS----------SD
cNT1 MADNTQRQRESISLTPMAHGLENMGAEFLESMEEGRLPHSHSSLPEGEGGLNKAE 55
ECOHU4748 KKSIS----------LRTVG
ECOHU4751 KKKIS----------LRTVG
ECNUPC RKKIR----------IRYVI
cNT1 RKAFSRWRSLQPTVQARSFCREHRQLFGWICKGLLSTACLGFLMVACLLDLQRAL 110
ECOHU4748 AALLLQ----IAIGGIM----------LYFPPGKWAVEQAALG
ECOHU4751 AALVLQ----VVIGGIM----------LWLPPGRWVAEKVAFG
ECNUPC QLLVIE----VLLAWFF----------LNSDVGLGFVKGFSEM
cNT1 ALLIITCVVLVFLAYDLLKRLLGSKLRRCVKFQGHSCLSLWLKRGLALAAGVGLI 165
ECOHU4748 V----------HKVMSYSD---------ACGA----------FIFGSL
ECOHU4751 V----------HKVMAYSD---------AGGA----------FIFGSL
ECNUPC F----------EKLLGFAN---------EGTA----------FVFGS-
cNT1 LWLSLDTAQRPEQLVSFAGICVFLVLLFAGSGHRAVSWRAVSWGLGLQFVLGLF 220
ECOHU4748 V----------GPKMDVLFD----GAGFIGA---------FRVLPAIIFVTA
ECOHU4751 V----------GPKMDTLFD----GAGFIFG---------FRVLPAIIFVTA
ECNUPC ----------NDQGL------AEFFF----------LKVLCPIVFISA
cNT1 VIRTEPGFIAFQWLGDQIQVFLSYTEAGSSFVFGEALVKDVFAFQVLPIIIFFSC 275
ECOHU4748 LISLLYYIGVMGLLIRILGSIFQKALNISKIESFVAVTTIFLGQNEIPAIVKPFI
ECOHU4751 LVSLLYYIGVMGLLIRILGGIFQKALNISKIESFVAVTTIFLGQNEIPAIVKPFI
ECNUPC LIGILQHIRVLPVIIRAIGHLLSKVNGMGKLESFNAVSSLILGQSENFIAYKDIL
cNT1 VMSVLYYLGLMQWVILKIAWLMQVTMGTSATETLSVAGNIFVSQTEAPLLIRPYL 330
ECOHU4748 DRMNRNELFTAICSGMASIAGSMMIGYAGMGVPIDYLLAASLMAIPGGILFARIL
ECOHU4751 DRLNRNELFTAICSGMASIAGSTMIGYAALGVPVEYLLAASLMAIPGGILFARIL
ECNUPC GKISRNRMYTMAATAMSTVSMSIVGAYMTMLEP-KYVVAALVLNMFSTFIVISIL
cNT1 ADMTLSEVHVVMTGGYATIAGSLLGAYISFGIDAASLIAASVMAAPCALALSKLV 385
ECOHU4748 SP-ATEPSQVTFENLSFSETPPKSFIEAAASGAMTGLKIAAGVATVVMAFVAIIA
ECOHU4751 SP-ATESSQVSFNNLSFTETPPKSIIEAAATGAMTGLKIAAGVATVVMAFVAIIA
ECNUPC NPYRVDASEENIQMSNLHE--GQSFFEMLGEYILAGFKVAIIVAAMLIGFIALIA
cNT1 YPEVEESKFRSENGVKLTYGDAQNLLEAASAGAAISVKVVANIAANLIAFLAVLA 440
ECOHU4748 LINGIIGGIGGWFGFANASLESIFGYVLAPLAWIMGVDWSDANLAGSLIGQKLAI
ECOHU4751 LINGIIGGVGGWFGFEHASLESILGVVLAPLAWIMGVDWSDANLAGSLIGQKLAI
ECNUPC ALNADFATVTGWFGY-SISFQGILGYIFYPIAWVMGVPSSEALQUGSIMATKLVS
cNT1 FVNAALSWLGDMVDIQGLSFQLICSYVLRPVAFLMGVAWEDCPVVAELLGIKFFL 495
ECOHU4748 NEFVAYLSFSPYLQ--TGGT----------LEVKTIAIISFALCGFANFGSIGVV
ECOHU4751 NEFVAYLNFSPYLQ--TAGT----------LDANTVAIISFALCGFANFGSIGVV
ECNUPC NEFVAMMDLQ---K--IAST----------LSPRAEGIISVFLVSFANFSSIGII
cNT1 NEFVAYQELSQYKQRRLAGAEEWLGDKKQWISVRAEILTTYALGFANFSSIGIM 550
ECOHU4748 VGAFSAISPKRAPEIAQLGLRALAAATLSNLMSATIAGFF----------
ECOHU4751 VGAFSAVAPHRAPEIAQLGLRALAAATLSNLMSATIAGFF----------
ECNUP AGAVKGLNEEQGNVVSRFGLKLVYGSTLVSVLSASIAALV----------
cNT1 LGGLTSLVPQRRSDFSQIVLRALITGAFVSLLNACVAGILYVPRGVEVDCVSLLN 605
ECOHU4748 ----------IGLA----------*
ECOHU4751 ----------IGLA----------*
ECNUPC ----------L----------*
cNT1 QTVSSSSFEVYLCCRQVFQSTSSEFSQVALDNCCRFYNHTVCT* 648

Figure 2.7. Alignment of cNT1 with NupC, ECOHU4748 and ECOHU4751 (GCG PILEUP analysis). Predicted transmembrane domains of cNT1 are marked by bars below the sequence alignment. Amino acids identical in all four proteins are indicated by asterisks. Gaps were introduced to optimise the sequence alignment.

```
ECOHU4748 -------------------------------------------------------
ECOHU4751 -------------------------------------------------------
ECNUPC    -------------------------------------------------------
cNT1      MADNTQRQRESISLTPMAHGLENMGAEFLESMEEGRLPHSHSSLPEGEGGLNKAE 55
CONSENSUS .......................................................

ECOHU4748 -------------------------------------------------------
ECOHU4751 -------------------------------------------------------
ECNUPC    -------------------------------------------------------
cNT1      RKAFSRWRSLQPTVQARSFCREHRQLFGWICKGLLSTACLGFLMVACLLDLQRAL 110
CONSENSUS .......................................................

ECOHU4748 -------------------------------------------------------
ECOHU4751 -------------------------------------------------------
ECNUPC    -------------------------------------------------------
cNT1      ALLIITCVVLVFLAYDLLKRLLGSKLRRCVKFQGHSCLSLWLKRGLALAAGVGLI 165
CONSENSUS .......................................................

ECOHU4748 -----------MDVMRSVLGMVVLLTIAFLLSVNKKKISLRTVGAALVLQVVIGGI
ECOHU4751 -----------MDIMRSVVGMVVLLAIAFLLSVNKKSISLRTVGAALLLQIAIGGI
ECNUPC    ----------MDRVLHFVLALAVVRILALLVSSDRKKIRIRYVIQLLVIEVLLAWF
cNT1      LWLSLDTAQRPEQLVSFAGICVFLVLLFAGSKHHRAVSWRAVSWGLGLQFVLGLF 220
CONSENSUS ...........DV..SV.GM.VLL..AFL.S...K.IS.R.V...L.LQV..G..
                      *4        *          *     * * 5

ECOHU4748 MLWLPPGRWVAEXVAFGVHKVMAYSDAGSAFIFGSLVGPKMDVLFDGAGFIFAFR
ECOHU4751 MLYFPPGKWAVEQAALGVHKVMAYSDADSAFIFGSLVGPKMDTLFDGAGFIFGFR
ECNUPC    FLNSDVGLGFVKGFSEMFEKLLGFANEGTNFVFGSND--------QGLAEFFFLK
cNT1      VIRTEPGFIAFQWLGDQIQVFLSYTEAGSSFVFGEALVKD----------VFAFQ 275
CONSENSUS .L...PG..........V.K...Y.DAGS.F.FGS...........G...IF.F.
                *                  6 * ***                *

ECOHU4748 VLPAIIFVTALISLLYYIGVMGLLIRILGSIFQKALNISKIESFVAVTTIFLGQN
ECOHU4751 VLPAIIFVTALVSLLYYIGVMGLLIRILGGIFQKALNISKIESFVAVTTIFLGQN
ECNUPC    VLCPIVFISALIGILQHIRVLPVIIRAIGHLLSKVNGMGKLESFNAVSSLILGQS
cNT1      VLPIIIFFSCVMSVLYYLGLMQWVILKIAWLMQVTMGTSATETLSVAGNIFVSQT 330
CONSENSUS VLP.IIFV.ALISIL..IGVM...IR..G..FQK.L..SK.ESF.AV..IFLGQ.
          **  * * 7     *      *             * 8           *

ECOHU4748 EIPAIVKPFIDRMNRNELFTAICSGMASIAGSMMIGYAGMGVPIDYLLAASLMAI
ECOHU4751 EIPAIVKPFIDRLNRNELFTAICSGMASIAGSTMIGYAALGVPVEYLLAASLMAI
ECNUPC    ENFIAYKDILGKISRNRMYTMAATAMSTVSMSIVGAYMTM-LEPKYVVAALVLNM
cNT1      EAPLLIRPYLADMTLSEVHVVMTGGYATIAGSLLGAYISFGIDAASLIAASVMAA 385
CONSENSUS E.P..VKPF...M.RNELFT....GMA.IAGS.M..Y..MGV...YL.AAS.MA.
          *                            9 *    *         ** 10

ECOHU4748 PGGILFARILSP-ATEPSQVTFEN-LSFSETPPKSFIEAAASGAMTGLKIAAGVA
ECOHU4751 PGGILFARILSP-ATESSQVSFNN-LSFTLTPPKSIIEAAATGAMTGLKIAAGVA
ECNUPC    FSTFIVISILNPYRVDASE---ENIQMSNLHEGQSFFEMLGEYILAGFKVAIIVA
cNT1      PCALALSKLVYP-EVEESKFRSENGVKLTYGDAQNLLEAASAGAAISVKVVANIA 440
CONSENSUS P....F..L..P...E.S....EN...F......SF.EAA..G.M..LK.AA.VA
                    *     *     *           *        *   *

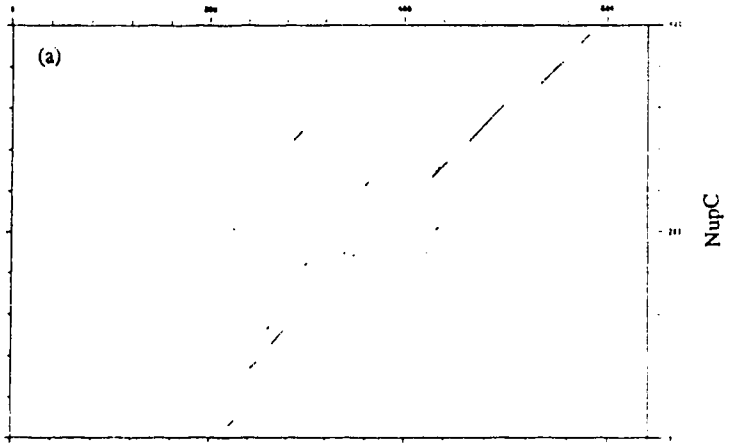
ECOHU4748 T-VVMAFVAIIALINGT[illegible]GIGGWFGFANASLESIFFGYVLAPLAWIMGVDWSDA
ECOHU4751 T-VVMAFVAIIALINGIIGGVGGWFGFEHASLESI-LGYVLAPLAWIMGVDWSDA
ECNUPC    AMLI-GFIALIAALNADFATVTGWFGY-SISFQGI-LGYIFYPIAWVMGVPSSEA
cNT1      ANLI-A[illegible]LAVLAFVNAALSWLGDMVDIQGLSFQLIC-SYVLRPVAFLMGVAWEDC 495
CONSENSUS .....AFVALLALIN.....VGGWFGF...S...I.LGYVL.P.AWVMGV.WSDA
               * *11* *             *   *  *12 * *  ***

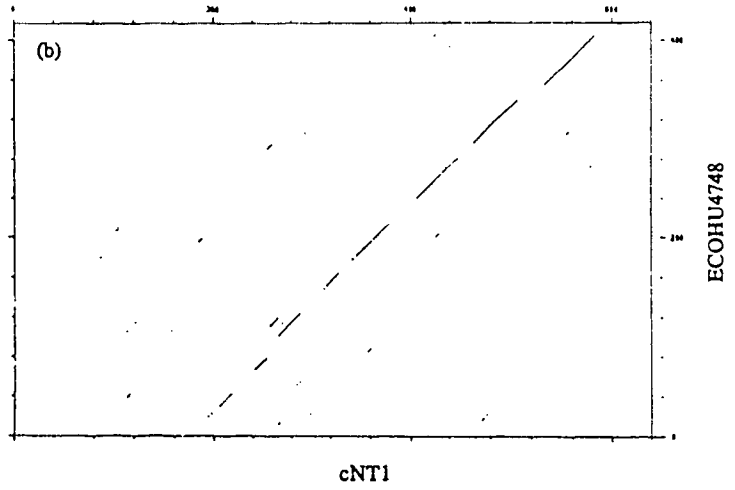
ECOHU4748 NIAGSLIGQKLAINEFVAYLSFSPYLQTGGT------------LEVKTIAIISFA
ECOHU4751 NLAGSLIGQKLAINEFVAYLNFSPYLQTAGT------------LDAKTVAIISFA
ECNUPC    IQUGSIMATKLVSNEFVAMMDLQKIAST---------------LSPRAEGIISVF
cNT1      PVVAELLGIKFFLNFFVAYQELSQYKQRRLAGAEEWLGDKKQWISVRAEILTTYA 550
CONSENSUS ...G...G.KL..NEFVAYL..S.Y.QT...............L......IISFA
                 *   *****

ECOHU4748 LCGFANFGSIGVVVGAFSAISPKRAPEIAQLGLRALAAATLSNLMSATIAGFFIG
ECOHU4751 LCGFANFGSIGVVVGAFSAVAPHRAPEIAQLGLRALAAATLSNLMSATIAGFFIG
ECNUPC    LVSFANFSSIGX-IAGVKGLNEEQGNVVSRFGLKLVYGSTLVSVLSASIAALVL-
cNT1      L[illegible]FANFSSIGIMLGGLTSLVPQRRSDFSQIVLRALITGAFVSLLNACVAGILYV 605
CONSENSUS LCGFANF.SIG..VG.F....P.R..EI.QLGLRAL...TL..L.SA.IAGFF..
          *  **** ***13                 *        14  *  *

ECOHU4748 --------------------------------------------------------
ECOHU4751 --------------------------------------------------------
ECNUPC    --------------------------------------------------------
cNT1      PRGVEVDCVSLLNQTVSSSSFEVYLCCRQVFQSTSSEFSQVALDNCCRFYNHTVCT 661
CONSENSUS ........................................................
```

Figure 2.8. Homology of cNT1 with NupC, ECOHU4748 and ECOHU4751 (GCG COMPARE and DOTPLOT analysis). a, cNT1 and *E.coli* NupC. b, cNT1 and ECOHU4748. c, cNT1 and ECOHU4751. The comparisons were performed using a 21-residue window and a stringency of 11.


(a)
NupC
cNT1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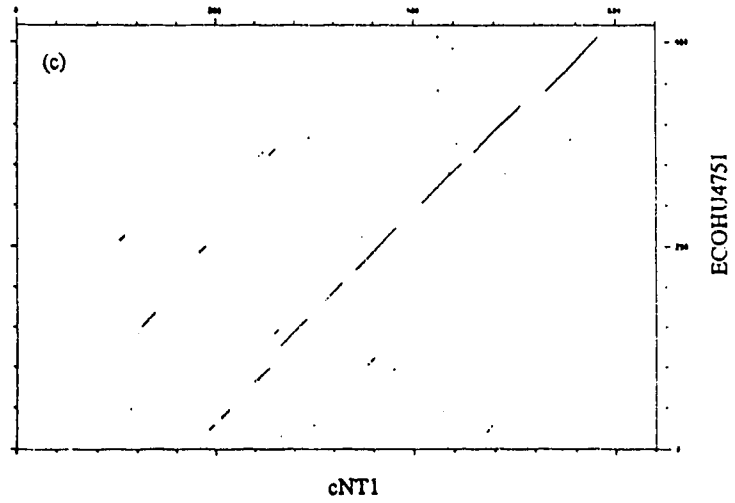
ECOHU4748
cNT1


(c)
ECOHU4751
cNT1

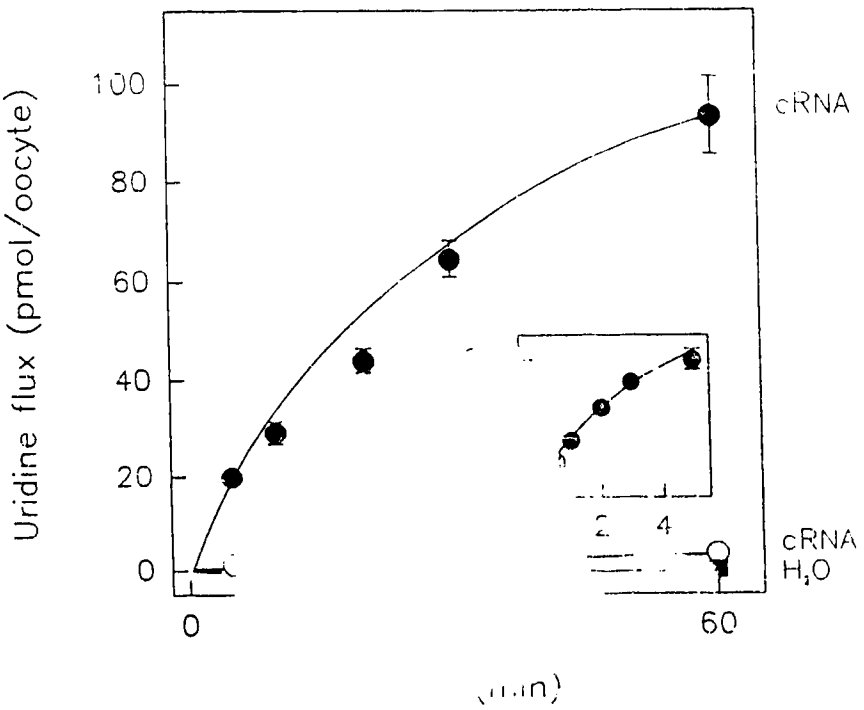


Figure 2.9. Time-course of uridine uptake in *Xenopus* oocytes. Oocytes were injected with either 10 nl of cNT1 cRNA (1 ng/nl) or 10 nl of water and incubated for 3 days at 18°C in MBM. Each value represents the mean ± SEM of 8-12 oocytes. Fluxes (10 μM, 20°C) were determined in transport buffer containing 100 mM NaCl (solid symbols) or 100 mM choline chloride (open symbols). The inset shows a time-course of uridine uptake by cRNA-injected oocytes measured over 30 s - 5 min.

10. Michalak, M., Quackenbush, E.J., and Letarte, M. (1986). Inhibition of Na^+/Ca^{2+} exchanger activity in cardiac and skeletal muscle sarcolemmal vesicles by monoclonal antibody 44D7. *J. Biol. Chem.* **261**, 92-95.

11. Wells, R.G., Lee, W.-S., Kanai, Y., Leiden, J.M., and Hediger, M.A. (1992). The 4F2 antigen heavy chain induces uptake of neutral and dibasic amino acids in *Xenopus* oocytes. *J. Biol. Chem.* **267**, 15285-15288.

12. Bertran, J., Magagnin, S., Werner, A., Markovich, D., Biber, J., Testar, X., Zorzano, A., Kühn, L.C., Palacin, M., and Murer, H. (1992). Stimulation of system y^+-like amino acid transport by the heavy chain of human 4F2 surface antigen in *Xenopus laevis* oocytes. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5606-5610.

13. Deves, R., Chavez, P., and Boyd, C.A.R. (1992). Identification of a new transport system (y^+L) in human erythrocytes that recognizes lysine and leucine with high affinity. *J. Physiol.* **454**, 491-501.

14. Lo, A.W.I., Qureshy, S.A., Harvey, C.M., and Young, J.D. (1993). Cation and neutral amino acid interactions with a novel lysine transporter (system y^+L) in human erythrocytes. *Biochem. Cell Biol.* **71**, Axiv.

15. Harvey, C.M., Muzyka, W.R., Yao, S. Y.-M., Cheeseman, C.I., and Young, J.D. (1993). Expression of rat intestinal L-lysine transport systems in isolated oocytes of *Xenopus laevis*. *Am. J. Physiol.* **265**, G99-G106.

16. Yao, S.Y.M., Muzyka, W.R., Elliott, J.F., Cheeseman, C.I., and Young, J.D. (1994). Poly(A^+)RNA from the mucosa of rat jejunum induces novel Na^+-dependent and Na^+-independent leucine transport activities in oocytes of *Xenopus laevis*. *Mol. Membr. Biol.* **11**, 109-118.

17. Kyte, J., and Doolittle R.F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105-132.

18. Kenelly, P.J., and Kerbs, E.G. (1991). Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **260**, 15555-15558.

19. Holland, E.C., and Drickamer, K. (1986). Signal recognition particle mediates the insertion of a transmembrane protein which has a cytoplasmic NH_2 terminus. *J. Biol. Chem.* **261**, 1286-1292.

20. Huang, Q.Q., Yao, S.Y.M., Ritzel, M.W.L., Paterson, A.R.P., Cass, C.E., and Young, J.D. (1994). Cloning and functional expression of a complementary

DNA encoding a mammalian nucleoside transport protein. *J. Biol. Chem.* **269**, 17757-17760.

21. Kanai, Y., Smith, C.P., and Hediger, M.A. (1993). A family of neurotransmitter transporters: the high-affinity glutamate transporters. *FASEB* **7**, 1450-1459.

22. Wang, C.D., Buck, M.A., and Fraser, C.M. (1991). Site-directed mutagenesis of α_{2A}-adrenergic receptors: identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol. Pharmacol.* **40**, 168-179.

23. Bertran, J., Werner, A., Chillaron, J., Nunes, V., Biber, J., Testar, X., Zorzano, A., Estivill, X., Murer, H., and Palacin, M. (1993). Expression cloning of a human renal cDNA that induces high affinity of L-cystine shared with dibasic amino acids in *Xenopus* oocytes. *J. Biol. Chem.* **268**, 14842-14849.

24. Lee W.-S., Wells, R., G., Sabbag, R.V., Mohandas, T.K., and Hediger, M.A. (1993). Cloning and chromosomal localization of a human kidney cDNA involved in cystine, dibasic, and neutral amino acid transport. *J. Clin. Invest.* **91**, 1959-1963.

25. Hediger, M.A., Kanai, Y., Lee, W.-S., and Wells, R.G. (1993). Identification of a new family of proteins involved in amino acid transport. In *Molecular biology and function of carrier proteins.* The Rockefeller University Press, pp301-314.

26. Kilberg, M S., Stevens, B.R., and Novak, D.A. (1993). Recent advances in mammalian amino acid transport. *Annu. Rev. Nutr.* **13**, 137-165.

27. Wright, E.M. (1994). Cystinuria defect expresses itself. *Nature Genetics* **6**, 328-329.

28. Christensen, H.N. (1994). Is the board range amino acid transporter which is induced by a renal microvillar cDNA clone the cystinuria gene. *Nutrition Rev.* **52**, 210-212.

29. Landschulz, W.H., Johnson, P.F., and McKnight, S.L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* **240**, 1759-1764.

30. Buckland, R., and Wild, F. (1989). Leucine zipper motif extends. *Nature* **338**, 547.

31. Muzyka, W.R. (1994). Characterization and expression of rat intestinal L-lysine transport systems. Master thesis, University of Alberta.

32. Wellner, D., and Meister, A. (1981). A survey of inborn errors of amino acid metabolism and transport in man. *Ann. Rev. Biochem.* **50**, 911-968.

33. Huang, Q.-Q., Harvey, C.M., Paterson, A.R.P., Cass, C.E. and Young, J.D. (1993). Functional expression of Na^+-dependent nucleoside transport systems of rat intestine in isolated oocytes of *Xenopus laevis*. Demonstration that rat jejunum expresses the purine-selective system N1 (*cif*) and a second novel system N3 having board specificity for purine and pyrimidine nucleosides. *J. Biol. Chem.* **268**, 20613-20619.

34. Veyhl, M., Spangenberg, J., Püschel, B., Poppe, R., Dekel, C., Fritzsch, G., Haase, W., and Koepsell, H. (1993). Cloning of a membrane-associated protein which modifies activity and propertites of the Na^+-D-glucose cotransporter. *J. Biol. Chem.* **268**, 25041-25053.

CHAPTER 6

General Discussion

This Thesis describes the use of the *Xenopus* oocyte expression system to isolate and functionally characterise cDNAs from rat jejunum encoding a concentrative Na^+-dependent nucleoside transporter protein (cNT1) and an amino acid/nucleoside/ monocarboxylate transport regulator protein (4F2hc). The cloning and functional expression of an *Escherichia coli* homolog of cNT1 (NupC) is also described.

6.1 FUNCTIONAL CHARACTERISATION OF cNT1

6.1a. Pyrimidine and Purine Nucleoside Transport

In the intestine, dietary nucleosides, including considerable amounts of dietary nucleotides which are converted into nucleosides before absorption (1), are taken up by enterocytes *via* concentrative, Na^+-dependent nucleoside transporters in the brush border membrane prior to intracellular metabolism (2-3). Na^+-dependent cNT1, with its N2-type transport characteristics (2), represents a potential uptake mechanism for pyrimidine nucleosides and adenosine.

Both uridine (a pyrimidine nucleoside) and adenosine (a purine nucleoside) are high-affinity permeants of cNT1 (apparent K_m values 37 μM and 26 μM, respectively) as judged by expression experiments in oocytes injected with cNT1 cRNA or water (Chapter 2). The adenosine apparent K_m value of 26 μM is in good agreement with an apparent K_i value of 29 μM determined for adenosine inhibition of cNT1-mediated uridine influx. Conversely, uridine inhibited cNT1-mediated adenosine influx, confirming that adenosine and uridine share a common transport route. However, the V_{max} for cNT1-mediated adenosine influx (0.07 pmol/oocyte.min^{-1}) was only 0.33% that of uridine (21 pmol/oocyte.min^{-1}), giving V_{max}/K_m ratios for the two nucleosides of 0.003 and 0.57, respectively, a difference of 190-fold. Similar behaviour has been described in this laboratory for L-arginine and L-alanine interactions with amino acid transport system asc_2 in horse erythrocytes (5,6) (Table 1.2), where the two amino acids have the same apparent K_m value of 14 mM, but the V_{max} for L-arginine transport is only 1.3% that for L-alanine. In *trans*-experiments, system asc_2-mediated L-alanine efflux from preloaded cells is stimulated by extracellular L-alanine, but inhibited by L-arginine (6). Kinetically, this is a consequence of a decreased mobility of the loaded carrier and it is likely that the different characteristics of cNT1-mediated transport of adenosine and uridine have a similar origin. Because of its low V_{max}, cNT1 (system N2) is unlikely to be physiologically significant with respect to adenosine transport. Adenosine inhibition of the transporter is also unlikely to be physiologically important because *in vivo* concentrations of adenosine are typically much lower than its apparent K_i value for inhibition of cNT1-mediated uridine influx.

With respect to cation interactions, it was established that removal of extracellular Na^+ resulted in a linear concentration-dependence for cNT1-mediated uridine transport. This suggests an ordered binding mechanism in which Na^+ binds to the transporter first, increasing the protein's affinity for the nucleoside permeant. The large pyrimidine nucleoside fluxes observed in oocytes injected with cNT1 cRNA will greatly facilitate kinetic dissection of the cNT1 translocation mechanism by conventional tracer methods

(7), while the system's Na^+-dependence will permit parallel application of steady-state and presteady-state electrophysiological techniques (8,9).

6.1b. Potential Role of cNT1 in Nucleoside Analog Transport

Nucleoside analogs are widely used as anticancer agents or as chemotherapeutic agents for inhibiting bacteria, fungi and viruses (10,11). Pyrimidine nucleoside analog drugs used against the human immuno-deficiency virus (HIV) include 3'-azido-3'-deoxythymidine (AZT), 2',3'-dideoxycytidine (ddC) and 2',3'-dideoxyinosine (ddI) (12). The common structural feature of these nucleoside analogs is the absence of the 3'-hydroxyl group. While this is responsible for the chain-terminating actions of the corresponding 5'-triphosphates, it also greatly reduces their ability to be transported by equilibrative nucleoside transport processes (13,14). To exert their therapeutic effect, these analogs must first cross the cell membrane. Most previous studies of AZT and ddC membrane permeability have focused on non epithelial cells. AZT is not transported by equilibrative nucleoside transporters and is considered to enter cells mainly by passive diffusion across the lipid bilayer of the plasma membrane (13,14). ddC has been reported to be a low-affinity equilibrative nucleoside transporter (*es*) substrate in human erythrocytes (15,16).

The results presented in Chapter 3 demonstrate that cNT1 is more tolerant than equilibrative nucleoside transporters of structural modifications at the 3'-hydroxyl group of the ribose ring, allowing the transporter to accept both AZT and ddC as permeants. Consistent with the tissue distribution of system N2, transcripts of cNT1 are also present in kidney. Thus, the human counterpart of cNT1 might be involved in the intestinal absorption and renal handling of AZT and ddC. On the other hand, slow entry of AZT and ddC into HIV-infected cells may reduce their therapeutic effectiveness (14,15). For example, AZT and ddC enter human immune effector cells including lymphocytes, macrophages and bone marrow cells mainly by passive diffusion and achievable intracellular concentrations of their active metabolites (AZT 5'-triphosphate and ddC 5'-triphosphate) in human lymphocytes *in vitro* are very much lower than the apparent K_m values of these nucleoside analog triphosphates for HIV reverse transcriptase (14). Potential long term applications of cNT1 and its human homolog(s) in anti-AIDS therapy may involve gene therapy (17,18) based upon the premise that introduction of the cNT1 gene into HIV-target cells by gene transfer may increase achievable intracellular concentrations of anti-HIV drugs (AZT and ddC), leading to more effective inhibition of HIV replication. As well, the availability of a cDNA encoding a nucleoside transporter tolerant of the sugar structural modifications that confer antiviral activity (cNT1) in combination with an efficient expression system (the *Xenopus* oocyte) may provide a useful tool in structure/activity studies and new drug design.

6.2. Na^+ AND NUCLEOSIDE RECOGNITION/BINDING SITES IN cNT1 AND NupC

Most mammalian secondary active solute transport sys. s are Na^+-dependent, while both H^+ and Na^+ are the major coupling cations in bacteria. In mammalian cells,

the transmembrane Na^+-gradient is generated by the Na^+/K^+ATPase. In bacteria, such as *E. coli*, H^+ and Na^+-gradients are driven either by primary H^+ or Na^+-pumps, or indirectly by the Na^+/H^+ antiporter (19). Some bacterial cotransporter proteins exclusively utilise H^+ (*e.g.* the Lac permease of *E. coli*), or Na^+ (*e.g.* the PutP proline transporter of *E. coli*), or (H^+ + Na^+) (*e.g.* the GltT glutamate transporter of *B. stearothermophilus* and *B. caldotenax*), or H^+ interchangeably with Na^+ (*e.g.* the MelB melibiose transporter of *E. coli*).

Results presented in Chapters 2 and 4, demonstrate that cNT1 is a Na^+-dependent, pH-independent nucleoside transporter, while the characteristics of its bacterial homolog NupC are consistent with the use of H^+ as the primary coupling cation. The substrate specificities of cNT1 and NupC are the same. Sequence comparisons between cNT1 and NupC reveal significant similarities in their C-terminal regions where the nucleoside binding site may therefore be located and some of the conserved residues in these regions may be involved in nucleoside recognition/translocation. Conserved amino acids are particularly concentrated within TM11, TM12 and TM13 and in a five residue-motif of the presumed extracellular loop connecting TMs 12 and 13. The striking C-terminal homology of cNT1 and NupC contrasts with other previously described Na^+/solute cotransporter families where N-terminal amino acid residues are more frequently conserved than C-terminal ones (20).

A motif of five amino acids residues (G---AxxxxLxxxGR) is common to the amino acid sequences of a number of Na^+-linked cotransport systems and has been proposed as a putative sodium binding (SOB) domain (21). This SOB motif is found in the Na^+/glucose cotransporter (SGLT) family (*e.g.* SGLT1 and SNST1, but not the pantothenate carrier), the glutamate transporter family (including the *E. coli* H^+/glutamate symporter GltP), the Na^+/Pi cotransporter and the *E.coli* Na^+/glutamate symporter (20). Two putative SOB motifs were identified at the N- and C-termini of cNT1, and no SOB motif was found in NupC. The possible functional significance of such SOB motifs is, however, uncertain. For example, it has been shown recently that the unique positively charged amino acid residue in the SOB motif of the *E. coli* Na^+/proline symporter (Arg376) is not important for sodium binding (22). Also, SOB motifs are present in a number of Na^+-independent transporters and absent from Na^+/Cl^--dependent neurotransmitter transporters (22).

Two recent reports show that single amino acid substitutions can have profound effects on the cation specificity of secondary active transporter proteins (23,24). Substitution of residues Asp51 of the *E. coli* melibiose carrier (MelB) with a glutamate abolishes the ability of the transporter to utilise Na^+ /Li^+ for melibiose cotransport (23). Conversely, substitution of Asp120 with a glutamate abolishes the ability of the transporter to couple melibiose transport to H^+ (23). An Ala58->Asn58 substitution enabled the H^+/melibiose symporter of *Klebsiella pneumoniae* to couple sugar transport to Na^+ instead of H^+ (24). It is possible that similar minor structural differences might account for the different cation specificities of cNT1 and NupC, although larger structural domains (*e.g.* within the C-terminal domains of the proteins) may also be important.

6.3. A NOVEL FAMILY OF TRANSPORTER PROTEINS

cNT1 shows no significant amino acid sequence similarity to proteins of mammalian origin including the SGLT family, to which the N3-type Na^+-dependent nucleoside transporter SNST1 belongs (25). In contrast, the amino acid sequence of cNT1 is homologous to a bacterial H^+-coupled nucleoside symporter, NupC (26) and to two bacterial proteins (ECOHU4748 and ECOHU4751) of undetermined function. cNT1 and its bacterial homologs share 27-34% amino acid sequence identity. Highly conserved regions of amino acid sequence are clustered in the C-terminal half of cNT1, especially in TM11, TM12 and TM13, while least conserved regions of sequence were found in the N-terminal half of the protein and in hydrophilic loops connecting TMs, except for the loop between TM12 and TM13. It is evident, therefore, that mammalian cNT1 and bacterial NupC, ECOHU4748 and ECOHU4751 are evolutionarily related proteins. Since cNT1 is more highly homologous to ECOHU4748/51 (34% identity) than to NupC (27% identity), ECOHU4748/51 may represent previously unknown *E. coli* nucleoside transporters, a possibility which could be tested by functional expression in *Xenopus* oocytes or other heterologous expression systems.

On the basis of sequence similarity, Saier and co-workers have grouped over 175 procaryote and eucaryote transporter cDNAs into two superfamiles, the major facilitator superfamily (MFS)(27) and the sodium/solute symporter superfamily (SSSS)(20). MFS consists of five known transporter families including the drug-resistance protein family, the sugar-facilitator (GLUT) family, the Kerbs cycle intermediate facilitator family, the phosphorylated carbohydrate antiporter family and the H^+-coupled oligosaccharide symporter family (27). SSSS contains eleven families: the Na^+-dependent SGLT family, the Na^+/Cl^--neurotransmitter transporter family, the dicarboxylate transporter family (including GLT-1, GLAST and EAAC1), the inorganic phosphate transporter family, the galactoside transporter family, the citrate transporter family, the bacterial Na^+/alanine symporter family, the bacterial Na^+/glutamate symporter family (which presently contains only the GltS of *E. coli.*), the bacterial Na^+/branched chain amino acid transporter family, the bile acid transporter family and the NaCl transporter family (20). MFS includes mainly uniporters, antiporters and H^+/solute symporters but no known obligatory Na^+/solute symporter, while SSSS contains mainly Na^+-dependent symporters. The relationship, if any, of the cNT1 transporter family to the MFS and SSSS superfamilies remains to be determined.

6.4. FUTURE TRENDS OF cNT1

Because of their potential clinical significance, an important priority of future research will be the isolation of cDNAs encoding human homolog(s) of cNT1. This can be achieved by homology-based PCR amplification (28) and/or high-stringency hybridisation screening of human intestinal/kidney cDNA libraries, followed by expression in *Xenopus* oocytes to define the functional characteristics of cloned cDNAs. Future functional studies of cNT1 in oocytes will include electrophysiological

measurements of transporter-generated currents (29). Electrophysiological studies will also be required to determine if NupC has any ability to transport Na^+ (Chapter 4).

Although the *Xenopus* oocyte expression system is ideal for functional studies, the amounts of protein produced are much too small for most structural and other types of mechanistic study. Overexpression of cNT1 and homologous proteins in other heterologous systems (*e.g. E. coli*, *Saccharomyces cerevisae*, CHO cells, baculovirus expression in *Spodoptera frugiperda*) may produce sufficiently large amounts of purified proteins in stable form for biochemical and biophysical studies (30-34). The baculovirus system has been applied successfully to both GLUT1 (34) and SGLT1 (33).

The putative transmembrane topology of cNT1 needs to be tested by the generation of antipeptide antibodies corresponding to hydrophilic regions of predicted extracellular and intracellular domains. For example, the topology of the glucose transporter GLUT1 was verified using this approach (35). In addition, extracellular loops of cNT1 could be studied by using recombinant DNA technology to introduce the haemagglutinin (HA) epitope tag and factor Xa protease cleavage sites into each of the predicted linker regions of cNT1 (36). Extracellular location of HA epitopes in cNT1 constructs expressed in oocytes or other eucaryotic cells would then be demonstratable not only by immunocytochemistry, but also by virtue of their accessibility to cleavage by extracellular protease in intact, unpermeabilised cells. Topology models of cNT1 might also be investigated by gene fusion techniques using alkaline phosphatase (37), ß-galatosidase (38), ß-lactamase (39) or acetylCoA-carboxylase (40). For example, the 12 transmembrane domain model of *E. coli* Lac permease has been verified by a series of Lac permease-alkaline phosphatase gene fusions (41).

Identification of regions of the cNT1 sequence involved in substrate/cation recognition and transport will be facilitated by the similarities and differences in the primary structures of cNT1 and NupC. Initial targets for site-directed mutagenesis experiments would be conserved charged/polar residues within potential transmembrane domains. Functions of broader regions of the nucleoside transporters could be tested by making chimaeras between the N-terminal and C-terminal portions of the bacterial and mammalian proteins, or by swapping single transmembrane segments, followed by functional characterisation in oocytes. The finding that NupC can be expressed in oocytes (Chapter 4), suggests that cNT1/NupC chimaeras are likely to be functional in this system. Proximity relationships between potential TMs may be studied by site-directed pyrene labeling (42,43). This requires a functional transporter devoid of cysteine residues and would be more appropriate for NupC (1 cysteine) than cNT1 (20 cysteines).

Ultimately, high-quality three-dimensional crystals of cNT1 will be required for high-resolution structure determination by X-ray crystallography (44). However, thus far only a few membrane proteins have been successfully obtained in well-ordered crystalline forms, most notably the photosynthetic reaction centre of *Rhodopseudomonas viridis* (45). Two-dimensional crystals have been prepared for one transporter, the erythrocyte Cl^-/HCO_3^- exchange protein, but the resulting 20Å resolution model generated by electron microscopy did not reveal structural details of the protein (46). A more immediately accessible approach to study cNT1 secondary and tertiary structure will be

through the application of nuclear magnetic resonance (NMR) spectroscopy (47) and Fourier-transform infrared spectroscopy (FTIR) (48). Membrane proteins, including the erythrocyte GLUT1 glucose transporter and *Halobacterium halobium* bacteriorhodopsin, have been studied by FTIR and found to be predominantly α-helical in structure (48). Structures of synthetic peptides corresponding to individual TM segments of the erythrocyte anion exchanger have been studied by NMR (49).

6.5. IS 4F2hc A TRANSPORTER OR A REGULATOR?

In Chapter 5, I described the isolation and functional characterisation of a cDNA encoding rat jejunal 4F2hc. The deduced amino acid sequence of this rat protein shows 76% and 89% identity to human SV-40 transformed fibroblast and mouse pre B-cell/macrophage 4F2hc, respectively. In addition, rat jejunal 4F2hc is homologous to the D2(rBAT) group of intestinal/renal proteins (27% amino acid identity) which induce Na^+-independent uptake of neutral and cationic amino acids (system $b^{o,+}$) (Table 1.2) when expressed in *Xenopus* oocytes (50-52). Expression of rat jejunal 4F2hc cRNA in oocytes induced Na^+-dependent transport of L-leucine with the transport characteristics of system y^+L (Table 1.2).

Unlike previously studied transporter proteins with multiple TMs (53-56), 4F2hc and D2(rBAT) are type II membrane glycoproteins with a cytoplasmic N-terminus, a single TM and a glycosylated extracellular C-terminus. The fact that 4F2hc and D2(rBAT) have single TMs does not *a priori* exclude the possibility that they function as transporters. However, my functional studies of rat jejunal 4F2hc and rat renal D2(rBAT) in oocytes indicate that, in addition to amino acids, these proteins also induce Na^+-dependent uptake of pyruvate and uridine, but not choline. Competition experiments showed that D2(rBAT)-mediated uridine influx in oocytes was inhibited by both pyrimidine and purine nucleosides (*ie* system N3-type activity) (Table 1.1) whereas rat 4F2hc-mediated uridine flux was inhibited by pyrimidine nucleosides and guanosine. This latter result suggests induction of a previously unknown Na^+-dependent nucleoside transporter subtype. In contrast, Na^+-dependent nucleoside transporter cNT1 cDNA only induced influx of uridine in oocytes and had no effect on the permeability of other solutes. These results are incompatible with a transport function for 4F2hc/D2(rBAT) and instead suggest that they act as transport activators. Recently, a cDNA encoding a potential regulatory subunit (RS1) of the rabbit intestinal Na^+-dependent glucose transporter (SGLT1) was isolated and characterised functionally in oocytes (57). Although RS1 and D2(rBAT)/4F2hc are not related in amino acid sequence, the predicted structures of RS1 and D2(rBAT)/4F2hc are rather similar, with single TMs and multiple *N*-linked glycosylation sites. Therefore, 4F2hc (and D2(rBAT)) may modulate nutrient transporter function in a manner analogous to RS1.

6.6. POTENTIAL PHYSIOLOGICAL ROLE(S) OF 4F2hc

The 4F2 cell surface antigen is a disulfide-linked heterodimer composed of an 85 kDa glycosylated heavy chain (4F2hc) and a 40 kDa non-glycosylated light chain (4F2lc).

The former is recognised by the monoclonal antibody (mab4F2) (58). Northern analyses with a mouse pre B-cell/macrophage 4F2hc cDNA demonstrated widespread expression in different tissues including the kidney, brain, lung, liver and testis (59). It has been reported that 4F2 may be involved in cell proliferation or activation (60) as well as Na^+/Ca^{2+} exchange in cardiac and skeletal muscle sarcolemma (61). However, the precise role of 4F2 in mammalian cells is still unknown.

The recent discovery that rBAT is a cystinuria gene (62) suggests that this family of proteins (D2(rBAT)/4F2hc) may play an important physiological role in amino acid transport in mammalian cells. Since 4F2hc does not mediate cystine uptake in oocytes (63), it is unlikely to be involved in cystinuria. On the other hand, 4F2hc's ubiquitous cellular distribution suggests that it may have a more generalised amino acid transport function than D2(rBAT) which is expressed only in intestine and kidney epithelial cells. Potentially, 4F2hc may be involved in other disorders of amino acid transport such as lysine malabsorption syndrome (64). Amino acid residues of rat jejunal 4F2hc equivalent to those of human rBAT mutated in cystinuria will be key residues for future site-directed mutagenesis studies to investigate 4F2hc structure/function relationships. The finding that 4F2hc and D2 induce multiple transport activities in oocytes suggests that the physiological roles of these proteins may not be limited to amino acid transport. Similarly, it has been found that the R1 protein is not specific for SGLT1 and activates at least one other member of the SGLT family (57).

An understanding of the physiological role of 4F2hc in membrane transport will require cDNA cloning of 4F2lc. As well, it will be important to isolate cDNAs encoding potential transporter proteins regulated by 4F2hc. This could be achieved by functional expression cloning in *Xenopus* oocytes where pools of clones are screened for transport activity in oocytes coinjected with 4F2hc cRNA. Reconstitution of recombinant 4F2 (and D2(rBAT)) into proteoliposomes either alone or in combination with candidate transporters regulated by these proteins will be an important step towards defining their biochemical actions and physiological functions.

6.7. BIBLIOGRAPHY

1. Uauy, R. (1989). Dietary nucleotides and requirements in early life. In *Textbook of Gastroenterology and Nutrition in Infancy* (ed. Lebenthal, E.), Raven Press, New York, pp265-280.

2. Vijayalakshimi, D. and Belt, J.A. (1988) Sodium-dependent nucleoside transport in mouse intestinal epithelial cells. Two transport systems with differing substrate specificities. *J.Biol. Chem.* **263**, 19419-19423.

3. Roden, M., Paterson, A.R.P., and Turnheim, K. (1991). Sodium-dependent nucleoside transport in rabbit intestinal epithelium. *Gastroenterology* **100**, 1553-1562.

4. Bronk, J.R., and Hastewell, J.G. (1988). The transport and metabolism of naturally occurring pyrimidine nucleosides by isolated rat jejunum. *J. Physiol.* **395**, 349-361.

5. Fincham, D.A., Mason, D.K., paterson, J.Y., and Young, J.D. (1987). Heterogeneity of amino acid transport in horse erythrocytes: a detailed kinetics analysis of inherited transport variation. *J. Physiol.* **389**, 385-409.

6. Fincham, D.A., Mason, D.K., and Young, J.D. (1988). Dibasic amino acid interactions with Na^+-independent transport system asc in horse erythrocytes. Kinetic evidence of functional and structural homology with Na^+-dependent system ASC. *Biochim. Biophys. Acta* **937**, 184-194.

7. Stein, W.D. (1986). In *Transport and Diffusion across Cell Membranes* (eds. Stein, W.D., and Lieb, W.R.), Academic Press, London.

8. Umbach, J.A., Coady, M.J., and Wright, E.M. (1990). Intestinal Na^+/glucose cotransporter expressed in *Xenopus* oocytes is electrogenic. *Biophys. J.* **57**, 1217-1224.

9. Boorer, K.J., Loo, D.D.F., and Wright, E.M. (1994). Steady-state and presteady-state kinetics of the H^+/hexose cotransporter (STP1) from *Arabidopsis thaliana* expressed in *Xenopus* oocytes. *J. Biol. Chem.* **269**, 20417-20424.

10. Perigaud, C.G., Gossselin, G., and Imbach, J.-L. (1992). Nucleoside analogues as chemotherapeutic agents:a review. Nucleosides and Nucleotides 11, 903-945.

11. Handschumacher, R.E., and Cheng, C.Y. (1993) in *Cancer Metabolism* (eds. Holland, E., Frei, E., Bast, R.C., Kufe, D.W., Morton, D.L., and Weichselbaum, R.R.), Lea & Febiger, Philadelphia, pp712-732.

12. Clumeck, N. (1993). Current use of anti-HIV drugs in AIDS. *J. Antimicrob. Chemother.* **32**, supp.A 133-138.

13. Zimmerman, T.P., Mahony, W.B., and Prus, K.L. (1987). 3'-Azido-3'-deoxythymidine, an unusual nucleoside analogue that permeates the membrane of human erythrocytes and lymphocytes by non-facilitated diffusion. *J. Biol. Chem.* **262**, 5748-5754.

14. Chan, T.C.K., Shapper, L., Redmond, R., and Pennington,K.L. (1993). Permeation and metabolism of anti-HIV and endogenous nucleosides in human immune effector cells. *Biochem. Pharmacol.* **46**, 273-278.

15. Ullman, B., Coons, T., Rockwell, S., and McCartan, K. (1988). Genetic analysis of 2',3'-dideoxycytidine incorporation into cultured human T lymphoblasts. *J. Biol. Chem.* **263**, 12391-12396.

16. Domin, B.A., Mahony, W.B., and Zimmerman, T.P. (1993). Membrane permeation mechanisms of 2',3'-dideoxynucleosides. *Biochem. Pharmacol.* **46**, 725-729.

17. Kerr, W.G., and Mule, J.J. (1994). Gene therapy: current and future prospects. *J. Leukoc. Biol.* **56**, 210-214.

18. Yu, M., Peoschla, E., Wong-Staal, F. (1994). Progress towards gene therapy for AIDS. *Gene Ther.* 1, 13-26.

19. Poolman, B., and Konings, W.N. (1993). Secondary solute transport in bacteria. *Biochim. Biophys. Acta* **1183**, 5-39.

20. Reizer, J., Reizer, A., and Saier, M.H., Jr. (1994). A functionally superfamily of sodium/solute symporters. *Biochim. Biophys. Acta* **1197**, 133-166.

21. Deguichi, Y., Yamato, I., and Anraku, Y. (1990). Nucleotide sequence of *gltS*, the Na^+/glutamate symport carrier gene of *Escherichia coli* B. *J. Biol. Chem.* **256**, 21704-21708.

22. Yamato, I., Kotani, M., Oka, Y., and Anraku, Y. (1994). Site-specific alteration of arginine 376, the unique positively charged amino acid residue in the mid-membrane-spanning regions of the proline carrier of *Escherichia coli*. *J. Biol. Chem.* **269**, 5720-5724.

23. Wilson, D.M., and Wilson, T.H. (1994). Transport properties of Asp-51 -> Glu and Asp-120-> Glu mutants of the melibiose carrier of *Escherichia coli*. *Biochim. Biophys. Acta* **1190**, 225-230.

24. Hama, H., and Wilson, T.H. (1994). Replacement of alanine 58 by asparagine enables the melibiose carrier of *Klebsiella pneumoniae* to couple sugar transport to Na^+. *J. Biol. Chem.* **269**, 1063-1067.

25. Pajor, A.M., and Wright, E.M. (1992). Cloning and functional expression of a mammalian Na^+/nucleoside cotransporter. A member of the SGLT family. *J. Biol. Chem.* **267**, 3557-3560.

26. Craig, J.E., Zhang, Y., and Gallagher, M.P. (1994). Cloning of the *nupC* of *Escherichia coli* encoding a nucleoside transport system, and identification of an adjacent insertion element, IS *186*. *Mol. Microbiol.* **11**, 1159-1168.

27. Marger, M.D., and Saier, M.H.,Jr. (1992). A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *TIBS* **18**, 13-20.

28. Vega-Saenz De Miera, E.C., and Lin, J.-W. (1992). Cloning of ion chanel gene families using the polymerase chain reaction. *Methods Enzymol.* **207**, 613-619.

29. Stuhmer, W. (1992). Electrophysiological recording from *Xenopus* oocytes. *Methods Enzymol.* **207**, 319-339.

30. Thorens, B., Sarkar, H.K., Kaback, H.R., and Lodish, H.F. (1988). Cloning and functional expression in bacteria of a novel glucose transporter present in liver, intestine, kidney, and ß-pancreatic islet cells. *Cell.* **55**, 281-290.

31. Picard, D. (1990). An inducible expression vector for both fission and budding yeast. *Gene* **86**, 257-261.

32. Harfst, E., and Johnstone, A.P. (1992). Characterization of the glutamine synthetase amplifiable eukaryotic expression system applied to an integral membrane protein - the human thyrotropin receptor. *Anal. Biochem.* **207**, 80-84.

33. Smith, C.D., Hirayama, B.A., and Wright, E.M. (1992). Baculovirus-mediated expression of the Na^+/glucose cotransporter in Sf9 cells. *Biochim. Biophys. Acta* **1104**, 151-159.

34. Yi, C.-K., Charalambous, B.M., Emery, V.C., and Baldwin, S.A. (1992). Characterization of functional human erythrocyte-type glucose transporter (GLUT1) expressed in insect cells using a recombinant baculovirus. *Biochem. J.* **283**, 643-646.

35. Davies, A., Ciardelli, T.L., Lienhard, G.E., Boyle, J.M., Whetton, A.D., and Baldwin, S.A. (1990). Site-specific antibodies as probes of the topology and function of the human erythrocyte glucose transporter. *Biochem. J.* **266**, 799-808.

36. Czech, M.P., Chawla, A., Woon, C.-W., Buxton, J., Armoni, M., Tang, W., Joly, M., and Corvera, S. (1993). Exofacial epitope-tagged glucose transporter chimeras reveal COOH-terminal sequences governing cellular localization. *J. Cell Biol.* **123**, 127-135.

37. Manoil, C., and Beckwith, J. (1986). A genetic approach to analyzing membrane protein topology. *Science* **233**, 1403-1408.

38. Froshauer, S., Green, G.N., Boyd, D., McGovern, K., and Beckwith, J. (1988). Genetic analysis of the membrane insertion and topology of MalF, a cytoplasmic membrane protein of *Escherichia coli*. *J. Mol. Biol.* **200**, 501-510.

39. Broome-Smith, J.K., Tadayyon, M., and Zhang, Y. (1990). Beta-lactamase as a probe of a membrane protein assembly and protein export. *Mol. Microbiol.* **4**, 1637-1644.

40. Cronan, J.E., Jr. (1990). Biotination of proteins *in vivo*. A post-translational modification to label, purify, and study proteins. *J. Biol. Chem.* **265**, 10327-10333.

41. Calamia, J., and Manoil, C. (1992). Membrane protein spanning segments as export signals. *J. Mol. Biol.* **224**, 539-543.

42. Jung, K., Jung, H., Wu, J., Privé, G.G., and Kaback, H.R. (1993). Use of site-directed fluorescence labeling to study proximity relationships in the lactose permease of *Escherichia coli*. *Biochemistry* **32**, 12273-12278.

43. Jung, K., Jung, H., and Kaback, H.R. (1994). Dynamics of lactose permease of *Escherichia coli* determined by site-directed fluorescence labeling. *Biochemistry* **33**, 3980-3985.

44. Michel, H. (1983). Crystallization of membrane proteins. *TIBS* **8**, 56-59.

45. Deisenhofer, J., and Michel, H. (1989). Nobel lecture. The photosynthetic reaction centre from the purple bacterium *Rhodopseudomonas viridis*. *EMBO J.* **8**, 2149-2170.

46. Wang, D.N., Sarabia, V.E., Reithmeier, R.A.F., and Kühlbrandt, W. (1994). Three-dimensional map of the dimeric membrane domain of the human erythrocyte anion exchanger, Band 3. *EMBO J.* **13**, 3230-3235.

47. Opella, S.J., Kim, Y., and McDonnell, P. (1994). Experimental nuclear magnetic resonance studies of membrane proteins. *Methods Enzymol.* **239**, 536-560.

48. Haris, P., and Chapman, D. (1992). Does Fourier-transform infrared spectroscopy provide useful information on protein structures. *TIBS* **17**, 328-333.

49. Gargaro, A.R., Bloomberg, G.B., Dempsey, C.E., Murray, M., and Tanner, M.J.A. (1994). The solution structures of the first and second transmembrane-spanning segments of Band 3. *Eur. J. Biochem.* **221**, 445-454.

50. Tate, S.S., Yan, N., and Udenfriend, S. (1992). Expression cloning of a Na^+-independent neutral amino acid transporter from rat kidney. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1-5.

51. Wells, R.G., and Hediger, M.A. (1992). Cloning of a rat kidney cDNA that stimulates dibasic and neutral amino acid transport and has sequence similarity to glucosidases. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5596-5600.

52. Bertran, J., Werner, A., Moore, M.L., Stange, G., Markovich, D., Biber, J., Testar, X., Zorzano, A., Palacin, M., and Murer, H. (1992). Expression cloning of a cDNA from rabbit kidney cortex that induces a single transport system for cystine and dibasic and neutral amino acids. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5601-5610.

53. Griffith, J.K., Baker, M.E., Rouch, D.A., Page, M.G.P., Skurray, R.A., Paulsen, I.T., Chater, K.F., Baldwin, S.A., and Henderson, P.J.F. (1992). Membrane transport proteins: implications of sequence comparisons. *Curr. Opin. Cell. Biol.* **4**, 684-695.

54. Wright, E.M., Hager, K.M., and Turk, E. (1992). Sodium cotransport proteins. *Curr. Opin. Cell Biol.* **4**, 696-702.

55. Henderson, P.J.F. (1993). The 12-transmembrane helix transporters. *Curr. Opin. Cell. Biol.* **5**, 708-721.

56. Maloney, P.C. (1994). Bacterial transporters. *Curr. Opin. Cell Biol.* **6**, 571-582.

57. Veyhl, M., Spangenberg, J., Püschel, B., Poppe, R., Dekel, C., Fritzsch, G., Haase, W., and Koepsell, H. (1993). Cloning of a membrane-associated protein which modifies activity and propertites of the Na^+-D-glucose cotransporter. *J. Biol. Chem.* **268**, 25041-25053.

58. Hemler, M.E., and Strominger, J.L. (1982). Characterization of the antigen recognized by the monoclonal antibody (4F2): different molecular forms on human T and B lymphoblastoid cell lines. *J. Immunol.* **129**, 623-628.

59. Parmacek, M.S., Karpinski, B.A., Gottasdiener, K.M., Thompson, C.B., and Leiden, J.M. (1989). Structure, expression and regulation of murine 4F2 heavy chain. *Nucleic Acid Res.* **17**, 1915-1931.

60. Yagita, H., Masuko, T., and Hashimoto, Y. (1986). Inhibition of tumor cell growth *in vitro* by murine monoclonal antibodies that recognize a proliferation-associated cell surface antigen system in rats and humans. *Cancer Res.* **46**, 1478-1484.

61. Michalak, M., Quackenbush, E.J., and Letarte, M. (1986). Inhibition of Na^+/Ca^{2+} exchanger activity in cardiac and skeletal muscle sarcolemmal vesicles by monoclonal antibody 44D7. *J. Biol. Chem.* **261**, 92-95.

62. Calonge, M.J., Gasparini, P., Chillaron, J., Chillon, M., allucci, M., Rousaud, F., Zelante, L., Testar, X., Dallapoccola, B., Silverio, F.D., Barcelo, P., Estivill, X., Zorzano, A., Nunes, V., and Palacin, M. (1994). Cystinuria caused by mutations in rBAT, a gene involved in the transport of cystine. *Nature Genet* **6**, 420-425.

63. Wells, R.G., Lee, W.-S., Kanai, Y., Leiden, J.M., and Hediger, M.A. (1992). The 4F2 antigen heavy chain induces uptake of neutral and dibasic amino acids in *Xenopus* oocytes. *J. Biol. Chem.* **267**, 15285-15288.

64. Wellner, D., and Meister, A. (1981). A survey of inborn errors of amino acid metabolism and transport in man. *Ann. Rev. Biochem.* **50**, 911-968.

APPENDIX 1

Reconstitution Studies of Amino Acid Transport System L in Rat Erythrocytes[1]

[1]A version of this chapter has been published. Yao, S.Y.M., George, R., and Young, J.D. 1993. Reconstitution studies of amino acid transport system L in rat erythrocytes. *Biochem. J.* **262**, 655-660.

A1.1. INTRODUCTION

Unlike most other classes of plasma membrane transport systems, no specific high-affinity inhibitors of mammalian amino acid transporters are known, and systems have been identified and characterised largely on the basis of substrate specificity and cation/anion requirements (1,2). This lack of suitable covalent or reversibly-binding probes together with a generally low membrane abundance of these systems has severely hampered attempts to purify amino acid transport proteins. At the present time, the only viable strategy for identification of relevant proteins in solubilised membrane extracts has been reconstitution of transport function into phospholipid vesicles. By this approach, enriched preparations of several Na^+-dependent amino acid transport proteins have been obtained (3-6), but no equivalent progress has been reported with respect to mammalian Na^+-independent amino acid transporters. cDNAs have been isolated recently from rat and rabbit kidney which, when expressed in *Xenopus* oocytes, elicit increases in Na^+-independent neutral and cationic amino acid transport activity (7-9). In contrast to a previously cloned murine fibroblast cationic amino acid transporter which has 14 putative transmembrane domains (622 amino acid residues) (10,11), these cDNAs (79% identity in nucleoside sequence) encode 683- and 677-amino acid type II membrane glycoproteins with single transmembrane regions. The encoded proteins are homologous to α-amylases and α-glycosidases, but have apparently no such enzymic activity.

Erythrocytes from different mammalian species express a diverse array of different Na^+-dependent and Na^+-independent amino acid transport systems and vary widely in their amino acid transport capacity (12,13). With respect to molecular studies of amino acid permeation, these cells have the additional advantage that they lack intracellular organelles and have a well defined membrane protein composition. Amino acid transport by erythrocytes has been most thoroughly and widely investigated in man (12,13). With respect to Na^+-independent amino acid transport, human erythrocytes have a high activity of system-L, a transporter with a wide tissue distribution which is selective for hydrophobic amino acids such as leucine and phenylalanine, and lower activities of two other Na^+-independent systems (T and y^+) (14-20). System T is selective for aromatic amino acids and y^+ transports dibasic amino acids.

In the present study, I have quantified the system-L transport capacity of erythrocytes from another mammalian species, the rat. The estimated membrane abundance of system-L in these cells is more than two orders of magnitude higher than that of system-L in human erythrocytes and approaches, or exceeds, that of the human erythrocyte glucose transporter (2.10^5 copies/cell). This Appendix also describes conditions for the reconstitution of the system-L transport protein.

A1.2. MATERIALS AND METHODS

A1.2a. Materials

L-[U-^{14}C]-leucine and other radioactive amino acids were obtained from Amersham. Cellulose nitrate filters (pore size 0.45 μm, HAWP 025 00) were obtained from Millipore. Crude egg-yolk phospholipid was prepared by the method of Dawson (1963) (21), assayed as described by Chen *etal.* (1956) (22) and stored at -70°C under N_2 in chloroform containing 0.1% (w/v) butylated hydroxytoluene. All other chemicals were obtained from Sigma, BDH or Merck. Protein molecular weight standards for SDS/polyacrylamide gel electrophoresis were obtained from Pharmacia LKB Biotechnology.

A1.2b. Uptake experiments (intact cells)

Rat and human blood was collected into heparin and washed 3 times in incubation medium (150 mM-NaCl, 15 mM-Mops and 5 mM-glucose, pH 7.4). The buffy coat was discarded. To deplete erythrocytes of intracellular amino acids, the cells were adjusted to a haematocrit of 10% in incubation medium and incubated for 2 h at 37°C, after which they were washed 3 further times in incubation medium and stored on ice at a haematocrit of 20% until required. Uptake experiments were performed by an n-dibutyl phthalate separation method used routinely in this laboratory (23). The stopping solution contained 2 mM phloretin (18). Blank values were obtained by mixing ice cold phloretin-treated cells with ice cold radiolabelled permeant, followed by immediate centrifugation through oil. Iso-osmolarity at different extracellular amino acid concentrations was maintained by adjusting the NaCl concentration of the incubation medium. The water content of rat erythrocytes, determined using ^3H_2O and the impermeant amino acid L-[^{14}C]glutamate as extracellular space marker, was 70.4% (v/v). Kinetic parameters were determined by ENZFITTER, a non-linear regression data analysis program (Elsevier-Biosoft, 1987).

A1.2c. Preparation of membranes

Rat erythrocyte ghosts were prepared as described previously (24) and depleted of extrinsic membrane proteins by treatment with 0.1 mM EDTA (pH 11.2) for 15 min on ice (24). Protein-depleted membranes were washed twice with ice-cold buffer containing 20 mM Na-MOPS (pH 7.4) and stored at -70°C until required.

A1.2d. Reconstitution

Reconstitution was by the procedure of McGivan and co-workers (25,26) and Poole & Halestrap (27), modified by the substitution of n-octyl glucoside for Mega-10 as solubilising detergent. Protein-depleted membranes (5 mg protein/ml) were solubilised for 5 min at 4°C in an equal volume of reconstitution buffer (20 mM-NaMops (pH 7.4), 1 mM-EGTA, 0.1 mM-dithiothreitol and 0.1 mM PMSF) containing 50 mM n-octyl-glucoside. Solubilised membranes were centrifuged at 150,000g for 20 min at 4°C, and the supernatant was assayed for protein content by the method of Peterson (28) after precipitation with trichloro acetic acid using bovine serum albumin as standard.

Liposomes were prepared from crude egg yolk phospholipid. Lipid was dried under a stream of nitrogen, re-dissolved in 1 ml diethyl ether and subsequently dried under N_2 to remove residual traces of chloroform. The dried lipid was suspended in 1 ml reconstitution buffer and sonicated at room temperature under N_2 until translucent.

Solubilised membrane proteins were mixed with sonicated liposomes at 4°C at a ratio of 1 mg protein/25 μmol lipid phosphate. Detergent was removed by passing the mixture (2 ml) through a Sephadex G-50 (coarse grade) column (1 x 30 cm) at 4°C equilibrated with reconstitution buffer. Turbid fractions collected in the void volume were pooled together and diluted 4-fold with reconstitution buffer. The mixture was frozen in liquid N_2 and thawed in a water bath at 25°C. Proteoliposomes were then collected by centrifugation at 50,000g for 30 min at 4°C. The white pellet of proteoliposomes was finally resuspended in a small amount of reconstitution buffer for the transport measurements. The size of proteoliposomes was determined using a BI-90 Particle Sizer (Brookhaven Instruments Corp.).

A1.2e. Transport experiments (proteoliposomes)

Uptake of L-[^{14}C]-leucine by proteoliposomes was measured at 25°C. Briefly, 30 μl pre-warmed proteoliposome suspension (1 mg protein/ml) were incubated with an equal volume of pre-warmed reconstitution buffer containing L-[^{14}C]-leucine (0.2 mM final concentration, 5 μCi/ml). After a pre-determined time interval, uptake was terminated by adding 0.5 ml ice-cold reconstitution buffer containing 2 mM phloretin, followed by immediate filtration through a Millipore nitrocellulose filter (0.45 μm) on a manifold connected with a vacuum assembly. The filter was washed rapidly with 3 x 1 ml ice-cold aliquots of phloretin-containing reconstitution buffer and then dissolved in 5 ml Ready Safe liquid scintillation cocktail (Beckman Instruments, Inc.) for 2 hours before measurement of radioactivity. Blanks were processed identically to samples, except that 1 ml ice-cold phloretin-containing reconstitution buffer was mixed with proteoliposomes before addition of the radioactive solution and filtered immediately.

A1.2f. SDS/polyacrylamide gel electrophoresis

SDS/polyacrylamide gel electrophoresis in 1.5 mm thick slab gels was performed by the method of Thompson and Maddy (29) using the Laemmli buffer system (30). Gels were stained with Coomassie Blue and scanned at 633 nm by laser densitometry (Gelscan XL, Pharmacia) for comparison with molecular weight standards.

A1.3. RESULTS

A1.3a. Uptake of leucine, phenylalanine and valine by human and by rat erythrocytes

Figure A1.1 shows the ability of human erythrocytes to transport L-leucine, L-phenylalanine and L-valine at 37°C when the 3 amino acids were present at an initial extracellular concentration of 0.2 mM. In agreement with previous studies from this and other laboratories (14,16,18,20), amino acid uptake by human erythrocytes proceeded rapidly, reaching the equilibration value of 200 μmol/l cell water in the first 5-10 min of incubation for leucine and phenylalanine, and between 20-40 min incubation for valine. Previous experiments have shown that leucine and phenylalanine uptake by these cells is mediated largely (> 95%) by system L (14,16,18,20). Valine is also a system L permeant, but has a lower affinity for the transporter than the other two amino acids (14). The inset to Figure A1.1 shows that uptake of leucine, phenylalanine and valine by rat erythrocytes was considerably more rapid than in human cells, all 3 amino acids fully equilibrating with intracellular water within the first 10s of incubation at 37°C.

Rapid equilibration with intracellular water (< 30s) was also observed for leucine uptake by rat erythrocytes when I reduced the incubation temperature, first to 25°C and then to 10°C. To obtain measurable initial rates of leucine uptake in these cells for subsequent kinetic and inhibitor studies, it was necessary to decrease the incubation temperature to 1°C and to use short (10 s) incubation periods. At this temperature, the initial rate of 0.2 mM leucine uptake by rat erythrocytes was typically 180 μmol/l cell water per min or 30 μmol/l cell water in 10s.

A1.3b. Kinetic and inhibition characteristics of leucine transport by rat erythrocytes

Various nonradioactive amino acids (5 mM extracellular concentration) were tested as inhibitors of [^{14}C]leucine uptake (0.2 mM) by rat erythrocytes at 1°C. As expected, transport of leucine was strongly inhibited by L-phenylalanine, nonradioactive L-leucine and by L-valine (91, 86 and 80% respectively (means of triplicate determinations, SEM<2%)). In contrast, L-tryptophan, which is transported largely by system T in human erythrocytes (19), caused only modest (25%) inhibition of leucine uptake. In human erythrocytes, system L shows only partial stereoselectivity for L- *vs* D-leucine uptake (20). In agreement with this, 5 mM D-leucine caused substantial (73%) inhibition of L-leucine uptake by rat erythrocytes. In contrast, small neutral amino acids (glycine, L-alanine and L-proline), acidic (L-glutamate) and basic amino acids (L-lysine) caused only slight inhibition of leucine influx (4-9%). Transport was inhibited by phloretin, with an IC_{50} value of 0.25 mM (data not shown).

Figure A1.2A shows the concentration dependence of leucine influx into rat erythrocytes at 1°C over the range 0.05 - 10 mM initial extracellular concentration. Uptake was determined both in the absence and in the presence of excess nonradioactive phenylalanine (50 mM) to distinguish system L-mediated uptake from that occurring by other routes (low affinity uptake by other transporters and simple diffusion through the lipid bilayer). In the presence of phenylalanine, leucine uptake was linear with respect to concentration and at the highest concentration tested (10 mM) corresponded to 39%

of the total uptake. In contrast, phenylalanine-inhibitable leucine uptake was saturable and conformed to simple Michaelis-Menten kinetics giving apparent K_m and V_{max} values of 0.49 ± 0.05 mM and 600 ± 15 mmol/l cell water per h (Figure A1.2B). For comparison, kinetic parameters for leucine uptake via system-L in human erythrocytes at this temperature from the work of Hoare are K_m 0.28mM and V_{max} 2.0μmol/l cell water per min (Figure A1.2, ref.17). There is a relatively small 2.7-fold difference in K_m between the two species, but the expected dramatic difference in V_{max} (355-fold). Based upon these kinetic parameters, the calculated initial rates of 0.2 mM leucine influx by system-L in the two species at 1°C are 148 and 0.83 μmol/l cell water per min in rat and human cells, respectively, a ratio of 178-fold. To confirm this very large difference in transport capacity between the two species at 1°C, I measured the concentration dependence of system L-mediated leucine uptake by human erythrocytes under conditions identical to those used for rat erythrocytes in Figure A1.2, except that it was necessary to use a 1 h incubation period in order to achieve measurable uptake of amino acid into the cells. Kinetic parameters for phenylalanine-sensitive leucine uptake were in good agreement with those of Hoare (17), except that the apparent K_m value (0.43 ± 0.03mM) was almost identical to that obtained by me for rat erythrocytes at this temperature, and the V_{max} (1.33 ± 0.2μmol/l cell water per min) was slightly lower than that reported by Hoare (17). Using my values, the V_{max} difference between the two species is 451-fold (352-fold difference in initial rate at 0.2 mM extracellular amino acid concentration).

A1.3c. Reconstitution of rat erythrocyte system-L

The presence of high system L activity in rat erythrocytes makes this cell type a potential source of transporter for isolation and purification studies. I therefore investigated conditions for the solubilisation of system-L from rat erythrocyte membranes and its reconstitution in a functional state into phospholipid vesicles. My approach towards reconstitution of system L was to use modifications of a gel-filtration, freeze-thaw protocol devised originally for the erythrocyte monocarboxylate transporter (27) and amino acid transport systems from rat liver and bovine kidney plasma membranes (25,26). Transport (0.2 mM leucine, 25°C, 30s incubation) was assayed by rapid filtration of reconstituted egg phospholipid vesicles through nitrocellulose filters and expressed as nmol/mg reconstituted membrane protein.

Before solubilisation, membranes were first treated with 0.1mM EDTA (pH 11.2) to remove cytoskeleton and other peripheral proteins. SDS/polyacrylamide gel electrophoresis determined that this protein-depletion procedure resulted in loss from the membranes of bands 1,2,4.2,5 and 6 (nomenclature of Steck (31)). Subsequent extraction of these membranes with 50 mM n-octyl-glucoside solubilised 64% of the integral membrane protein and the SDS/polyacrylamide gel electrophoresis profile of the solubilised preparation was undistinguishable from that of the starting protein-depleted membranes. Under standard reconstitution conditions (please see below), ≥ 90% of the solubilised protein was incorporated into proteoliposomes which had a mean diameter of 560nm.

Figure A1.3 shows that optimal reconstitution of system-L transport activity was achieved at lipid:protein ratios of 25-35. A lipid:protein ratio of 25:1 was used in all

subsequent experiments. Under these conditions, approximately one-half of the total uptake of leucine was inhibited by 2 mM phloretin. Leucine is a lipophilic amino acid. As a control to distinguish between transport of leucine into liposomes and binding of amino acid to the surface of vesicles, I confirmed that the presence of external sucrose (50 and 250 mM) in the extravesicular medium decreased leucine uptake by reconstituted proteoliposomes, indicating that uptake was into an osmotically-active space (Figure A1.4). To demonstrate that phloretin-sensitive L-leucine uptake was mediated by system-L, I tested the effects of different nonradioactive amino acids (5 mM) as inhibitors of this component of leucine uptake by reconstituted proteoliposomes. In agreement with the results for intact rat erythrocytes, uptake was markedly inhibited by L-phenylalanine and nonradioactive L-leucine (97 and 77%, respectively), and only to a very much smaller extent by glycine and L-alanine (20 and 10%, respectively) (Table A1.1). In contrast, amino acids had no effect on the phloretin-insensitive component of leucine uptake (data not shown). Two other inhibitors of system-L in intact cells (MK 196 and PCMBS) (32) were also effective inhibitors of phloretin-sensitive leucine transport in reconstituted proteoliposomes (see also Table A1.1). Since leucine is a relatively hydrophobic amino acid, the residual phloretin-insensitive component of leucine uptake is most likely to represent simple diffusion of amino acid across the lipid bilayer. This was confirmed in the experiment shown in Figures A1.5A and A1.5B which demonstrate that this component of leucine uptake corresponds to that seen with heat-treated membrane proteins and also with liposomes prepared in the absence of membrane extract.

A1.4. DISCUSSION

In terms of transport activity, the dominant amino acid transport system present in human erythrocytes is Na^+-independent system-L (20). As illustrated in Figure A1.1, transport of leucine by this route in human erythrocytes is rapid, with an initial rate of ~ 120 μmol/l cell water per min (0.2 mM extracellular leucine, 37°C). Kinetically, the transporter conforms to the simple carrier model of Lieb and Stein (33-35). The system is also present in erythrocytes of some other mammalian species (*eg.* rabbit), but not ruminants, and shares functional similarities with equivalent transporters in a wide range of other cell types and tissues (1,2). With respect to transport capacity (V_{max}), system-L ranks fourth behind 3 other human erythrocyte equilibrative transport systems; the Cl^-/HCO_3^- exchange transporter (10^6 copies per cell), the glucose transporter (2.10^5 copies/cell) and the nucleoside transporter (10^4 copies per cell) (24,36,37).

If system-L has a turnover number equivalent to those determined for the human erythrocyte glucose and nucleoside transport systems (35), then the estimated membrane abundance of L-transport proteins in rat erythrocytes would be $\sim 2.10^4$ copies/cell based upon V_{max} values for zero-trans influx or $\sim 1 \times 10^4$ copies/cell based upon V_{max} values for equilibrium-exchange influx. In a recent study, this laboratory has used equilibration values for leucine and lysine uptake by right-side-out vesicles to provide a more direct estimate of the membrane abundance of amino acid transport system-L and system-y^+ in human erythrocytes (38). It was found that all of the intravesicular space was accessible to leucine, as judged by comparisons with uridine uptake via the nucleoside transporter, while only 28% of the total intravesicular space was accessible to lysine uptake by system-y^+. Since human erythrocyte membranes generate an average of $\sim 10^4$ vesicles/cell, these data provide independent evidence that system-L is present in human erythrocytes at $\geq 10^4$ copies/cell, while system-y^+ is present in smaller amounts (~ 300 copies/cell).

In the present series of experiments, I have demonstrated that rat erythrocytes have a dramatically higher system-L transport activity than human erythrocytes. This difference is a V_{max} effect, with essentially no difference in apparent K_m. While some of the measured (350-450 fold) difference in transport capacity between the two cell types a 1°C might be a consequence of differences in the temperature sensitivity or turnover number of the transporters in the two species (39), it seems likely that much of the difference can be attributed to differences in the numbers of copies of system-L transporter present in the two cell types. There are well established precedents for such species differences. For example, large species variations in erythrocyte nucleoside transport activity have been shown to correlate directly with the membrane abundance of nucleoside transporter ligand binding sites (40). On the basis of these arguments, it can be estimated that the number of copies of system-L in rat erythrocyte membranes approaches, or exceeds, that of the human erythrocytes glucose transport system (2.10^5 copies/cell) which accounts for ~5% of the total integral membrane protein (36). However, compared with human erythrocytes, those from rat exhibit low glucose and nucleoside transport activity (41,42). For these different reasons, rat erythrocytes represent a unique potential source of system L transporter for isolation and purification

studies.

In common with the situation for other mammalian amino acid transporters, no specific, high-affinity inhibitors or other ligands of system L are available. Reconstitution of transporter into liposomes followed by assays of transport activity is the only means presently available to detect system-L after its extraction from the plasma membrane. Previously, there have been two reports of reconstitution of system L activity, both in hepatic cells (26,46). The present study using rat erythrocyte membranes is the first to report reconstitution of an erythrocyte amino acid transporter.

ADDENDUM

The experiments described in this Appendix were performed with the objective of undertaking biochemical purification of the system L amino acid transporter. These efforts were not successful because L-leucine transport activity was lost during chromatographic fractionation of n-octyl-glucoside solubilised rat erythrocyte membrane extracts (data not presented). A DEAE-cellulose fraction prepared in the course of these experiments was, however, enriched in a previously unknown 73 kDa protein which was subsequently purified to homogeneity by preparative SDS-polyacrylamide gel electrophoresis. Automated Edman degradation of the protein (Alberta Peptide Institute, University of Alberta) allowed identification of the first 33 residues of its N-terminal domain: GCDLNVGLITGAIITAVLAVFGGILMPVGDLLI. This N-terminal sequence is 84% identity to the N-terminal sequence of a human membrane glycoprotein named CD36 (43). CD36 is a leucocyte differentiation antigen which occurs in a wide range of tissues and plays major roles in adhesion phenomena, signal transduction and hematopathology (43). In particular, CD36 is thought to play an important role in infection with the human malaria parasite, *P. falciparum* (44). Adult erythrocytes have previously been considered to be negative for CD36, but a recent report demonstrates that human erythrocytes also express CD36 which may act as a receptor for rosetting of *P. falciparum*-infected erythrocytes (45). However, no erythroid CD36 cDNAs have been isolated. Potentially, the N-terminal amino acid sequence of the 73kDa protein could be used to design a degenerate oligonucleotide probe to screen an appropriate (*eg.* bone marrow or reticulocyte) cDNA library for cDNAs encoding rat or human erythrocyte CD36.

TABLE A1.1. Inhibition of L-leucine uptake by reconstituted proteoliposomes from rat erythrocyte membranes

Inhibitor	L-leucine uptake (nmol/mg protein)	
Control	2.23 ± 0.10	(100%)
L-Leucine (5 mM)	0.52 ± 0.07	(23%)
L-Phenylalanine (5 mM)	0.06 ± 0.06	(3%)
L-Alanine (5 mM)	2.01 ± 0.39	(90%)
Glycine (5 mM)	1.78 ± 0.17	(80%)
MK196 (2 mM)	0.37 ± 0.05	(17%)
PCMBS (2 mM)	0.45 ± 0.05	(20%)

Various nonradioactive amino acids and other compounds were tested as inhibitors of phloretin-sensitive L-[^{14}C]leucine transport (0.2 mM) in reconstituted proteoliposomes at 25°C (30s incubation). Nonradioactive amino acids and [^{14}C]leucine were added to proteoliposomes simultaneously. MK 196 and PCMBS were preincubated with proteoliposomes for 15 min at 20°C before addition of permeant. Values are means (± SEM) of triplicate determinations. Numbers in parentheses are % control.

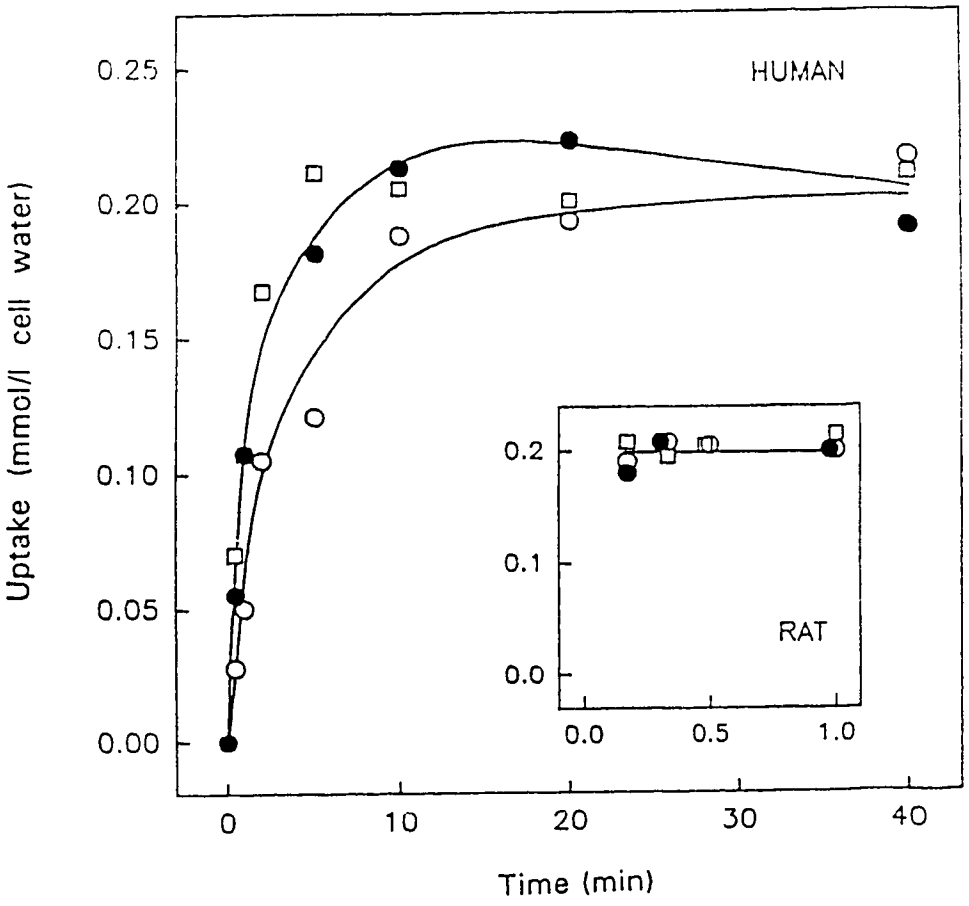


Figure A1.1. Time courses of L-leucine, L-phenylalanine and L-valine uptake in human and rat erythrocytes. Uptake of L-leucine (●), L-phenylalanine (□) and L-valine (○) by human and by rat erythrocytes at 37°C and 0.2 mM initial extracellular amino acid concentration. Values are means of triplicate determinations.

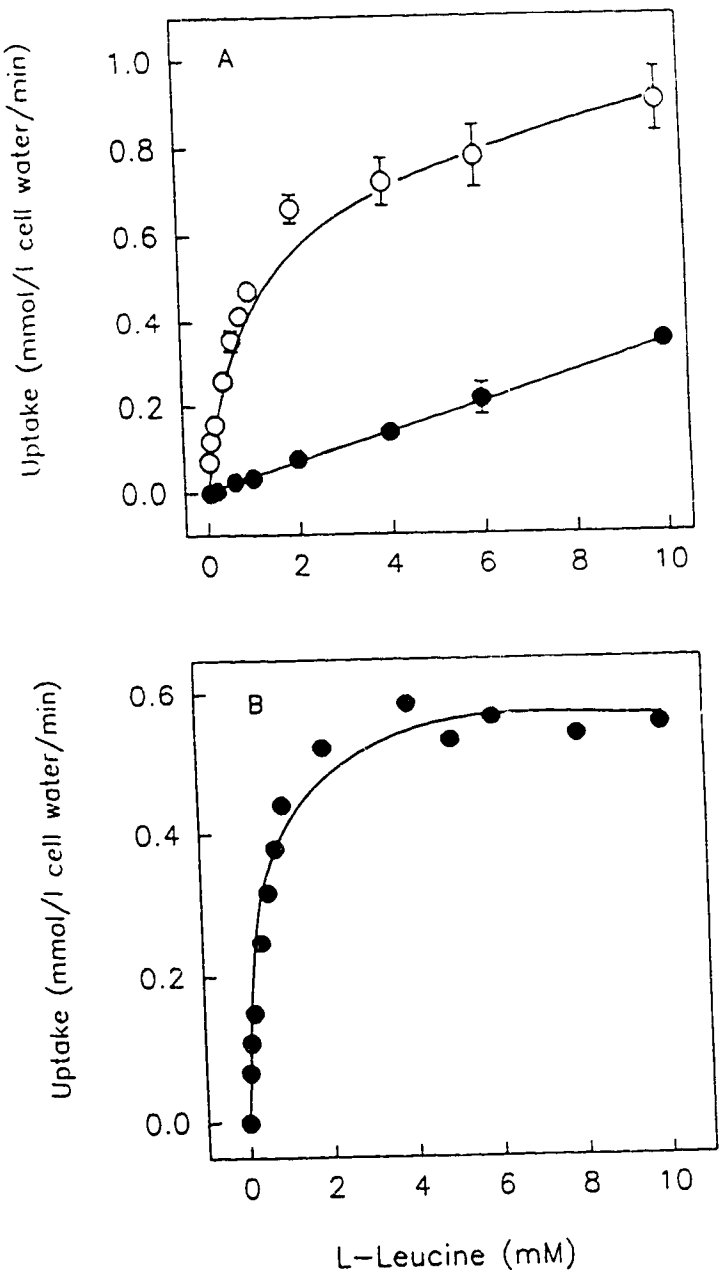


Figure A1.2. Concentration dependence of L-leucine uptake by rat erythrocytes. Initial rates of L-[^{14}C]leucine uptake by rat erythrocytes at 1°C (10s incubation) were measured in the absence (○) and in the presence (●) of 50 mM nonradioactive L-phenylalanine (A). Each data point is the mean (± SEM) of triplicate determinations. B, the phenylalanine-sensitive component of leucine uptake.

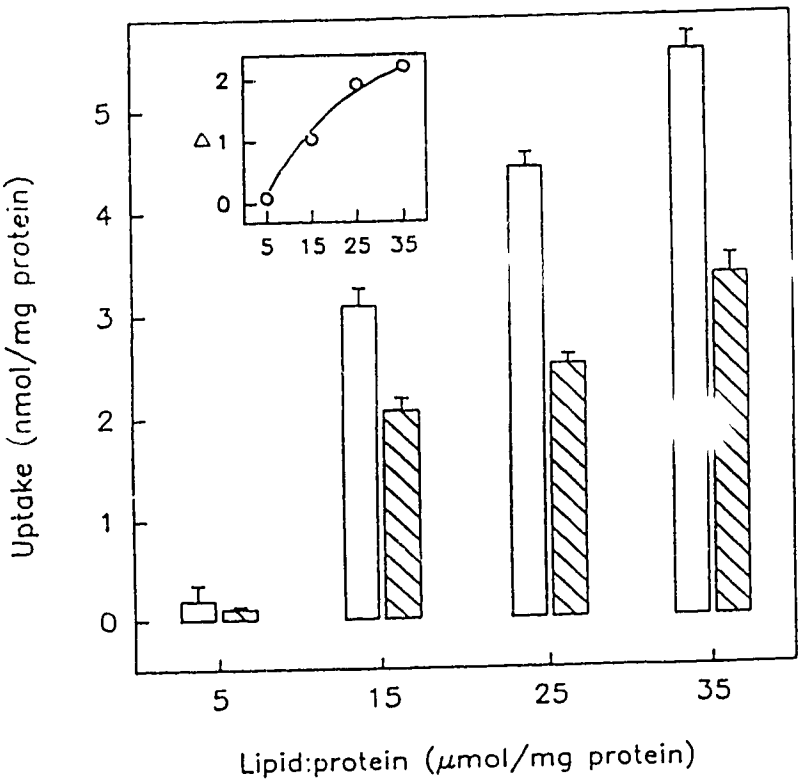


Figure A1.3. Reconstitution of rat erythrocyte system L into proteoliposomes. Uptake of L-[^{14}C]leucine (0.2 mM, 25°C, 30 s incubation) into proteoliposomes reconstituted at different lipid:protein ratios was measured in the absence (open bars) and in the presence of 2 mM phloretin (hatched bars) (15 min preincubation at 25°C) as described in the text. Values are means (± SEM) of triplicate determinations. The inset shows the effect of lipid:protein ratio on the phloretin-sensitive component of uptake. Please see text for other experimental details.

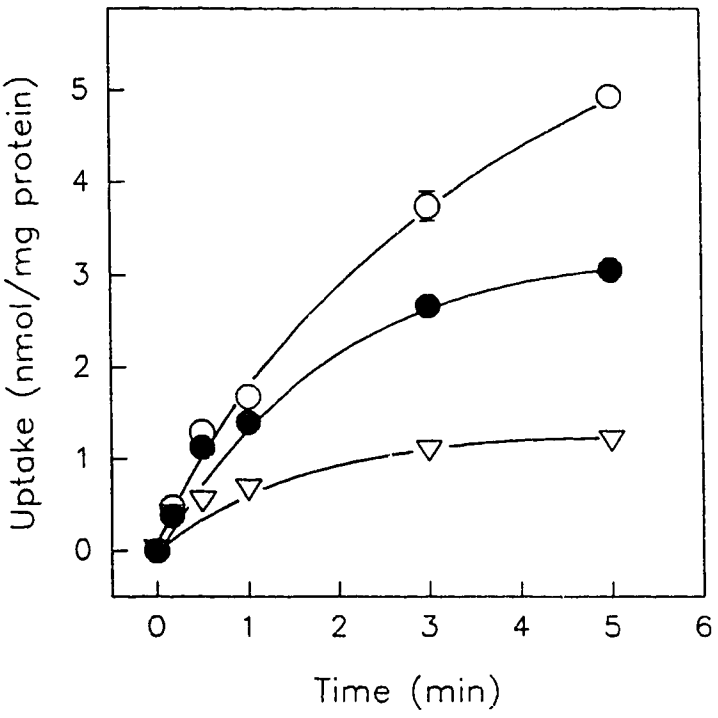


Figure A1.4. Effect of sucrose on L-leucine uptake by reconstituted proteoliposomes. Time-courses of L-[^{14}C]leucine uptake (0.2 mM, 25°C) by reconstituted proteoliposomes was measured in normal reconstitution buffer (○) and in buffer containing 50 mM (●) and 250 mM (▽)sucrose. Values are means (± SEM) of triplicate determinations. Please see text for other experimental details.

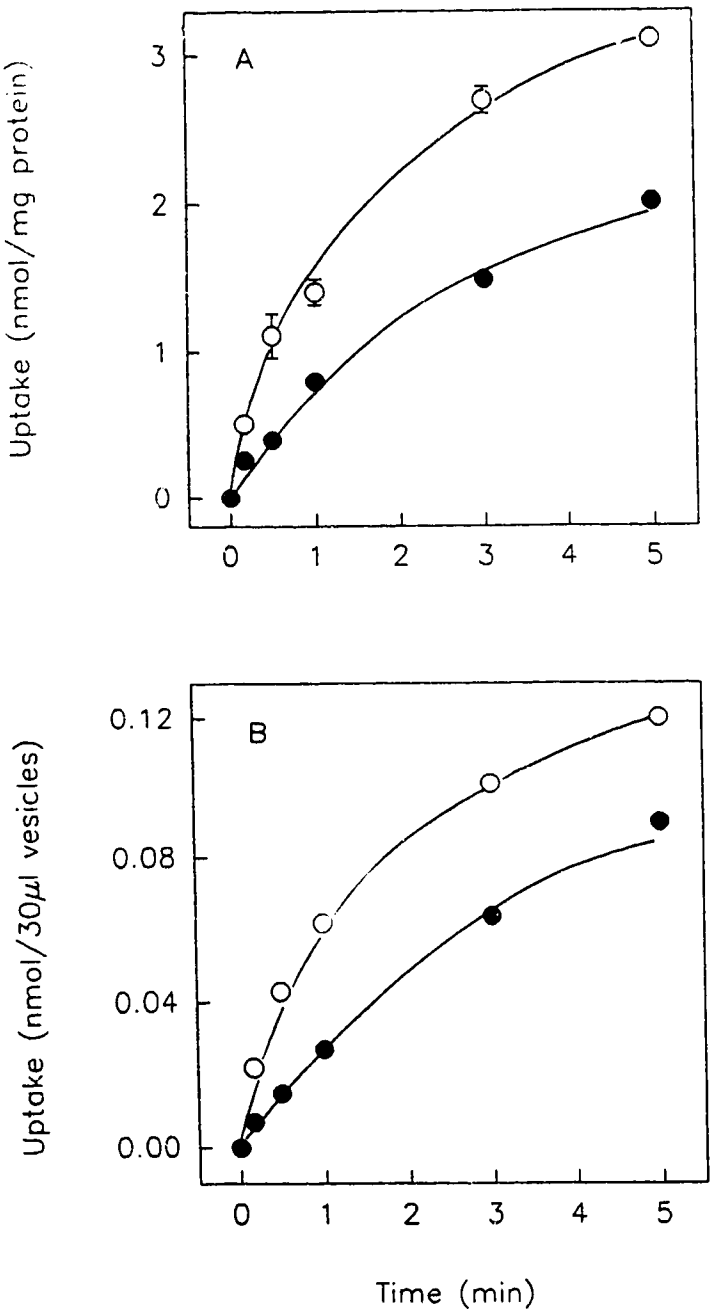


Figure A1.5. L-leucine uptake by reconstituted proteoliposomes, liposomes reconstituted with heat-inactivated membrane extract and protein-free liposomes. A, time-courses of L-[^{14}C]leucine uptake (0.2 mM, 25°C) by normal proteoliposomes (○) and liposomes reconstituted with heat-treated (100°C for 4 min) membrane extract (●). B, time course of L-leucine uptake measured in normal proteoliposomes (○) and protein-free liposomes (●). Values are means (± SEM) of triplicate determinations. Please see text for other experimental details.

A1.5. BIBLIOGRAPHY

1. Christensen, H. N. (1979). Exploiting amino acid structure to learn about membrane transport. *Adv. Enzymol.* **49**, 41-101.

2. Christensen, H. N. (1984). Organic ion transport during seven decades, the amino acids. *Biochem. Biophys. Acta* **779**, 255-269.

3. Bracy, D. S., Schenerman, M. A., and Kilberg, M. S. (1987). Solubilization and reconstitution of hepatic system A-mediated amino acid transport. Preparation of proteoliposomes containing glucagon-stimulated transport activity. *Biochem. Biophys. Acta* **899**, 51-58.

4. Radian, R, Bendahan, A., and Kanner, B.I. (1986). Purification and identification of the functional sodium-and chloride-coupled γ-aminobutyric acid transport glycoprotein from rat brain. *J. Biol. Chem.* **261**, 15437-15441.

5. McCormick, J. I., and Johnstone, R. M. (1988). Simple and effective purification of a Na^+-dependent amino acid transport system from Ehrlich ascites cell plasma membrane. *Biochemistry* **85**, 7877-7881.

6. Tamarappoo, B. K., and Kilberg, M. S. (1991). Functional reconstitution of the hepatic system N amino acid transport activity. *Biochem. J.* **274**, 97-101.

7. Tate, S.S., Yan, N., Udenfriend, S. (1992). Expression cloning of a Na^+-independent neutral amino acid transporter from rat kidney. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1-5.

8. Wells, R.G., and Hediger, M.A. (1992). Cloning of a rat kidney cDNA that stimulates dibasic and neutral amino acid transport and has sequence similarity to glucosidases. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5596-5600.

9. Bertran, J., Werner A., Moore, M.L., Stange, G., Markovich, D., Biber, J., Testar, A., Zorzano, A., Palacin, M., and Murer, H. (1992). Expression cloning of a cDNA from rabbit kidney cortex that induces a single transport system for cyctine and dibasic and neutral amino acids. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5601-5605.

10. Kim, J.W., Closs, E.I., Abritton, L.M., and Cunningham, J.M. (1991). Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature* **352**, 725-728.

11. Wang, H., Kavanaugh, M.P., North, R.A., and Kabat, D. (1991). Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter.

Nature **352**,729-731.

12. Young, J. D., and Ellory, J. C. (1977). In *Membrane Transport in Red Cells* (eds. Ellory, J. C., and Lew, V. L.), Academic press, London, pp301-325.

13. Harvey, C. M., and Ellory, J. C. (1989). In *Methods in Enzymololgy* (eds. Fleischer, S., and Fleischer, B.), **173**, pp122-160.

14. Winter, C. G., and Christensen, H. N. (1964). Migration of amino acids across the membrane of the human erythrocyte. *J. Biol. Chem.* **239**, 872-878.

15. Gardner, J. D., and Levy, A. G. (1972). Transport of dibasic amino acids by human erythrocytes. *Metab. Clin. Exp.* **21**, 413-431.

16. Hoare, D. G. (1972a). The transport of L-leucine in human erythrocytes: a new kinetic analysis. *J. Physiol.* **221**, 211-329.

17. Hoare, D. G. (1972b). The temperature dependence of the transport of L-leucine in human erythrocytes. *J. Physiol.* **221**, 331-348.

18. Rosenberg, R. (1981). L-leucine transport in human red blood cells: a detailed kinetic analysis. *J. Membrane Biol.* **62**, 79-83.

19. Rosenberg, R., Young, J. D., and Ellory, J. C. (1980). L-tryptophan transport in human red blood cells. *Biochem. Biophys. Acta* **598**, 375-384.

20. Young, J. D., Jones, S. E. M., and Ellory, J. C. (1980). Amino acid transport in human and sheep erythrocytes. *Proc. R. Soc. Lond. B B.* **209**, 355-375.

21 Dawson, R. M. C. (1963). On the mechanism of action of phospholipase A. *Biochem. J.* **88**, 414-423.

22. Chen, P. S., Toribara, T. Y., and Warner, H. (1956). Microdetermination of phosphorus. *Anal. Chem.* **11**, 1756-1758.

23. Young, J. D., and Ellory, J. C. (1982). In *Red Cell Membranes: A Methodological Approach* (eds. Ellory, J. C., and Young, J. D.) , Academic press, London, pp. 119-133.

24. Jarvis, S. M., and Young, J. D. (1980). Nucleoside transport in human and sheep erythrocytes. Evidence that nitrobenzylthioinosine binds specifically to functional nucleoside-transport sites. *Biochem. J.* **190**, 331-339.

25. Lynch, A. M., and McGivan, J. D. (1987). A rapid method for the

reconstitution of Na^+-dependent neutral amino acid transport from bovine renal brush-border membranes. *Biochem. J.* **244**, 503-507.

26. Quesada, A. R., and McGivan, J. D. (1988). A rapid method for the functional reconstitution of amino acid transport systems from rat liver plasma membranes. *Biochem. J.* **255**, 963-969.

27. Poole, R. C., and Halestrap, A. P. (1988). Reconstitution of the L-lactate carrier from rat and rabbit erythrocyte plasma membrane. *Biochem. J.* **254**, 385-390.

28. Peterson, G. L. (1977). A simplification of the protein assay method of Lowry *et al* which is more generally applicable. *Anal. Biochem.* **83**, 346-356.

29. Thompson, S., and Maddy, A.A. (1982). In *Red Cell Membranes - A Methodological Approach* (eds. Ellory, J.C., and Young, J.D.), Academic Press, London, pp. 67-93.

30. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**, 680-685.

31. Steck, T.L. (1974). The organization of proteins in the human red blood cell membrane. A review. *J. Cell Biol.* **62**, 1-19.

32. Wolowyk, M. W., Young, J. D., and Ellory, J. C. (1983). Inhibition of amino acid transport by loop diuretics. *Proc. West. Pharmacol. Soc.* **26**, 247-249.

33. Lieb, W. R., and Stein, W. D. (1974). Testing and characterization of the simple carrier. *Biochem. Biophys. Acta* **373**, 178-196.

34. Lieb, W. R. (1982). In *Red Cell Membranes: A Methodological Approach* (eds. Ellory, J. C., Young, J. D.), Academic Press, London, pp.135-164.

35. Stein, W. D. (1986). *Transport and Diffusion across Cell Membranes*, Academic press, London.

36. Lin, S., and Spudich, J. A. (1974). Biochemical studies on the mode of cytochalasin B. Cytochalasin B binding to red cell membrane in relation to glucose transport. *J Biol. Chem.* **249**, 5778-5783.

37. Wieth, J. O. (1979). Bicarbonate exchange through the human red cell membrane determination with [^{14}C]bicarbonate. *J. Physiol. (London)* **294**, 521-539.

38. Tse, C. M., Fincham, D. A., Ellory, J. C., and Young, J. D. (1991). Use of

membrane vesicles to estimate the numbers of system y^+ and system L amino acid transporters in human erythrocytes. *Biochem. J.* **277**, 565-568.

39. Ellory, J.C. & Willis, J.S. (1983). Adaptative changes in membrane-transport systems of hibernators. *Biochem. Soc. Trans.* **11**, 330-332.

40. Young, J. D., and Jarvis, S. M. (1983). Nucleoside transport in animal cells. *Rev. Biosci. Reports* **3**, 309-322.

41. Widdas, W. F. (1980). The asymmetry of the hexose transfer system in the human red cell membrane. *Curr. Top. Membr. Transp.* **14**, 165-223.

42. Jarvis, S. M. and Young, J.D. (1986). Nucleoside transport in rat erythrocytes: two components with differences in sensitivity to inhibition by nitrobenzylthioinosine and p-chloromercuriphenyl sulphonate. *J. Membr. Biol.* **4**.. 1-10.

43. Greenwalt, D.E., Lipsky, R.H., Ockenhouse, C.F., Ikeda, H., Tandon, N.N., and Jamieson, G.A. (1992). Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction, and transfusion medicine. *Blood* **80**, 1105-1115.

44. Ockenhouse, C.F., Tandon, N.N., Magowan, C., Jamieson, G.A., and Chulay, J.D. (1989). Identification of a platelet membrane glycoprotein as a *falcilarum* malaria sequestration receptor. *Science* **243**, 1469-1471.

45. van Schravendijk, M.R., Handunnetti, S.M., Barnwell, J.W., and Howard R.J. (1992). Normal human erythrocytes express CD36, an adhesion molecule of monocytes, platelets, and endothelial cells. *Blood* **80**, 2105-2114.

46. Mitsumoto, Y., and Mohri, T. (1991). Reconstitution of the L-leucine-H^+ cotransporter of the plasma membrane from Chang liver cells into proteoliposomes. *Biochem. Biophys. Acta* **1061**, 171-174.

APPENDIX 2

Poly(A)$^+$RNA from the Mucosa of Rat Jejunum Induces Novel Na^+-dependent and Na^+-independent Leucine Transport Activities in Oocytes of *Xenopus laevis*[1]

[1]A version of this chapter has been published. Yao, S.Y.M., Muzyka, W R., Elliott, J.F., Cheeseman, C.I., and Young, J.D. 1994. Poly(A)$^+$ RNA from the mucosa of rat jejunum induces novel Na^+-dependent and Na^+-independent leucine transport activities in oocytes of *Xenopus laevis*. *Mol. Membr. Biol.* 11, 109-118.

A2.1. INTRODUCTION

In the small intestine, absorption of the essential amino acids lysine and leucine is a consequence of net uptake from the lumen into epithelial cells and, subsequently, efflux across basolateral membranes towards the blood. These two stages of lysine and leucine transport have not been characterised clearly and there are unresolved questions of Na^+-dependence and reciprocal inhibitory interactions between the two amino acids (1-6). In most tissues, cationic amino acids are transported by amino acid transport system y^+ (7-9). This transporter, which is Na^+-independent with respect to cationic amino acid transport, exhibits a low-affinity interaction with neutral amino acids of intermediate size such as homoserine, but only when Na^+ is present in the external medium (10-13). High-affinity inhibition of lysine transport by large neutral amino acids such as leucine, as occurs in intestine, indicates involvement of systems other than y^+. Van Winkle and coworkers have described Na^+-independent (system $b^{o,+}$) and Na^+-dependent transporters (system $B^{o,+}$) from mouse blastocysts which transport both cationic and large neutral amino acids with high affinity (14-16). Identification of $b^{0,+}$ and $B^{0,+}$ in mammalian cells generally has been lacking, although there is clear evidence from perfusion and vesicle studies that leucine interacts with lysine transporters in the intestine (1,4-6). In a relatively recent review, lysine transport in intestine was still described in terms of y^+ (17).

Leucine transport in intestine is equally problematic (18,19). Conventional systems responsible for leucine transport in most other mammalian cells (Na^+-dependent system A and Na^+-independent system L) do not, in contrast to intestinal leucine transporters, interact with cationic amino acids (7,8). The presence of a $b^{0,+}$-type transporter in intestinal brush border membranes has been invoked in order to account for this interaction (14), but the extent to which intestinal leucine and lysine transport are Na^+-dependent or Na^+independent has not been defined unequivocally, and is complicated by dietary and species variation as well as by differences down the length of the intestine (3,4,6,20).

Several mammalian amino acid transporters have been cloned and sequenced (21-31). These include a Na^+-independent cationic amino acid transporter from mouse fibroblasts with y^+-like characteristics (ecoR)(22,23, Chapter 1, Section 1.5e). This protein has 14 putative transmembrane domains (622 amino acid residues) and shows sequence homology with proton-dependent cationic and neutral amino acid transporters from *S. cerevisiae* (32-35). Initially cloned as an ecotropic retrovirus receptor of unknown function (36), its identity as an amino acid transporter was established by functional expression in *Xenopus* oocytes (22,23). Oocytes microinjected with ecoR cRNA exhibit large increases in cationic, but not neutral amino acid transport and voltage clamp studies have demonstrated a homoserine-dependent Na^+-current, consistent with the established (homoserine + Na^+) interaction with y^+ (23). Significant message for y^+ has been identified in neonatal rat intestine, but not in adult jejunum, the major site of amino acid absorption in rat intestine (37).

cDNAs have been isolated recently from rat and rabbit kidney which, when expressed in *Xenopus* oocytes, elicit large increases in neutral/cationic amino acid

transport activity (38-40, Chapter 1, Section 1.5f). In contrast to ecoR, these cDNAs (79% identity in nucleotide sequence) encode 683- and 677-amino acid type II membrane glycoproteins with single transmembrane regions. The encoded proteins are homologous to alpha-amylases and alpha-glucosidases, but have apparently no such enzymic activity (39,40). The characteristics of transport activity induced by the rat (D2) and rabbit (rBAT) clones were of $b^{0,+}$-type (*ie* high affinity Na^+-independent transport of both neutral and cationic amino acids). Messenger RNA equivalent to D2 and rBAT was detected in Northern blots of kidney and intestine, and not other tissues (39,40).

The rat and rabbit D2/rBAT sequences exhibit significant homology to the human and mouse 4F2 heavy-chain (4F2hc) cell surface antigens, type II glycoproteins which are widely expressed in proliferating cells (41,42). Injection of cRNA from human fibroblast 4F2hc into Xenopus oocytes led to a small (2-fold) increase in amino acid transport activity (43,44) that has been incorrectly identified as y^+-like (43) and which more closely resembles another transporter that this laboratory (45) and others (46) have identified kinetically in human erythrocytes. This system, which has been given the designation y^+L (46), has the novel ability to catalyse high-affinity transport of leucine in a Na^+-dependent manner and high-affinity transport of lysine in the absence of Na^+. An apparently similar route of transport may occur in placenta (47) which has also been reported to exhibit system $b^{0,+}$ activity (48). The fact that the D2,rBAT/4F2hc family of proteins have a structure which predicts a single transmembrane domain does not *a priori* exclude the possibility that they function as transport proteins or constitutive elements of transport proteins. A homooligomeric K^+ channel with subunits containing a single transmembrane region has been cloned recently (49), as has a volume-activated Cl^- channel with no potential transmembrane helices (50). It is perhaps more likely, however, that these proteins function as specific activators of mammalian and oocyte amino acid transporters (39,43,51,52). D2 mRNA is strongly expressed in the rat kidney proximal tubule S3 segment which is consistent with a role of D2 in kidney amino acid reabsorption (53).

In other studies, Murer and colleagues (54) have investigated the properties of Na^+-independent arginine and leucine fluxes induced in Xenopus oocytes microinjected with mRNA from rabbit intestinal mucosa. Transport of arginine was inhibited by leucine and *vice versa*. Approximately one-half of the jejunum mRNA-stimulated arginine uptake and three-quarters of the induced leucine transport were blocked by hybrid-depletion of the mRNA with antisense oligonucleotide complementary to rBAT cDNA, suggesting that rBAT is a major determinant of Na^+-independent neutral/cationic amino acid transport in rabbit intestine. These authors did not investigate the characteristics of Na^+-dependent leucine transport activity in oocytes injected with rabbit intestinal mRNA. This laboratory has used functional expression in *Xenopus* oocytes to study the characteristics of lysine transport in the jejunum of the rat (55). Na^+-dependent as well as Na^+-independent components of expressed lysine transport activity were identified in oocytes microinjected with rat jejunal mRNA, and both were inhibited by leucine with high affinity. In contrast to D2 and rBAT, however, the Na^+-independent component of induced lysine transport, which represented the majority (62%) of the expressed transport activity, was not inhibited by alanine or by phenylalanine. In parallel

with the properties of system y^+L in human erythrocytes, however, leucine inhibition of the flux was reduced substantially in the absence of sodium (55).

To investigate further the properties of neutral/cationic amino acid transport in the small intestine and the relationship of these activities to D2/rBAT and 4F2hc, this Appendix describes a study of the characteristics of leucine transport in oocytes microinjected with rat jejunal mRNA. My results show that, in contrast to lysine influx, the majority (80%) of expressed leucine transport activity was Na^+-dependent. Lysine was inhibitory, but not alanine or phenylalanine. The Na^+-independent component of induced leucine transport (20% of the total flux) was also sensitive to lysine inhibition and was reduced by half in the presence of alanine and phenylalanine. Experiments utilising PCR demonstrated the presence of both D2 and 4F2hc message in rat jejunum. However, hybrid-depletion of jejunal mRNA with an antisense oligonucleotide complementary to D2 had no effect on the expression of Na^+-linked leucine transport activity and only reduced the smaller Na^+-independent component of leucine transport by 40%. In parallel experiments, hybrid-depletion of jejunal mRNA with an antisense oligonucleotide complementary to 4F2hc had no effect on either Na^+-dependent or Na^+-independent leucine transport activity. Therefore, mRNA from rat jejunum encodes both Na^+-dependent and Na^+-independent neutral/cationic amino acid transport activities unrelated to the D2/4F2hc glycoproteins.

A2.2. MATERIALS AND METHODS

A2.2a. Isolation and fractionation of mRNA

Total RNA from the jejunal mucosa of adult male Sprague-Dawley rats was extracted using caesium chloride/guanidium thiocyanate centrifugation as described previously (55). In brief, jejunal intraluminal scrapings were frozen in liquid nitrogen and ground into a fine powder before being homogenised in guanidium thiocyanate (4M). The homogenate was centrifuged (5,000 *g*, 10 min., 4°C) and the resulting supernatant was layered onto a cushion of 5.7 M caesium chloride (*d* 1.7 g/ml) in 0.01M EDTA (pH 7.5) and centrifuged (111,000 *g*, 24 hrs, 20°C) in a Beckman SW 28 rotor to yield RNA pellets. Purification of poly(A)$^+$RNA (mRNA) was achieved using a PolyATract purification kit (Promega). Purified mRNA was dissolved in RNAase-free water (1 μg/μl). Size-fractionation of mRNA was accomplished by non-denaturing sucrose gradient centrifugation (56). A 150 μg sample of mRNA was heated to 65°C and chilled on ice before being loaded onto a 5-25% (w/v) sucrose density gradient. After centrifugation (97,000 *g*, 20hrs, 4°C), 0.5ml fractions were collected, precipitated in 3 volumes of ice-cold ethanol and 0.1 volumes of 3M sodium acetate. The fractions were then washed with 80% (v/v) ethanol, dried and resuspended in RNAase-free water at a concentration of 1 μg/μl. The size range of mRNA in different fractions was examined by electrophoresis on a denaturing 1% (w/v) agarose gel containing formaldehyde (0.66 M). Ethidium bromide (100ug/ml)-labelled RNA fractions were resolved by electrophoresis at room temperature at a constant voltage of 40 V/cm for 11.5 h. Gel lanes were scanned by laser densitometry (Gelscan XL, Pharmacia) and the sizes mRNAs in different fractions calculated by reference to BRL RNA molecular weight standards (GIBCO/BRL).

A2.2b. Oocyte preparation and injection

Stage VI oocytes were prepared as described in Section 2.2d of Chapter 2 and injected (Inject+Matic system) with either 50 nl of rat intestinal mRNA (1 ng/nl) or 50 nl of water. Injected oocytes were incubated at 18°C in MBM for 5 days with a daily change of medium before the assay of L-leucine transport activity.

For antisense hybrid-depletion experiments (39,40,54), rat jejunal mRNA was denatured by heating at 65°C for 10 min, then hybridised to one of four synthetic oligonucleotides (DNA Synthesis Service, Department of Microbiology, University of Alberta) in 50 mM NaCl for 15 min at 42°C (1 μg mRNA and 0.25 μg oligonucleotide/ul): a) sense D2 oligonucleotide, corresponding to nucleotide positions 76-94 of rat D2 cDNA (5'CAAAGACAAGAGAGACTC-3'); b) antisense D2 oligonucleotide, complementary to nucleotide positions 286-301 of rat D2 cDNA (5'GTAGCGAGCCTGGCCA-3'); c) sense 4F2hc oligonucleotide, corresponding to nucleotide positions 795 to 811 of mouse 4F2hc cDNA (5'GGTGTGGATGGTTTCCA-3') ; d) antisense oligonucleotide, complementary to nucleotide positions 1146 to 1162 of mouse 4F2hc cDNA (5'GTCCCTGGCAGAGTGAA-3'). Oocytes were injected with mRNA/oligonucleotide mixtures or control mRNA as described above.

A2.2c. PCR amplification

Single-stranded cDNA was reverse transcribed from rat jejunal poly $(A)^+$RNA using oligo$(dT)_{12-18}$ as a primer. The primers used for PCR amplification of D2 and 4F2 were the same two pairs of sense and antisense oligonucleotides synthesised for the antisense hybrid depletion experiments described in the previous section. Reaction mixtures (100 μl) contained 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 25ng cDNA, 100 pmol of each primer and 2.5 units of Taq polymerase (Perkin-Elmer). Amplification was accomplished by incubating at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. After 30 cycles, 15 μl of each reaction mixture was separated on a 2 % (w/v) non-denaturing agarose gel containing 0.5μg/ml ethidium bromide. The amplification products were subcloned into pCR™II vector (Invitrogen). Positive clones were isolated and sequenced by the Department of Biochemistry (University of Alberta) DNA Sequencing Laboratory using the *Taq* DyeDeoxy™ terminator cycle sequencing with an automated Model 373A (Applied Biosystems) DNA Sequencer.

A2.2d. Transport assays

Inward fluxes of L-leucine into injected oocytes were measured by conventional tracer techniques (55). The initial rate of leucine uptake (0.2 mM, 20°C) was traced with L-[U-^{14}C]leucine (2.5 μCi/ml)(Amersham) using a 10 min incubation period (influx was linear with time for 30 min). Assays were performed in 1.5 ml Eppendorf microcentrifuge tubes (5-10 oocytes/tube, incubation volume 0.1 ml) using transport buffers containing 100 mM NaCl or 100 mM choline chloride; 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.5). At the end of the flux, extracellular label was removed by six rapid ice-cold washes in choline chloride transport buffer; all washes were completed within 1 min. Individual oocytes were dissolved in 5% (w/v) sodium dodecyl sulphate (SDS) for quantitation of oocyte-associated [^{14}C]leucine by liquid scintillation counting (LS 6000 IC, Beckman Canada Inc.). All leucine influx values cited are means $\pm$ SEM of 5 or more assays each performed with a single oocyte.

For competition experiments, oocytes were exposed to nonradioactive L-amino acids (0.25 - 1 mM final concentration) at the same time as [^{14}C]leucine. Kinetic constants for leucine influx were determined by non-linear regression analysis (ENZFITTER, Elsevier-Biosoft). To minimise variability, each experiment used oocytes from a single animal. Each protocol was performed with a minimum of two different batches of mRNA.

A2.3. RESULTS

A2.3a. Endogenous leucine transport in oocytes

Basal influx of L-leucine (0.2 mM) in water-injected *Xenopus* oocytes was measured both in NaCl transport buffer and in choline chloride transport buffer. In the representative experiment shown in Table A2.1, the majority (91%) of leucine influx was Na^+-dependent. When Na^+-independent leucine influx (uptake measured in choline chloride transport buffer) was assayed in the presence of competing nonradioactive amino acids (1 mM) as described in Section A2.2, the order of inhibition was leucine (56% inhibition) >alanine (30%) >phenylalanine (17%) >lysine (4%). In contrast, the pattern of inhibition for Na^+-dependent leucine influx, calculated as the difference in uptake in NaCl and choline chloride transport buffer, was leucine (86%) >lysine (73%) >phenylalanine (31%) >alanine (26%). Thus, while nonradioactive leucine was the most effective inhibitor of both components of [^{14}C]leucine influx, lysine selectively inhibited Na^+-dependent leucine influx. Phenylalanine and alanine caused intermediate inhibition of the two components of endogenous leucine transport influx.

A2.3b. Leucine transport in mRNA-injected oocytes

Xenopus oocytes injected with rat intestinal mRNA expressed higher leucine (0.2mM) influx than water-injected oocytes. In the representative experiment shown in Figure A2.1, leucine influx into mRNA-injected oocytes in NaCl transport buffer was 12.4 $\pm$ 2.2 pmol/oocyte in 10 min, compared with 3.5 $\pm$ 0.5 pmol/oocyte in 10 min for water-injected oocytes. In the absence of Na^+ ions, leucine influx into mRNA-injected and water-injected oocytes was 2.4 $\pm$ 0.6 and 0.5 $\pm$ 0.1 pmol/oocyte in 10 min. Therefore, expressed Na^+-dependent influx of leucine (10.0 $\pm$ 2.3 pmol/oocyte in 10 min) was 3.2-fold higher than endogenous Na^+-dependent leucine influx, while expressed Na^+-independent leucine influx (2.4 $\pm$ 0.6 pmol/oocyte in 10 min) was 4.8-fold higher than the corresponding endogenous Na^+-independent uptake of leucine. In five separate experiments with different batches of mRNA, Na^+-dependent and Na^+-independent leucine influx were increased 5.1 $\pm$ 1.6 and 4.6 $\pm$ 0.4-fold, respectively, compared to control oocytes injected with water. The mean ratio of induced Na^+-dependent (11.1 $\pm$ 1.3 pmol/oocyte in 10 min): Na^+-independent (3.6 $\pm$ 1.3 pmol/oocyte in 10 min) leucine influx was 3.1:1.

A2.3c. Kinetic and competition studies

The experiments described above demonstrate that oocytes microinjected with rat jejunal mRNA express both Na^+-dependent and Na^+-independent components of leucine influx, the former representing 75% of the total expressed transport activity. Figure A2.2 shows the concentration dependence of these two components of influx measured over the leucine concentration range 0.1-1.0 mM, and corrected for the contribution of endogenous leucine uptake. Both Na^+-dependent and Na^+-independent leucine influx conformed to simple Michaelis-Menten kinetics, giving apparent K_m values of 0.20 $\pm$ 0.04 and 0.10 $\pm$ 0.05 mM, respectively. Apparent V_{max} values were 15.9 $\pm$ 1.1 and 3.6 $\pm$ 0.5 pmol/oocyte in 10 min, respectively, a ratio of 4.2:1.

Previously, it has been demonstrated that microinjection of *Xenopus* oocytes with rat jejunal mRNA results in induction of Na^+-independent L-lysine transport activity and, as well, a smaller increase in Na^+-dependent lysine influx (55). Both activities were inhibited, with high affinity, by leucine. Concentrations of leucine required for half maximal inhibition of expressed Na^+-dependent and Na^+-independent lysine (0.2mM) influx were 0.3 and 0.8 mM, respectively. This reciprocal inhibitory interaction between leucine and lysine was investigated further by measuring expressed Na^+dependent and Na^+-independent [^{14}C]leucine (0.2 mM) influx in the presence of increasing concentrations of nonradioactive lysine in the range 0.25 to 5mM (Figure A2.3). Lysine was an effective inhibitor of induced leucine influx, abolishing Na^+-dependent leucine uptake completely and 81% of Na^+-independent leucine uptake. Concentrations of lysine required to cause half-maximal inhibition of the two components of expressed leucine influx were 0.21 and 0.05 mM, respectively. Assuming competitive inhibition and using the apparent K_m values for leucine influx derived from the data presented in Figure A2.2, the calculated K_i values for lysine inhibition of Na^+-dependent and Na^+-independent leucine influx are 0.10 and 0.02 mM, respectively.

Figure A2.4 extends these competition studies by comparing the abilities of 1 mM leucine, lysine, phenylalanine and alanine to inhibit the two components of expressed leucine influx. In agreement with preceding experiments, nonradioactive leucine inhibited both fluxes completely, while lysine abolished all Na^+-dependent leucine influx and all but a small residual component (20%) of Na^+-independent leucine influx. Phenylalanine and alanine caused partial inhibition of Na^+-independent leucine influx (35 and 30% inhibition, respectively), but had no significant effect on Na^+-dependent leucine influx. A higher concentration of phenylalanine and alanine (10 mM) gave essentially identical results to those obtained with 1 mM phenylalanine and alanine (data not shown).

A2.3d. PCR and antisense hybrid-depletion experiments

As described in Section A2.1, the family of D2,rBAT/4F2hc glycoproteins induce leucine uptake in *Xenopus* oocytes injected with their cRNA. In order to investigate whether the mRNAs of these or related proteins are present in rat jejunum, I performed conventional PCR amplification using rat jejunal cDNA as template and primers based upon conserved regions of the rat kidney D2 and mouse pre-B cell/macrophage 4F2hc nucleotide sequences. As shown in Figure A2.5 (lane a) a single PCR product with size of ~350 bp was amplified from rat jejunal cDNA with 4F2hc primers and a second of ~200 bp with D2 primers (lane b): expected sizes of the respective PCR products, based upon the mouse 4F2hc and rat D2 sequences, are 226 and 368 bp. The deduced amino acid sequences are shown in Figure A2.6. For 4F2hc there was 82% identity in amino acid sequence with mouse 4F2hc (42), while for D2 there was 100% identity with the corresponding rat kidney sequence (38,40). The nucleotide sequences were 88 and 100% identical, respectively (not shown).

The role of intestinal D2 and 4F2hc-like proteins in the expression of leucine transport activities in *Xenopus* oocytes was investigated by antisense hybrid-depletion (39,40,54). Either sense or antisense oligonucleotides were hybridised with rat jejunal mRNA, before being microinjected into oocytes for assay of expression of Na^+-

dependent and Na^+-independent leucine influx (0.2 mM). Figure A2.7 shows that D2-antisense oligoneucleotide had no significant effect on expressed Na^+-dependent leucine influx, when compared to control mRNA-injected oocytes. In contrast, however, expressed Na^+-independent leucine influx was inhibited 49% by D2-antisense oligonucleotide. Since Na^+-independent leucine influx corresponds to only about 20% of the total induced leucine influx in mRNA-injected oocytes, < 10% of the total expressed leucine uptake was inhibited by D2-antisense oligonucleotide. Although small, the inhibition was specific because D2-sense oligonucleotide did not exhibit any inhibitory effect on either Na^+-dependent or Na^+-independent leucine influx (Figure A2.7). Figure A2.7 also shows that 4F2hc-antisense oligonucleotide had no significant effect on either component of expressed leucine influx.

A2.3e. mRNA size fractionation

In order to determine the size of the rat jejunal mRNA responsible for induced influx of leucine, the total rat jejunal poly(A)$^+$ RNA was size-fractionated on a 5-25% sucrose density gradient. Three mRNA fractions with size ranges of a) 2.0kb to 2.8kb (median 2.6kb), b) 1.5kb to 2.25kb (median 2.0kb) and c) 0.75kb to 1.6kb (median 1.1kb) were microinjected into oocytes and assayed for expression of Na^+-dependent and Na^+-independent leucine influx (0.2 mM). As shown in Figure A2.8, maximum expression of Na^+-dependent leucine influx was obtained with Fraction b, with substantial transport activity also in Fraction a. Maximum expression of Na^+-independent leucine influx was also found with Fraction b but, unlike Na^+-dependent leucine uptake, Fraction c gave more activity than Fraction a.

A2.4. DISCUSSION

I have employed functional expression in *Xenopus* oocytes to answer questions concerning the characteristics of intestinal leucine/lysine transport. Previously, it has been shown that oocytes express both Na^+-dependent and Na^+-independent lysine transport activity when microinjected with rat jejunal mRNA (55). Na^+-dependent lysine influx was inhibited by both leucine and alanine, while Na^+-independent lysine influx was inhibited by leucine and not by alanine. Phenylalanine had no effect on either component of transport and the ability of leucine to inhibit Na^+-independent lysine influx was markedly reduced when sodium in the transport buffer was replaced by choline.

The present series of experiments show that oocytes also express leucine transport activity when microinjected with rat jejunal mRNA. In contrast to lysine, where most of expressed transport activity was Na^+-independent, the majority (80%) of induced leucine transport activity was Na^+-dependent. Both the Na^+-dependent and Na^+-independent components of expressed leucine influx were saturable, giving apparent K_m values of 0.2 and 0.1 mM, respectively. Both were also inhibited by lysine (apparent K_i values 0.1 and 0.02 mM, respectively). Alanine and phenylalanine (1mM) had no effect on Na^+-dependent leucine transport, but gave partial inhibition of Na^+-independent leucine transport (30% and 35% respectively). These results indicate expression of at least three intestinal leucine transport systems: 1) a minor Na^+-independent activity inhibited by lysine, alanine and phenylalanine; 2) a Na^+-independent activity also inhibited by lysine and not by alanine and phenylalanine; 3) a major Na^+-linked activity inhibited by lysine but not by alanine or phenylalanine. In addition, there was a small residual component of Na^+-independent leucine transport activity which was insensitive to inhibition by lysine (Figures. A2.3b and A2.4b) and which may therefore reflect low-level expression of a neutral amino acid-selective transporter such as systems A or L (7,8).

It is my interpretation that Na^+-dependent leucine transport system 2 corresponds to Na^+-independent component of expressed lysine transport activity subject to Na^+-dependent inhibition by leucine. It is not the same as the minor Na^+-dependent component of lysine transport, because alanine was not inhibitory. Therefore, I have evidence for the expression of a transport system which functions in a Na^+-dependent mode with leucine as permeant and in a Na^+-independent mode with lysine as permeant. I did not find a component of leucine influx corresponding to the component of lysine transport that was Na^+-dependent, suggesting that leucine is a better inhibitor of the system than it is a permeant. Conversely, I did not previously detect a component of lysine transport corresponding to leucine transport system 1, most likely because of its small magnitude and its high apparent affinity for lysine.

An important difference between the leucine/lysine transport activities induced by jejunal mRNA and that induced by the kidney D2/rBAT glycoproteins is that the former were, with the exception of leucine transport system 1, not inhibited by phenylalanine or alanine. The activities therefore have different characteristics from the D2/rBAT system, suggesting that different proteins are involved. This conclusion is supported by previous comparisons of cystine transport in oocytes microinjected with jejunal and

kidney mRNA. This amino acid is a permeant on the D2/rBAT system and has K_m and V_{max} values broadly comparable to leucine and arginine (40). Oocytes injected with rat and rabbit kidney mRNA show similar increases in transport for all three amino acids. This is not the case for intestine, where levels of cystine transport in mRNA-injected oocytes are low (Figure 1 in reference 40) and small compared with the leucine/lysine transport activities expressed in our studies with oocytes injected with jejunal mRNA. Therefore, if D2-type activity is expressed in oocytes microinjected with mRNA from rat jejunum, it is only a minor component of total transport activity. Leucine transport induced by D2/rBAT is Na^+-independent, while the majority of leucine influx in oocytes microinjected with rat jejunal mRNA was Na^+-dependent.

The presence of D2/rBAT message in enterocytes has been inferred from Northern blots of intestinal mRNA probed with kidney D2/rBAT cRNA (39,40). The reported regional distribution of D2 in rat intestine (duodenum > ileum > jejunum) is opposite to that of amino acid transport activity (jejunum > ileum > duodenum) (39). In this study, I have used PCR to confirm the occurrence of D2 in rat jejunum and to show that the nucleotide and amino acid sequences of rat jejunum and kidney D2 are identical. Previously, it has been demonstrated that an antisense oligonucleotide (nucleotides 285-302) against the rat kidney sequence blocks expression of phenylalanine-inhibitable cystine transport in oocytes microinjected with rat jejunal mRNA (39,40,57). Therefore, I tested this oligonucleotide (which was one of the primers used in my PCR) for its ability to block oocyte expression of jejunal leucine transport activity. The antisense oligonucleotide had no effect on Na^+-dependent leucine influx, but reduced the smaller Na^+-independent component of expressed leucine transport activity by 40%. This was a specific effect because no inhibition was observed with the corresponding sense oligonucleotide used in the PCR. It is probable that the antisense-oligonucleotide-inhibited leucine flux corresponds to that part of Na^+-independent leucine transport which is sensitive to inhibition by alanine and phenylalanine. This component of transport (leucine system 1) is the smaller of the two Na^+-independent leucine transport activities and is $<10\%$ of the total expressed leucine influx. In contrast, Murer and co-workers (54) have recently reported that rBAT accounts for the majority of expressed Na^+-independent leucine transport activity (identified as system $b^{0,+}$) in oocytes microinjected with mRNA from rabbit jejunum.

The major component of leucine transport induced by rat jejunal mRNA was Na^+-dependent and has, in part, the characteristics of y^+L. As detailed in the Section A2.1, an apparently similar transport system exists in human erythrocytes and possibly also in placenta (45-47). It has also been reported that low-level system y^+L-like activity is also induced in oocytes by cRNA encoding the human 4F2hc glycoprotein (43,44). This protein, which is structurally related to D2/rBAT, is widely distributed in proliferating cells, but its occurrence in intestine is unknown. In parallel with my D2 experiments, I therefore used PCR to test for the presence of 4F2hc or 4F2hc-related sequences in rat jejunum. Using oligonucleotide primers designed from conserved regions of the mouse 4F2hc sequence, rat jejunal cDNA gave a PCR product of the predicted size. The encoded amino acid sequence was 82% identical to mouse pre-B cell/ macrophage 4F2hc and 71% identical to human SV40-transformed fibroblast 4F2hc. The extent to which

these differences reflect species variation and/or tissue-specific isoforms of the protein remains to be established. In contrast to the situation with hybrid depletion employing the D2 antisense oligonucleotide, the corresponding 4F2hc antisense oligonucleotide had no significant effect on expression of either Na^+-dependent or Na^+-independent leucine transport activity. Therefore, although 4F2 or a closely related protein is present in rat jejunum, it does not account for the induction of y^+L-type transport activity in oocytes microinjected with mRNA from that tissue. An important difference between the y^+L activity in human erythrocytes and that induced by rat jejunal mRNA is that the former is inhibited by phenylalanine, and to a lesser extent, alanine (45). The extent to which these two amino acids inhibit 4F2hc-induced amino acid transport activity has not been reported.

To address the question of whether or not I might be activating endogenous oocyte transporters by some other mechanism, I undertook an investigation of the characteristics of leucine transport in collagenase-treated oocytes injected with water instead of jejunal mRNA (Table A2.1). Although the majority of endogenous leucine transport was Na^+-dependent, phenylalanine and alanine were approximately half as effective as leucine and lysine in inhibiting the flux. This is in contrast to expressed leucine transport system 1 which was unaffected by the same concentration (1 mM) of phenylalanine (Figure A2.4). Endogenous Na^+-independent leucine transport differed from expressed leucine transport activity by being poorly inhibited by lysine. These differences, together with the known characteristics of leucine/lysine transport in the small intestine, are consistent with the conclusion that I have indeed expressed rat jejunal amino acid transporters in *Xenopus* oocytes.

The *Xenopus* oocyte system not only permits the functional characterisation of expressed transport activities but also provides a means of identifying and isolating cDNA clones encoding the transporter(s) responsible for these fluxes. Molecular cloning by functional expression in *Xenopus* oocytes was first applied to transport proteins in 1987 to clone the rabbit intestinal Na^+-linked glucose transporter (58). An increasing number of other transporters and ion channels have subsequently been cloned by this route (50,59-61). As described in this Appendix, I have size-fractionated total jejunal mRNA by sucrose gradient centrifugation and identified a size fraction (1.5-2.25 kb, peak 2.0 kb) inducing maximal leucine transport activity. Previously, it has been shown that the same size fraction encodes arginine-inhibitable lysine influx. Substantial leucine transport activity was also observed with mRNA of the size range 2.0-2.8 kb (peak 2.6 kb). Both size ranges are consistent with that expected for integral membrane proteins which function as transporters (62-65).

In summary, I have expressed multiple rat jejunal leucine/lysine transporters in *Xenopus* oocytes. The dominant leucine transport activity has, in part, the characteristics of amino acid transport system y^+L. In contrast to previous reports of induction of neutral/cationic amino acid transport activity in *Xenopus* oocytes by kidney or intestinal mRNA, the expressed transport activity does not seem to be related to either the D2/rBAT or 4F2hc glycoproteins, both of which I have shown to be present in rat jejunum.

TABLE A2.1. Inhibition of L-leucine uptake in oocytes injected with water

(pmol/oocyte.10min^{-1})

Inhibitor	NaCl medium	Choline Chloride medium	Difference
None	10.5 ± 0.5	0.90 ± 0.09	9.6 ± 0.50
L-ala	7.70 ± 0.1	0.63 ± 0.09	7.1 ± 0.13
L-phe	7.38 ± 0.3	0.75 ± 0.06	6.6 ± 0.31
L-leu	1.70 ± 0.1	0.40 ± 0.08	1.3 ± 0.13
L-lys	3.43 ± 1.8	0.86 ± 0.27	2.6 ± 1.82

Various nonradioactive amino acids were tested as inhibitors of L-[^{14}C]leucine transport (0.2 mM) in oocytes injected with water at 20°C (10 min incubation). Nonradioactive amino acids and [^{14}C]leucine were added to oocytes simultaneously. Values are means (±SEM).

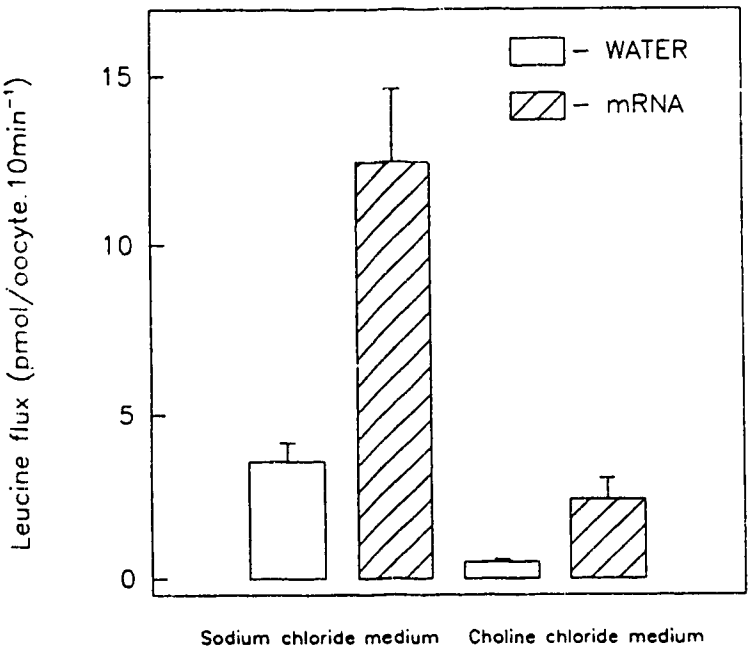


Figure A2.1. Influx of L-leucine into isolated oocytes of *Xenopus laevis*. Oocytes were injected with either 50nl of rat jejunal mRNA (1ng/nl) or 50nl of water and incubated for 5 days at 18°C in MBM. Fluxes (0.2mM leucine, 10 min at 20°C) were performed in transport buffer containing 100 mM NaCl or 100 mM choline chloride. Values are means ± SEM. All oocytes came from one frog.

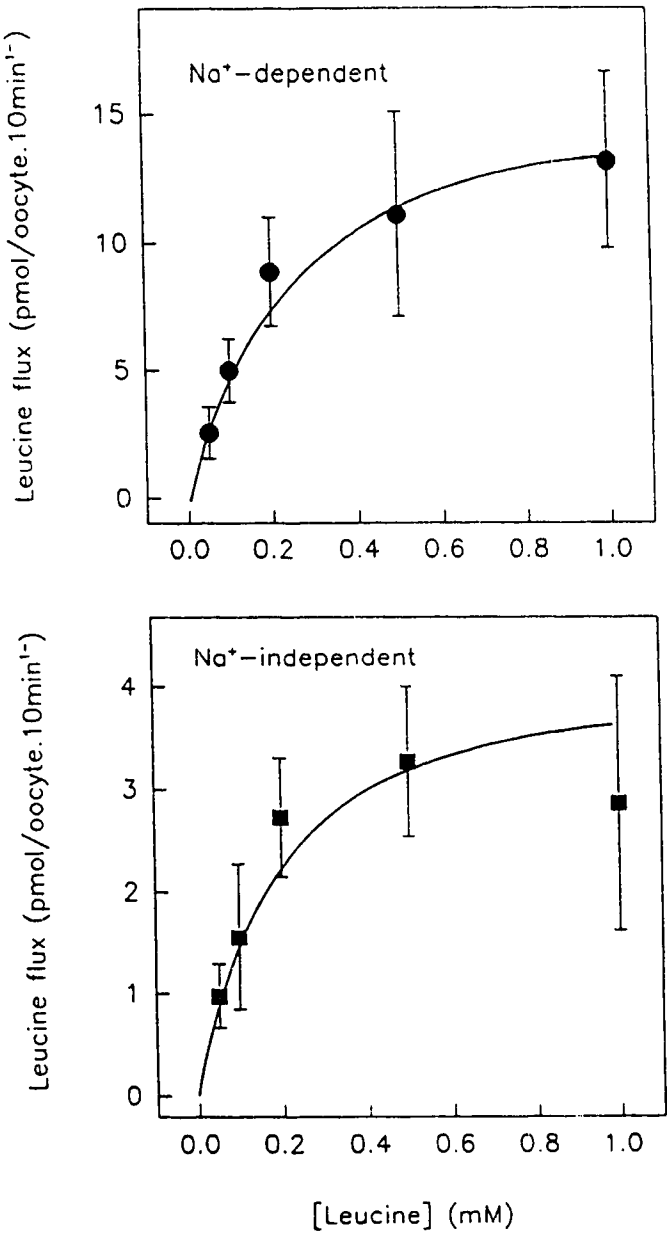


Figure A2.2. Concentration dependence of induced Na^+-dependent and Na^+-independent L-leucine influx in mRNA-injected oocytes. Na^+-independent influx of leucine (10 min, 20°C) was measured in choline chloride medium, while the Na^+-dependent component of influx was calculated as the difference in uptake in NaCl and choline chloride medium. Values are means ± SEM and have been corrected for endogenous leucine influx measured in water-injected oocytes. Please see text for other experimental details.

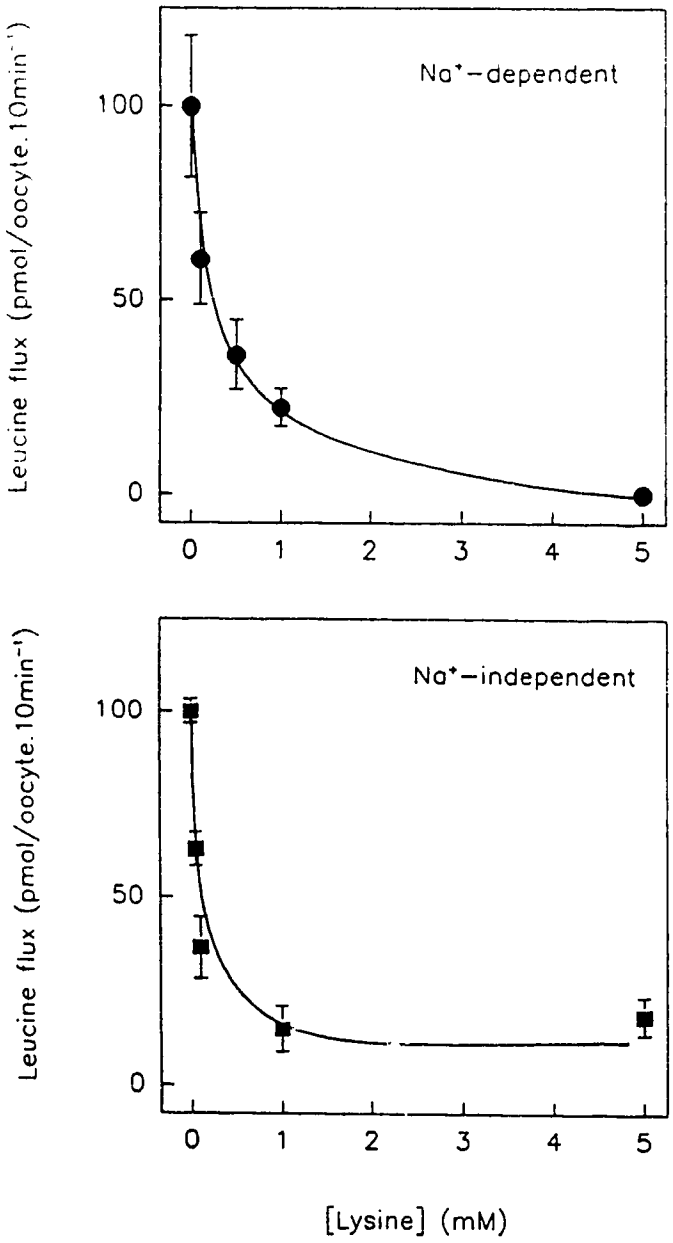


Figure A2.3. L-Lysine inhibition of induced Na^+-dependent and Na^+-independent L-leucine influx in oocytes injected with rat jejunal mRNA. Influx of 0.2 mM leucine (10 min, 20°C) was measured in NaCl and choline chloride medium in the absence or in the presence of various concentrations of nonradioactive lysine (0.25-5 mM). Values (± SEM) for the Na^+-dependent and Na^+-independent components of induced leucine influx, corrected for the contribution of endogenous Na^+-dependent and Na^+-independent leucine uptake, were calculated as described in the legend to Figure A2.2 and are expressed as a percentage of the control uptake in the absence of lysine. Nonradioactive lysine was added to oocytes at the same time as permeant.

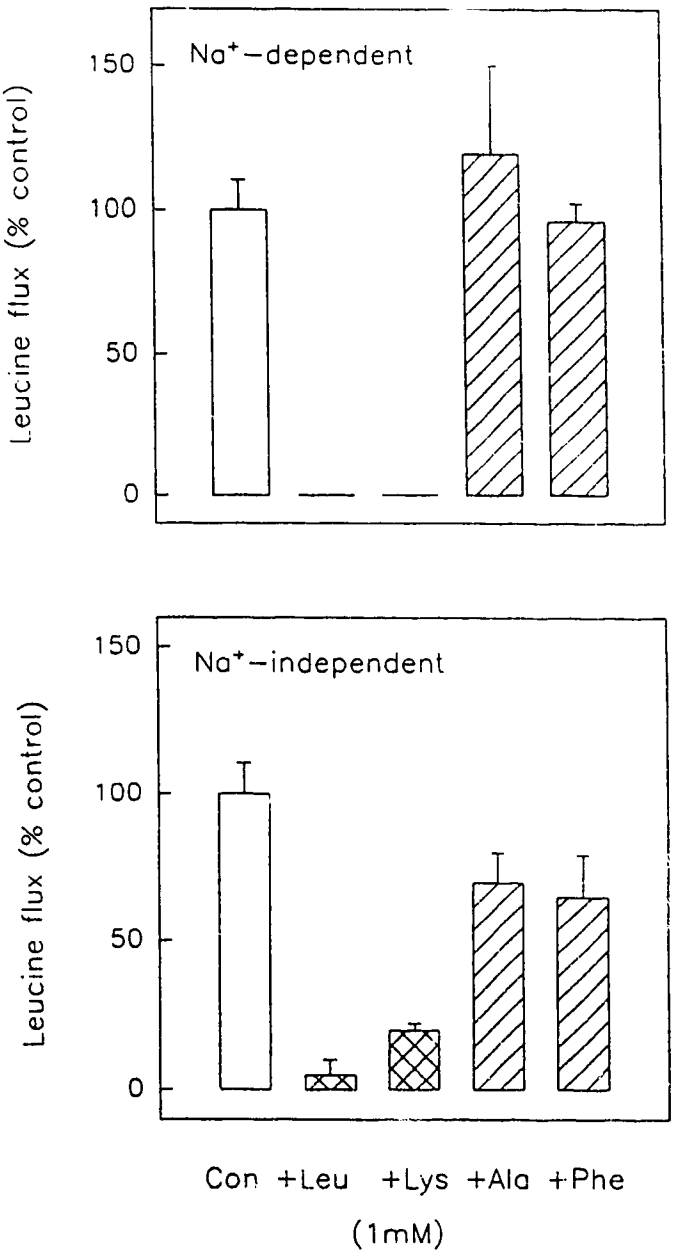


Figure A2.4. Amino acid inhibition of induced Na^+-dependent and Na^+-independent L-leucine influx in oocytes injected with rat jejunal mRNA. Influx of 0.2 mM leucine (10 min, 20°C) was measured in the absence or in the presence of 1 mM nonradioactive amino acids and corrected for the contribution of endogenous Na^+-dependent and Na^+-independent leucine uptake as described in the legend to Figure A2.2. Values (± SEM) are expressed as a percentage of the control uptake measured in the absence of inhibitor.

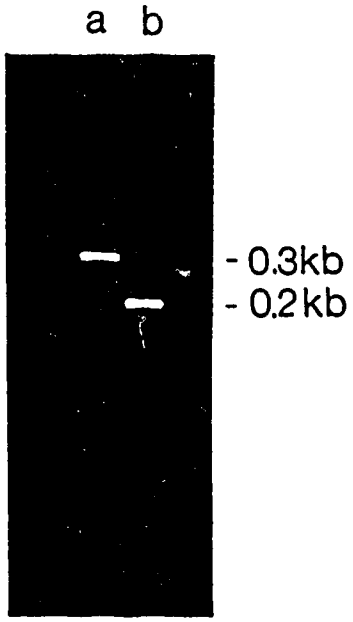


Figure A2.5. An agarose gel (2%(w/v)) of the 4F2hc and D2 PCR products from rat jejunum cDNA. Lanes (a) and (b) contain the PCR products amplified from rat jejunal cDNA with 4F2hc primers and D2 primers, respectively. Please see text for other experimental details.

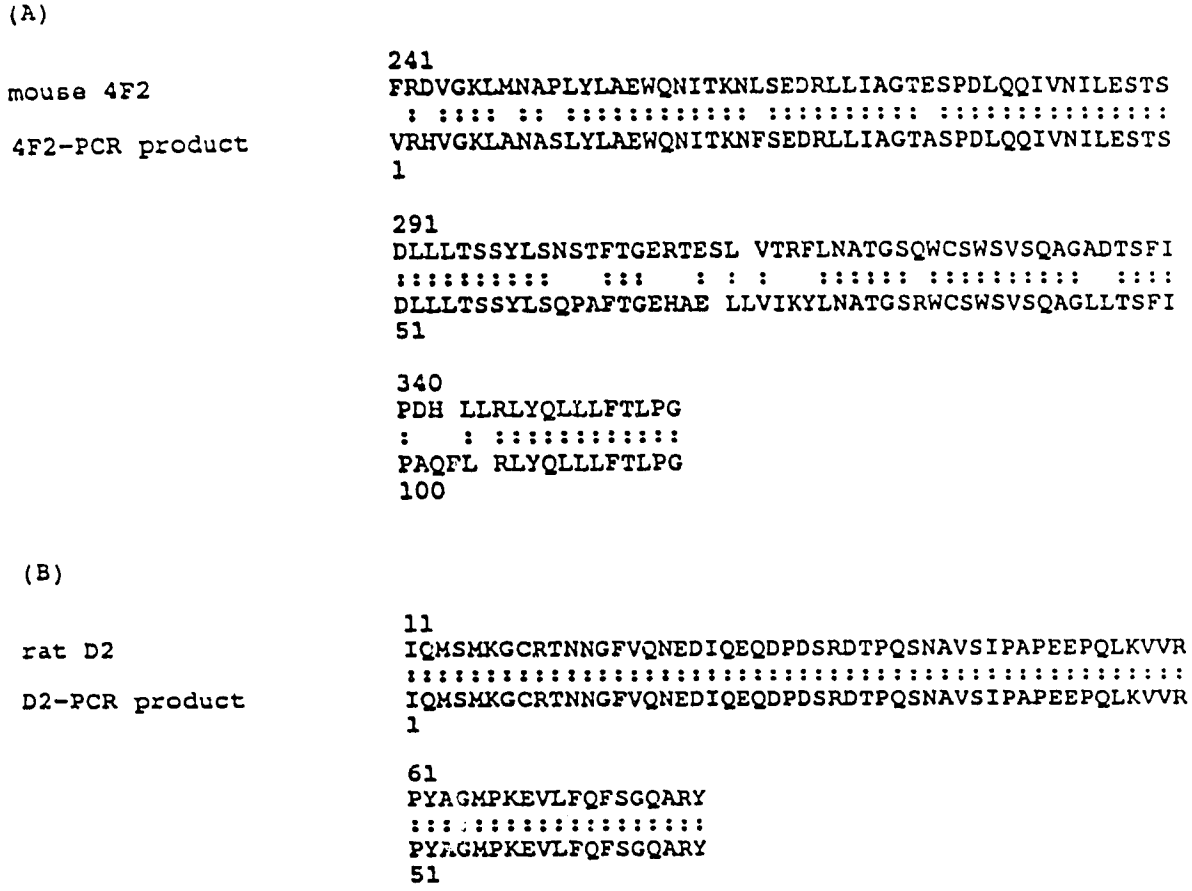


Figure A2.6. Deduced amino acid sequences of the 4F2hc and D2 PCR products from rat jejunum. (A) Alignment of the amino acid sequences (one letter code) of mouse pre-B cell/macrophage 4F2hc (top) and the rat jejunal 4F2hc-PCR product (bottom). (B) Alignment of amino acid sequences of rat kidney D2 (top) and the rat jejunal D2-PCR product (bottom). Gaps are indicated by spaces.

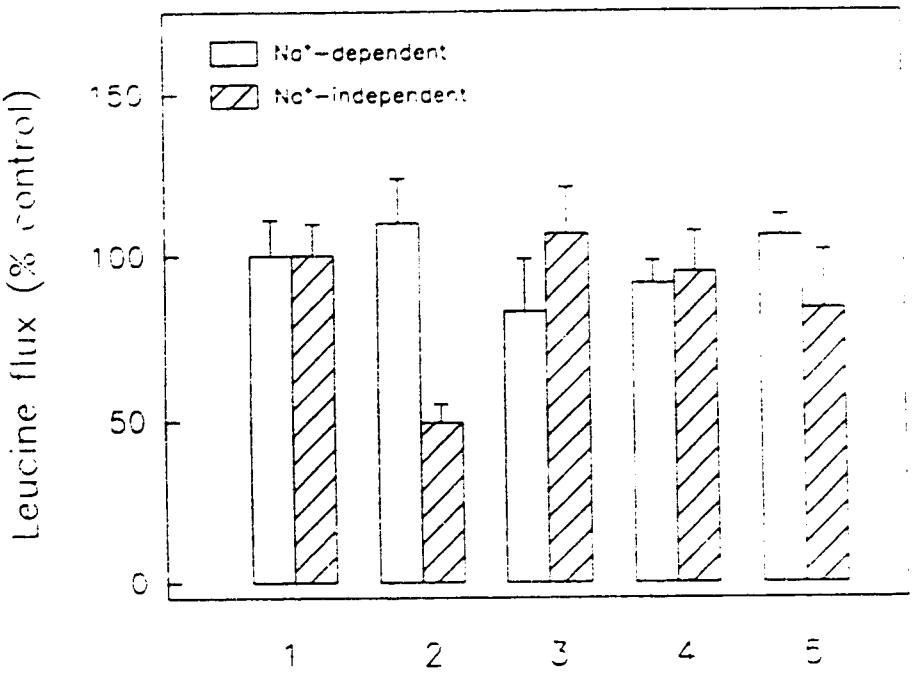


Figure A2.7. Effects of antisense hybrid-depletion on the expression of Na^+-dependent and Na^+-independent L-leucine influx in oocytes injected with rat jejunal mRNA. Rat jejunal mRNA was hybridized with the following oligonucleotides: (lane 2) antisense oligomers for D2; (lane 3) sense oligomers for D2; (lane 4) antisense oligomers for 4F2hc; (lane 5) sense oligomers for 4F2hc. The mixtures were injected into oocytes and assayed for expression of Na^+dependent and Na^+-independent leucine influx (0.2 mM, 10 min at 20°C) as described in the legend to Figure A2.2. Values (± SEM) were corrected for the contribution of endogenous Na^+-dependent and Na^+-independent leucine influx and are expressed as a percentage of the control uptake obtained from oocytes injected with rat jejunal mRNA (lane 1).

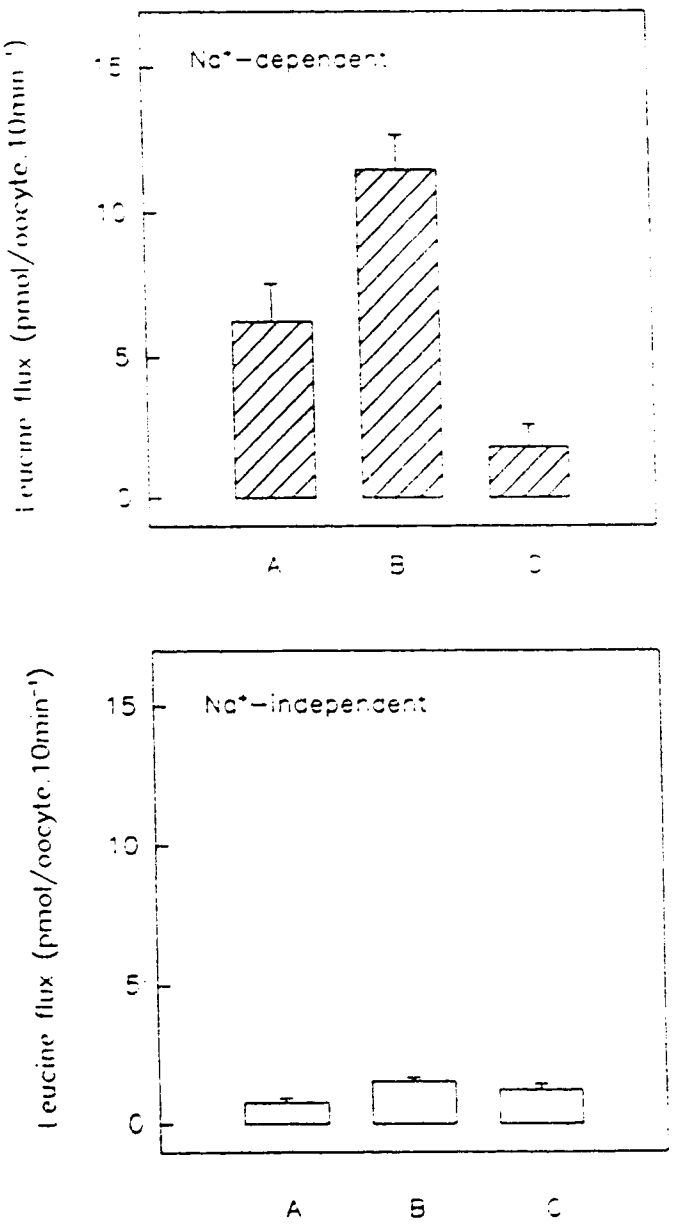


Figure A2.8. Expression of Na^+-dependent and Na^+-independent influx of L-leucine in oocytes injected with size-fractionated rat jejunal mRNA. Three mRNA fractions with size ranges (A) 2.0-2.8kb, (B) 1.5-2.25kb, (C) 0.75-1.6kb, estimated by laser densitometry, were injected into oocytes and assayed for expression of Na^+-dependent and Na^+-independent leucine influx (0.2 mM for 10 min at 20°C) as described in the legend to Figure A2.2. Values are means ± SEM and have been corrected for the contribution of endogenous Na^+-dependent and Na^+-independent leucine influx.

A2.5. BIBLIOGRAPHY

1. Munck, B.G. (1980). Lysine transport across the small intestine. *J. Memb.Biol.* **53**, 45-53.

2. Mircheff, A.K., Kippen, I., Hirayama, B, and Wright, E.M. (1982). Delineation of sodium-stimulated amino acid transport pathways in rabbit kidney brush border vesicles. *J. Memb. Biol.* **64**, 113-122.

3. Cassano, G., Leszczynska, B., and Murer, H. (1983). Transport of L-lysine by rat intestinal brush border membrane vesicles. *Pflugers Arch.* **397**, 114-120.

4. Munck, B.G. (1983). Comparative aspects of amino acid transport in guinea pig, rabbit and rat small intestine. In *Intestinal Transport*, (eds. M. Gilles-Ballien and R. Gilles), Springer, Berlin, pp.260-263.

5. Lawless, K., Maenz, D., and Cheeseman, C. (1987). Is leucine an allosteric modulator of the lysine transporter in the intestinal basolateral membrane? *Am. J. Physiol.* **253**, G637-642.

6. Munck, L.K. and Munck, B.K. (1992). Variation in amino acid transport along the rabbit small intestine. Mutual jejunal carriers of leucine and lysine. *Biochim. Biophys. Acta* **1116**, 83-90.

7. Christensen, H.N. (1979). Exploiting amino acid structure to learn about membrane transport. *Adv. Enzymol.* **49**, 41-101.

8. Christensen, H.N. (1984). Organic ion transport during seven deades the amino acid. *Biochim. Biophys. Acta* **779**, 255-269.

9. Young, J.D., Jones, S.E.M., and Ellory, J.C. (1981). Amino acid transport in human and in sheep erythrocytes. *Proc. R. Soc. B* **209**, 355-375.

10. Christensen, H.N., and Antonioli, J.A. (1969). A bicyclic amino acid to improve discriminations among transport systems. *J. Biol. Chem.* **244**, 1497-1504.

11. Christensen, H.N., and Handlogten, M.E. (1969). Reactions of neutral amino acid plus Na^+ with a cationic amino acid transport system *FEBS Letters* **3**, 14-17.

12. Christensen, H.N., Handlogten, M.E., and Thomas, E.L. (1969). Na^+-facilitated reactions of neutral amino acids with a cationic amino acid transport system. *Proc. Natl. Acad. Sci. U.S.A.* **63**, 948-955.

13. Thomas, E.L., Shao, T.C., and Christensen, H.N. (1971). Structural selectivity in interaction of neutral amino acids and alkali metal ions with a cationic amino acid transport system. *J. Biol. Chem.* **246**, 1677-1681.

14. VanWinkle, L.J., Campione, A.L., and Gorman, J.M. (1988). Na^+-independent transport of basic and zwitterionic amino acids in mouse blastocysts by a shared system and by processes which distiguish between these substrates. *J. Biol. Chem.* **263**, 3063.

15. VanWinkle, L.J., Campione, A.L., and Gorman, J.M. (1990). Inhibition of transport system $b^{0,+}$ in blastocysts by inorganic and cations yields insight into the structure of its amino acid receptor site. *Biochim. Biophys. Acta* **1025**, 215-233.

16. VanWinkle, L.J., Campione, A.L., and Farrington, B.H. (1990). Development of system $B^{0,+}$, and a broad-scope Na^+-dependent transporter of zwitterionic amino acids in preimplantation mouse conceptuses. *Biochim. Biophys. Acta* **1025**, 225-233.

17. White, M.F. (1985). The transport of cationic amino acids across the plasma membrane of mammalian cells. *Biochim. Biophys. Acta* **822**, 355-374.

18. Stevens, B.R., Ross, H.J., and Wright, E.M. (1982). Mutiple transport pathways for neutral amino acids in rabbit jejunal brush border vesicles *J. Memb. Biol.* **66**, 213-225.

19. Silbernagl, S. (1988). The renal handling of amino acids and oligopeptides. *Physiol. Rev.* **68**, 911-1007.

20. Wolfram, S., Giering, H., and Scharrer, E. (1984). Effect of feeding a high protein diet on amino acid uptake into rat intestinal brush border membrane vesicles. *Comp. Biochem. Physiol.* **78A**, 475-480.

21. Gustella, J., Nelson, N., Nelson, H., Czyzyk, L., Keyman, S., Miedel, M. C., Davidson, N., Lester, H.A., and Kanner, B.I. (1990). Cloning and expression of a rat brain GABA transporter. *Science* **249**, 1303-1306.

22. Kim, J.W., Closs, E.I., Albritton, L.M., and Cunningham, J.M. (1991). Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature* **352**, 725-728.

23. Wang, H., Kavanaugh, M.P., North, A., and Kabat, D. (1991). Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter. *Nature* **352**, 729-731.

24. Kanai, Y., and Hediger, M.A. (1992). Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature* **360**, 467-471.

25. Liu, Q.R., Lopez-Corcuera, B., Nelson, H., Mandiyan, S., and Nelson, N. (1992) Cloning and expression of a cDNA encoding the transporter of taurine and ß-alanine in mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 12145-12149.

26. Liu, Q.R., Lopez-Corcuera, B., Mandiyan, S., Nelson, H., and Nelson, N. (1993). Molecular characterization of four pharmacologically distinct γ-aminobutyric acid transporters in mouse brain. *J. Biol. Chem.* **268**, 2106-2112.

27. Pines, G., Danbolt, N.C., Bjoras, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-Mathison, J., Seeberg, E., and Kanner, B. I. (1992). Cloning and expression of a rat brain L-glutamate transporter. *Nature* **360**, 464-467.

28. Smith, K.E., Borden, L.A., Hartig, P.R., Branchek, T., and Weinshank, R. L. (1992). Cloning and expression of a glycine transporter reveal colocalisation with NMDA receptors. *Neuron* **8**, 927-935.

29. Storck, T., Schulte, S., Hofmann, K., and Stoffel, W. (1992). Structure, expression and functional analysis of a Na^+-dependent glutamate, aspartate transporter from rat brain. *Proc. Natl. Acad. Sci., U.S.A.* **89**, 10955-10959.

30. Amara, S.G., and Kuhar, M.J. (1993). Neurotransmitter transporters: recent progress. *Ann. Rev. Neuro.* **16**, 73-93.

31. Kong, C-T., Yet, S.F., and Lever, J.E. (1993). Cloning and expression of a mammalian Na^+/amino acid cotransporter with sequence similarity to Na^+/glucose cotransporters. *J. Biol. Chem.* **268**, 1509-1512.

32. Tanaka, J.I., and Fink, G.R. (1985). The histidine permease gene (HIP1) of *Saccharomyces cerevisiae*. *Gene* **38**, 205-214.

33. Hoffmann, W. (1985). Molecular characterisation of the CAN1 locus in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **260**, 11831-11837.

34. Vandenbol, M., Jauniaux, J.C., and Grenson, M. (1989). Nucleotide sequence of the *Saccharomyces cerevisiae* PUT4 proline-permease-encoding gene: similarities between CAN1, HIP1 and PUT4 permeases. *Gene* **83**, 153-159.

35. Jauniaux, J.C., and Grenson, M. (1990). GAP1, the general amino acid permease gene of *Saccharomyces cerevisiae*-nucleotide sequence protein similarity

with the other baker yeast amino acid permeases and nitrogen catabolite repression. *Euro. J. Biochem.* **190**, 39-44.

36. Albritton, L.M., Tseng, L., Scadden, D., and Cunningham, J.M. (1989). A putative murine ecotropic reteovirus receptor gene encodes a mutiple membrane-spanning protein and confers susceptibility to virus infection. *Cell* **57**, 659-666.

37. Puppi, M., Noel, R.A., and Henning, S.J. (1992). Expression of the mRNA for the Na-dependent cationic amino acid transport. in the rat small intestine. *Physiologist* **35**, A-15.

38. Kate, S.S., Yan, N., and Udenfriend, S. (1992). Expression cloning of a Na^+-independent neutral amino acid transporter from rat kidney. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1-5.

39. Wells, R.G., and Hediger, M.A. (1992). Cloning of a rat kidney cDNA that stimulates dibasic and neutral amino acid transport and has sequence similarity to glycosidases. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5596-5600.

40. Bertran, J., Werner, A., Moore, M. L., Stange, G., Markovich, D., Biber, J., Testar, A., Zorzano, A., Palacin, M., and Murer, H. (1992). Expression cloning of a cDNA from rabbit kidney cortex that induces a single transport system for cystine and dibasic neutral and amino acids. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5601-5605.

41. Teixeira, S., CiGrandi, S., and Kuhn, L.C. (1987). Primary structure of human 4F2 antigen heavy chain predicts a transmembrane protein with a cytoplasmic NH_2 terminus. *J. Biol. Chem.*, **262**, 9574-9580.

42. Parmacek, M.S., Karpinski, B.A., Gottasdiener, K.M., Thompson, C.B., and Leiden, J.M. (1989). Structure, expression and regulation of murine 4F2 heavy chain. *Nucleic Acids Res.* **17**, 1915-1931.

43. Bertran, J., Magagnin, S., Weiner, A., MArkovich, D., Biber, J., Testar, X., Zorzano, A., Kuhn, L.C., Palacin, M., and Murer, H. (1992). Stimulation of system y^+-like amino acid transport by the heavy chain of human 4F2 surface antigen in *Xenopus laevis* oocytes. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5606-5610.

44. Wells, R.G., Lee, W.-S., Kanai, Y., Leiden, J.M., and Hedison, M.A. (1992). The 4F2 antigen heavy chain induces uptake of neutral and dibasic amino acids in *Xenopus* oocytes. *J. Biol. Chem.* **267**, 15285-15288.

45. Devés, R., Chávez, P., and Boyd, C.A.R. (1992). Identification of a new transport system (y^+L) in human erythrocytes that recognizes lysine and leucine with high affinity. *J. Physiol.* **454**, 491-501.

46. Lo, A.W.I., Qureshy, S.A., Harvey, C.M., and Young, J.D. (1993). Cation and neutral amino acid interactions with a novel lysine transporter (system y^+L) in human erythrocytes. *Biochem. Cell Biol.* **71**, Axiv.

47. Eleno, N., Devés, R., and Boyd, C.A.R. (1992). Discrimination between two lysine transporters in human placental brush-border membrane vesicles by altering membrane potential. *J. Physiol.* **452**, 349P.

48. Furesz, T.C., Moe, A.J., and Smith, C.H. (1991). Two cationic amino acid transport systems in human placental basal plasma membranes. *Am. J. Physiol.* **261**, C246-C252.

49. Takumi, T., Ohkubo, H., and Nakanishi, S. (1988). Cloning of a membrane protein that induces a slow voltage-gated potassium current. *Science* **242**, 1042-1045.

50. Paulmichl, M., Li, Y., Wickman, K., Ackerman, M., Peralta, E., and Clapham, D. (1992). New mammalian chloride channel identified by expression cloning. *Nature* **356**, 238-241.

51. Campa, M.J., and Killerg, M.S. (1989). Characterization of neutral and cationic amino acid transport in *Xenopus* oocytes. *J. Cell. Physiol.* **141**, 238-241.

52. Mackenzie, B., Taylor, P.M., Harper, A.A., and Rennie, M.J. (1993). Ion dependence and transport stoichiometry of system $B^{0,+}$ amino acid transport in *Xenopus* oocytes determined by tracer uptakes and evoked currents. *J. Physiol.* **459**, 40P.

53. Kanai, Y., Stelzner, M.G., Lee, W.-S., Wells, R.G., Brown, D., and Hediger, M.A. (1992). Expresion of mRNA (D2) encoding a protein involved in amino acid transport in S3 proximal tubule. *Am. J. Physiol.* **263**, F1087-1093.

54. Magagnin, S., Bertran, J., Werner, A., Biber, J., Palacin, M., and Murer, H. (1992). Poly(A)$^+$ RNA from rabbit intestinal mucosa induces $b^{0,+}$ and y^+ amino acid transport activities in *Xenopus laevis* oocytes. *J. Biol. Chem.* **267**, 15384-15390.

55. Harvey, C.M., Muzyka, W.R., Yao, S.Y.M., Cheeseman, C.I., and Young, J. D. (1993). Expression of rat intestinal L-lysine transport systems in isolated oocytes of *Xenopus laevis*. *Am. J. Physiol.* **265**, G99-106.

56. McNamara, P.D., Rea, C.T., and Segal, S. (1991). Expression of rat jejunal cystine carrier in *Xenopus* oocytes. *J. Biol. Chem.* **266**, 986-989.

57. Hediger, M.A., Coady, M.J., Ikeda, T.S., and Wright, E.M. (1987). Expression cloning and cDNA sequencing of the Na^+/glucose co-transporter. *Nature* **330**, 379-381.

58. Hagenbuch, B., Stieger, B., Foguet, M., Lubbert, H., and Meir, P.J. (1991). Functional expression cloning and characterization of the hepatocyte Na^+/bile acid cotransport system. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10629-10633.

59. Kwon, H.M., Yamauchi, A., Uchida, S., Pre[illegible]n, A.S., Garcia-Perez, A., Burg, M.B., and Handler, J.S. (1992). Cloning o[illegible]e cDNA for a Na^+/myo-inositol cotransporter, a hypertonicity stress protein. *J. Biol. Chem.* **267**, 6297-6301.

60. Gamba, G., Miyanoshita, A., Lombardi, M., and Herbert, S.C. (1992). Cloning, functional characterization and tissue distribution of the thiazide-sensitive Na^+:Cl^- transporter of the winter flounder (*Pseudopleuronectes americanus*) urinary bladder. *J. Gen. Physiol.* **100**, 8a

61. Hediger, M.A., Ikeda, T., Coady, M.J., Gendersen, C.B., and Wright, E.M. (1987). Expression of size-selected mRNA encoding the intestinal Na[illegible]ucose cotransporter in *Xenopus laevis* oocytes. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2634-2637.

62. Su, T.Z., Logsdon, C.D., and Oxander, D.L. (1992). Chinese hamster ovary mRNA-dependent, Na^+-independent L-leucine transport in *Xenopus* oocytes. *Mol. Cell. Biol.* **5**, 4470.

63. Tarnuzzer, R.W., Campa, M.J., Qian, N.X., Engelberg, E., and Kilberg, M. (1990). Expression of the mammalian system A neutral amino acid transporter in *Xenopus* oocytes. *J. Biol. Chem.* **265**, 13914-13917.

64. Coady, M.J., Pajor, A.M., Toloza, E.M., and Wright, E.M. (1990). Expression of mammalian renal transporters in *Xenopus laevis* oocytes. *Arch. Biochem. Biophys.* **283**, 130-134.

65. Baxter-Gabbard, K.L. (1972). A simple method for the large-scale preparation of sucrose gradients. *FEBS Letters* **20**, 117-119.