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Nucleoside Transporters in Mammalian Cells

by

Marci Eileen Schaner

DISSERTATION

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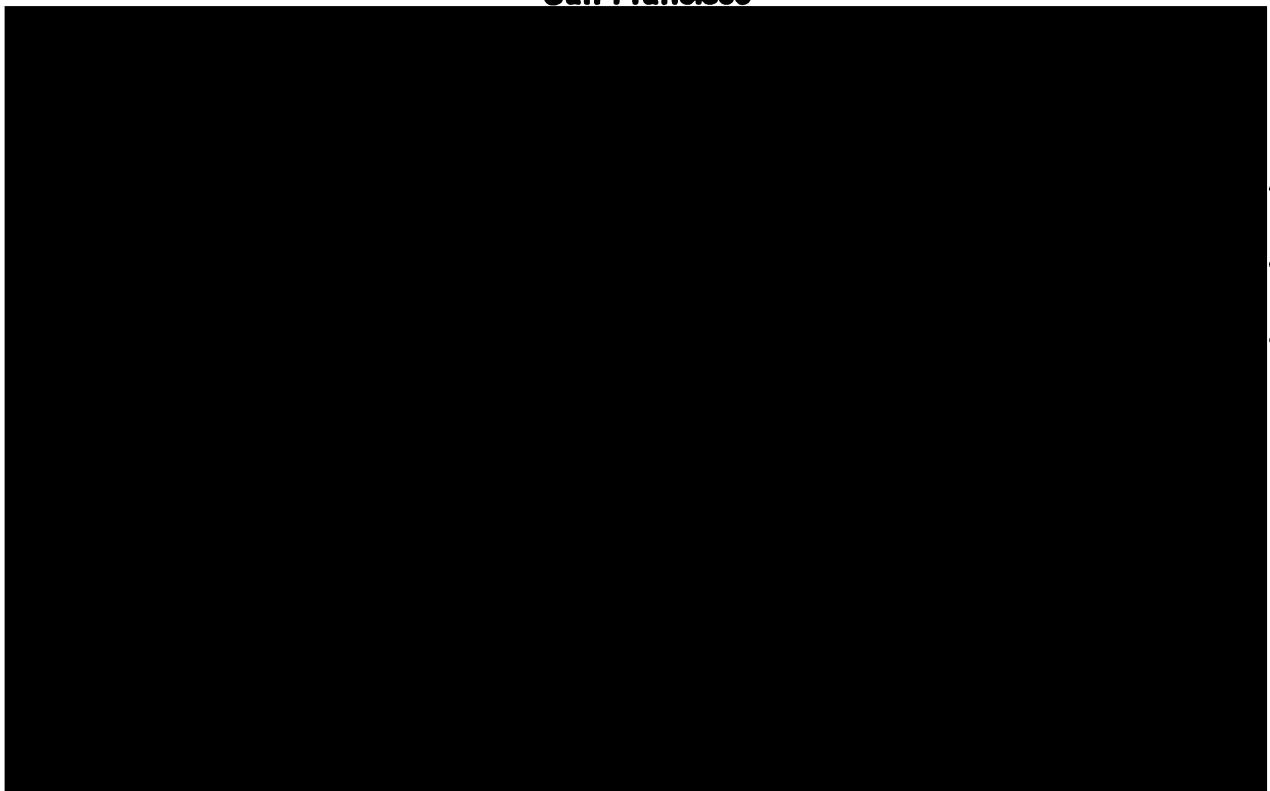
In

Pharmaceutical Chemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

In memory of my Grandparents

Dr. Morris Schaner and Minnie Schaner

and my Great-grandfather

John Autilio

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ABSTRACT

NUCLEOSIDE TRANSPORTERS IN MAMMALIAN CELLS

Marci Eileen Schaner

Nucleosides and nucleoside analogs exert many physiological and clinical effects and are important therapeutic agents in cancer and antiviral chemotherapy. Because these compounds are hydrophilic, nucleoside transporters are critical for the movement of nucleosides across cells membranes. During the course of this dissertation research, there was significant growth in the field of nucleoside transport; specifically a number of mammalian nucleoside transporters were cloned. The overall goal of the research presented in this dissertation was to characterize both endogenous and cloned nucleoside transport processes in mammalian cells and tissues.

Studies in the first section of this dissertation describe the establishment of a mammalian expression system for cloned nucleoside transporters. The second section is focused on the use of this expression system to determine the kinetics and functional characteristics of cloned nucleoside transporters. In the final section, antibodies to study the transport proteins are described. The development and application of a transient expression system for the purine-selective nucleoside transporter, SPNT, in HeLa cells enabled kinetic characterization and the rapid screening of a number of nucleoside analogs for interaction with the cloned rat purine-selective nucleoside transporter. Next, this mammalian expression system was modified and applied to study the kinetics and drug interactions of the cloned human homolog of SPNT, hSPNT1. Interestingly, species differences between the rat and human homologs of SPNT were observed. The development of an epitope-tagged, pyrimidine-selective nucleoside transporter, cNT1.tag, that may have potential applications in the study of the trafficking and membrane topology of this transporter is described. Finally, antibodies specific for the cloned nucleoside transporters, SPNT and cNT1 were developed and the initial characterization of the cNT1 anti-peptide antibody is presented.

Kathleen M. Giacomini

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CHAPTER 1

NUCLEOSIDE TRANSPORTERS IN MAMMALIAN CELLS

Overall Goal

The overall goal of the research presented in this dissertation was to characterize nucleoside transport processes in mammalian cells and tissues. During the course of this research, the field of nucleoside transport underwent significant growth as the cDNAs encoding nucleoside transporters were isolated and the pool of molecular information grew accordingly. A review of the changes in the field of nucleoside transport is presented following a brief background defining the subtypes of nucleoside transporters. Next, the functional and molecular information detailing the different subtypes of nucleoside transporters is reviewed. Third, a review of the studies using expression systems and antibodies to study nucleoside transporters is presented. Finally, a summary and brief synopsis of each chapter is presented.

Background

Nucleosides and nucleoside analogs produce many physiological and clinical effects. Currently, nucleoside analogs, including zidovudine (AZT), lamivudine (3TC), and didanosine (ddI), and zalcitabine (ddC), are important drugs used in the treatment of patients infected with HIV (1, 2) and are often used in combination (2) with protease inhibitors (3). Nucleoside analogs are also clinically important in the treatment of a number of opportunistic infections associated with AIDS (Autoimmune Deficiency Syndrome) such as cytomegalovirus and herpes simplex virus (4, 5), and have been shown to be effective in the prevention of maternal-fetal transfer of HIV infection (1). Other nucleoside analogs, such as, cladribine (2CdA), gemcitabine (2',2'-difluorodeoxycytidine, dFdC), and fludarabine (FAMP) are important therapeutic agents in cancer chemotherapy (6, 7). The

endogenous nucleoside, adenosine, has significant cardiac effects, and is used clinically in the treatment of cardiac arrhythmias (8-11) (Table 1).

Because nucleosides are hydrophilic, specialized transporters are necessary for the movement of these compounds across cell membranes (12) (Figure 1, Table 2). Nucleoside transporters are broadly distributed in many tissues in the body, including choroid plexus (13, 14), kidney (15, 16), liver (17), intestine (18) and placenta (19, 20). These carrier proteins may be important in both the absorption and elimination of nucleosides in the body. Nucleoside transporters are critical for the salvage of endogenous purines and pyrimidines in the body, particularly in tissues devoid of *de novo* pathways for nucleoside synthesis, such as brain and muscle (21).

Two major classes of plasma membrane nucleoside transporters, in mammalian cells, have been described in the literature: equilibrative (12, 22, 23) and concentrative (13, 14, 16, 24-26). Equilibrative transporters are facilitative carriers that move substrates down their concentration gradient. This class of nucleoside transporters is further divided into two subtypes. Equilibrative sensitive (*es*), can be inhibited by nanomolar concentrations of the thiopurine, nitrobenzylthioinosine (NBMPR), whereas equilibrative insensitive (*ei*) is not sensitive to nanomolar concentrations of NBMPR. The concentrative nucleoside transporters are secondary active, Na^+-dependent carriers that transport nucleosides uphill, against their concentration gradient and are also divided into several subtypes (N1-N5) which are discussed later in this Chapter in the section entitled 'Na$^+$-dependent nucleoside transporters' (Table 2). Many clinically used nucleosides and nucleoside analogs interact with these transport proteins, however the underlying molecular mechanisms responsible for this interaction remain to be elucidated.

Table 1. Clinically used Nucleosides and Nucleoside Analogs

Acycloguanosine (acyclovir)

Ganciclovir

Azidothymidine (AZT, zidovudine)

2', 3'- Dideoxyinosine (ddI)

2', 3'- Dideoxycytidine (ddC)

Cytarabine (AraC)

2-Chloro-2'-deoxyadenosine(2CdA, Cladribine)

Lamivudine (3TC)

Floxidine (5-Fluoro-2'-deoxyuridine)

Adenosine

3

Figure 1. Structures of Nucleosides and Nucleoside Analogs

NUCLEOSIDES

PURINES
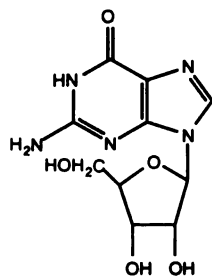
Guanosine
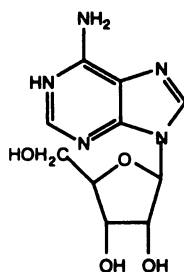
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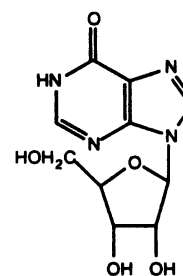
Inosine

PYRIMIDINES
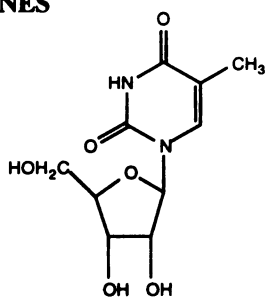
Thymidine
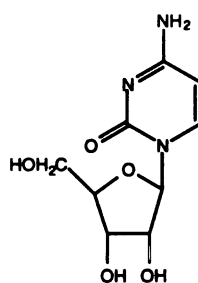
Cytidine
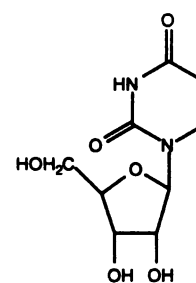
Uridine

NUCLEOSIDE ANALOGS

ddC
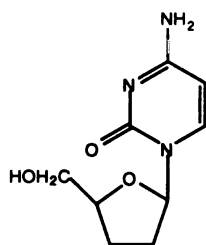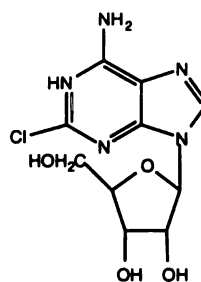
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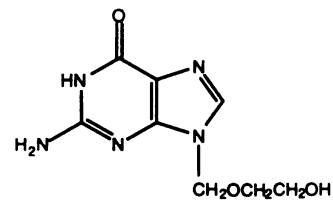
Acyclovir
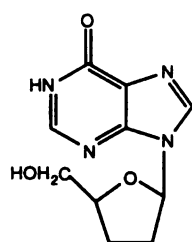
ddI
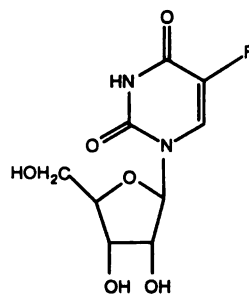
5-fluorouridine

AZT

4
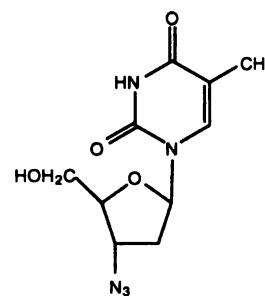
Table 2. Characterized Nucleoside Transport Systems

Transporter	Substrate selectivity	Sensitivity to NBMPR	Na$^+$ Dependency
N1	purine nucleosides, uridine	-	+
N2	pyrimidine nucleosides, adenosine	-	+
N3	purine and pyrimidine nucleosides	-	+
N4	pyrimidine nucleosides, guanosine, adenosine	-	+
N5	*adenosine, formycin B	+	+
es	purine and pyrimidine nucleosides	+	-
ei	purine and pyrimidine nucleosides	-	-

*limited data available

Overview of Recent Advances in the Field of Nucleoside Transport

There has been a dramatic increase in the information available on the molecular characteristics of nucleoside transporters in the past five years. In 1992, a Na^+-dependent, broadly selective nucleoside transporter, SNST1, was cloned from rabbit kidney by Pajor and Wright using a homology cloning approach, with probes to the Na^+-glucose transporters (27). The cDNA encoding SNST1 displayed significant sequence homology with the Na^+-glucose family of transporters (27). Subsequently, in 1994, Huang *et al.* employed a functional cloning approach, using *Xenopus laevis* oocytes, to isolate the cDNA encoding a Na^+-dependent, pyrimidine-selective nucleoside transporter, cNT1, from rat jejunum (28). In contrast to SNST1, this Na^+-dependent nucleoside transporter was not homologous to the Na^+-glucose transporters, but rather, displayed significant sequence homology to a previously cloned bacterial nucleoside transporter, nupC (28, 29). Another member of the cNT1, nupC family was identified in 1995, when a Na^+-dependent, purine-selective nucleoside transporter, SPNT, was cloned from rat liver (17) by expression cloning in *Xenopus laevis* oocytes. More recently, the human homologs of both SPNT (hSPNT1) and cNT1 (hCNT1) were cloned from human kidney (30, 31; Table 1) and the first human (20, 32) and rat (33) equilibrative nucleoside transporters were cloned. This laboratory reported information localizing the substrate binding region of the cloned transporters to transmembranes 8-9, giving new insight into the amino acids involved in substrate selectivity of nucleoside transporters (34). This increasing pool of information has created an exciting opportunity to dissect the underlying mechanisms of substrate binding and the driving forces of the transporters at the molecular level and to gain a greater understanding of functional data previously obtained.

Nucleoside Transporters

Na⁺-Dependent Nucleoside Transporters

There are two major subtypes of Na^+-dependent nucleoside transporters, purine and pyrimidine. In addition, several broadly- selective Na^+-dependent nucleoside transporters (N3-N5) have been described in the literature. The N1 subtype is purine-selective, and also transports uridine (17, 18) (Table 2). The rat cDNA encoding the N1-type nucleoside transporter was first described in 1995, and termed SPNT (17). Subsequently, its human homolog, hSPNT1 was cloned (30) (Table 3). Transcripts of SPNT (~ 2.9 kb) were present in the liver, jejunum, spleen, and heart, based on Northern blotting. Furthermore, two other transcripts (~6 and ~1 kb) were observed in brain, skeletal muscle, heart, jejunum, and spleen (Table 3) (17). Similarly, multiple transcripts of the human homolog, hSPNT1, were identified on Northern blotting. A large transcript (~4.4 kb) was identified in brain, heart, liver, skeletal muscle, kidney, intestine, pancreas, placenta, and lung. Two transcripts of expected size (2.6 and 2.4 kb) were detected in pancreas, kidney, liver, and skeletal muscle and in the intestine only the 2.6 kb band was detected. A small transcript (~1.6 kb) was also observed in heart and skeletal muscle (30). Functional characteristics of the cloned SPNTs are very similar to those of the N1-subtype previously characterized in tissue and cell studies.

The pyrimidine-selective subtype, N2, has also been cloned (cNT1 and hCNT1). In rat, transcripts of cNT1 were identified in kidney and jejunum (28). Tissue localization of the human homolog of cNT1, hCNT1, has not been determined to date. This subtype accepts pyrimidine nucleosides as well as adenosine. Both hSPNT1 and hCNT1 have been localized to human chromosome 15 (30, 31).

The broadly selective nucleoside transporter, N3, transports both purine and pyrimidine nucleosides. This broadly selective nucleoside transporter has been characterized in tissue slices from rabbit choroid plexus as well as in rabbit intestine (13, 14, 18). In 1992, Pajor and Wright (27) cloned a broadly selective nucleoside transporter,

Table 3. Characteristics of Cloned Nucleoside Transporters.

Name	Subtype	Tissue Distribution	Reference
cNT1	N2	intestine, kidney	(28)
SPNT	N1	liver, jejunum, spleen, heart	(17)
rENT1	*es*	intestine	(33)
hENT1	*es*	placenta	(32)
rENT2	*ei*	intestine	(33)
hCNT1	N2	kidney*	(31)
hSPNT1	N1	kidney, intestine, brain, heart, placenta	(30)
hENT2	*ei*	placenta, brain, ovarian tissue	(20)

* Extensive localization was not carried out

SNST1, using homology cloning to the Na⁺-glucose transporters. However, SNST1 does not display significant sequence homology to the cloned N1 and N2 subtypes. Furthermore, the cloned N1 and N2 subtypes do share significant sequence homology (17, 28, 30, 31). It is not clear if this cloned transporter represents the broadly selective nucleoside transporter previously characterized or if there is in fact a homologous member related to the cloned N1 and N2 subtypes. In recently published studies from this laboratory (34) it was determined that when transmembrane 8 (TM8) of N1 was transplanted into N2, the substrate-selectivity of N2 was switched from pyrimidine-selective, to broadly-selective (34). It remains to be determined whether a homologous member, related to N1 and N2, exists in nature. It is possible that the broadly-selective transporter "subtypes" represent polymorphisms or alternatively spliced variants of the cloned N1 and N2 transporters. Alternatively, the transporters may represent different gene products.

The other two subtypes of the Na⁺-driven nucleoside transporters are N4 and N5. N4 was characterized in human kidney and is similar to N2, except that it also interacts with guanosine (16, 26). The N5 subtype is the only member of the Na⁺-dependent nucleoside transporters which is inhibited by NBMPR (24).

Equilibrative nucleoside transporters

Until 1997, very little was known about the molecular identity of the equilibrative nucleoside transporters. The cloning of the *es* (hENT1) and *ei* (hENT2) subtypes of equilibrative nucleoside transporter from human placenta provided new information and confirmed that the two classes of nucleoside transporters, equilibrative and concentrative (Na⁺-dependent) do not share significant sequence homology (21, 30-33). The human placental hENT1 cDNA encodes a 456 amino acid glycoprotein which is predicted to have 11 membrane-spanning domains, with the N-terminus being cytosolic and the C-terminus extracellular (32). The protein encoded by hENT2 cDNA is 46% homologous to hENT2

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and is also 456 amino acids long (21). The rat homologs of both hENT1 and hENT2 (rENT1 and rENT2, respectively) have also been cloned (33).

Expression Systems Available for the Study of Cloned Nucleoside Transporters.

Xenopus laevis oocytes

The use of *Xenopus laevis* oocytes in the functional expression of Na^+-dependent nucleoside transporters (15, 24, 35-38) and its application to the cloning and subsequent functional characterization of the Na^+-dependent nucleoside transporters, cNT1 and SPNT (17, 28) has proven an invaluable tool. Briefly, either mRNA or cRNA (mRNA obtained by transcription of cDNA) is injected into the oocytes and the protein machinery in the oocyte processes and expresses the protein on the surface of the oocytes. Functional studies can then be carried out. This system was also utilized to clone and carry out initial characterization studies of the human homologs of these two carrier proteins, hSPNT1 and hCNT1 (30, 31).

Mammalian Expression Systems

Another area of major development over the past few years has been in methods available to express the cloned Na^+-dependent nucleoside transporters and to study drug interactions with these proteins. Two expression systems in mammalian cells have been described, one in COS-1 cells (39) and the other in HeLa cells (40). The pyrimidine-selective, Na^+-dependent nucleoside transporter, cNT1 was successfully expressed in both systems and displayed similar functional characteristics to those observed in previous studies in *Xenopus laevis* oocytes (28, 35). The HeLa expression system has also been applied to examine functional characteristics of the cloned purine-selective nucleoside transporters, rSPNT and hSPNT1. These studies have elucidated important species differences between the rat SPNT transporter and its human homolog, hSPNT1 in terms of

kinetics of interaction of endogenous nucleosides and synthetic nucleoside analogs. The development of transfection systems in several mammalian cell lines allows for another option when studying the functional characteristics of cloned transporters and provides a mammalian system which may be used to address questions regarding the regulation and sorting of cloned nucleoside transporters.

Species Differences Identified in Functional Studies of Cloned Nucleoside Transporters

The recent cloning of the human homologs of the rat N1- and N2- type nucleoside transporters has enabled the comparison of functional characteristics of the expressed proteins between species. In comparative studies between rSPNT and hSPNT1, uridine was found to have a higher affinity for the rSPNT (K_m= 20.6 μM in HeLa cells; 14 μM in oocytes) than for its human counterpart, hSPNT1 (K_m= 114 μM in HeLa cells; 80 μM in oocytes). Differences in the IC_{50} values for 2CdA were also observed when comparisons were made between rSPNT (IC_{50} = 13 μM) and hSPNT1 (IC_{50} = 479 μM) (Chapter 4). Data in HeLa cells transfected with the cDNA of hSPNT1 demonstrate that inosine is transported at a faster rate than adenosine, in comparative studies. This is in contrast to data obtained in HeLa cells transfected with rSPNT cDNA.

Antibodies

Na$^+$-Dependent Nucleoside Transporters

Very few studies have been published directly examining the Na$^+$-dependent nucleoside transport proteins expressed in either mammalian or amphibian expression systems (35, 39). In our laboratory we have developed *anti*- peptide antibodies to the N-terminal region of rCNT1 and an epitope-tagged construct of this transporter (rCNT.tag) (Chapters 5 and 6). Anti- peptide antibodies have been employed to study the expressed nucleoside transporters in transfected cells. In Chapter 6 data are presented, using

immunofluorescence confocal microscopy, indicating that the anti-cNT1 Ab is specific for the rCNT1 protein and may be used to further characterize the expressed nucleoside transporter. An epitope-tagged construct of rCNT1 has been expressed in both transfected cells (39) and *Xenopus laevis* oocytes (Chapter 5). A recent abstract by Hamilton *et al.* reported the identification of two bands on Western blot analysis (isolated from *Xenopus laevis* oocytes injected with rCNT1 cRNA), corresponding to 68 kDa and 77 kDa (41). Furthermore, two bands were also detected when an epitope-tagged cNT1 was examined by Western blotting (isolated from *Xenopus laevis* oocytes injected with rCNT.tag cRNA) (Chapter 5). These bands may be due to glycosylation of the protein. Following treatment of the membrane fractions with endoglycosidase F, Hamilton *et al* observed an increased mobility of the labeled bands on SDS/ PAGE gels. The conclusions suggest that the original topology prediction for cNT1 may be incorrect. Initially it was thought that both the N-and C-termini were intracellular (18, 41). However, the two potential glycosylation sites based on this prediction would be intracellular. These findings suggest that both termini are actually extracellular and that the original prediction of membrane topology may be erroneous. Further studies are needed to examine this question.

Equilibrative Nucleoside Transporters

Prior to the cloning of equilibrative nucleoside transporters from human placenta, antibodies were employed to examine the localization of the equilibrative nucleoside transporters in the human placenta (19). The polyclonal antibodies used in this study were raised against a purified human erythrocyte nucleoside transporter. These Abs were shown to cross-react with a band from the human syncytiotrophoblast which comigrated with the erythrocyte nucleoside transporter. Results from this study revealed that while a cross-reactive species does exist in brush border membrane vesicles isolated from human placenta (as determined by Western blotting) this band was not present in the basal membrane human placental vesicles (19). With the cloning of both subtypes of equilibrative

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nucleoside transporters (*es* and *ei*) it is now possible to determine the tissue distribution of the two proteins. Either the basal membrane vesicles express the *ei* subtype, which would not have been detected using the polyclonal antibodies, or, alternatively, another subtype of the es transporter is expressed in basal membrane vesicles. The development of specific antibodies designed against unique regions of *es* and *ei* will be critical in determining the distribution of equilibrative nucleoside transporters in the placenta and in other tissues.

Summary and Review of Chapters

Nucleosides and nucleoside analogs have a variety of clinical applications in the fields of cancer and infectious diseases (Table 4). An understanding of the mechanisms by which these compounds cross cell membranes is of critical importance. Initial information determining the characteristics of nucleoside transporters was based on functional studies in isolated cell and tissue preparations. In all such studies, valuable information was obtained regarding substrate selectivity and ion requirements for the nucleoside transporters; however, the problem of expression of multiple nucleoside transporters in a single experimental system created problems of substrate overlap. With recent advances in the field of nucleoside transport, including the cloning of both concentrative and equilibrative transporters as well as the establishment of mammalian expression systems and the development of antibodies which recognize the expressed nucleoside transporters, the opportunity exists to address many questions regarding the regulation, structure-function and posttranslational modifications of nucleoside transporters.

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Table 4. Clinical Applications for Nucleosides and Nucleoside Analogs

Compound	Clinical Applications	References
AZT	Used in combination therapy for patients infected with HIV Prevention of Maternal-Fetal Transmission of HIV	(2)
2CdA	Cancer chemotherapy: CLL (Chronic Lymphocytic Leukemia), hairy cell leukemia	(42-45)
ddI	Combination or mono-therapy for HIV infection	(2)
ddC	Combination or mono-therapy for HIV infection	(2)
Ara-C	Cancer chemotherapy	(46)
Gemcitabine (2',2'-difluorodeoxycytidine, dFdC)	Cancer chemotherapy	(7)
FAMP	CLL (Chronic Lymphocytic Leukemia),	(42)

A brief summary of the work presented in each chapter of this dissertation is presented below:

Chapter 2

Nucleosides and nucleoside analogs are currently given to HIV positive, pregnant women to prevent maternal-fetal transmission of HIV. Knowledge of the mechanisms of nucleoside transport across the placenta is critical in understanding the delivery and toxicity of these compounds to the fetus. The aim of this study was to examine placental nucleoside transport mechanisms using JAR cells (a human choriocarcinoma cell line) as a model system. JAR cells were plated on 12-well plates (0.5×10^6 cells/well) and the uptake of ^{3}H-thymidine was determined 2 days after plating. ^{3}H-Thymidine uptake was temperature dependent (22°C: 0.56 ± 0.04 pmol/mg protein; 4°C: 0.21 ± 0.02 pmol/mg protein) and was inhibited ($P<0.05$) by the metabolic inhibitor 2,4-dinitrophenol (250 µM), consistent with an energy dependent process. The uptake of ^{3}H-thymidine was saturable ($K_m = 113$ nM), Na$^+$-independent, and was selectively inhibited by both purines and pyrimidines. Nucleoside analogs [AZT, ddI, ddC (2 µM)] inhibited ($P<0.05$) thymidine uptake. These data suggest the presence of a nucleoside transporter in JAR cells which is unique, in terms of its substrate selectivity, kinetic characteristics, and ion dependency, from previously described nucleoside transporters. This transporter may play a role in the maternal-fetal transport of therapeutically important nucleoside analogs.

Chapter 3

The goal of this study was to develop a mammalian expression system for the cloned rat intestinal, Na$^+$-dependent, purine-selective nucleoside transporter (SPNT$_{int}$) and to study the interactions of nucleosides and nucleoside analogs with this transporter. Lipofection was used to transfect HeLa cells with a mammalian expression vector (pcDNA3) containing the cDNA insert encoding SPNT$_{int}$. Nucleoside transport activity

was measured using ^{3}H-inosine, ^{3}H-uridine, ^{3}H-dideoxyinosine (ddI), and ^{3}H-2-chloro-2'-deoxyadenosine (2CdA) as model substrates. Expression of SPNT$_{int}$ was observed between 36 and 90 h post-transfection, with maximal expression at 66 h. At 66 h, Na$^+$-stimulated uptake of ^{3}H-inosine in cells transiently transfected with SPNT$_{int}$ was approximately threefold greater than that in cells transfected with empty vector (p < 0.05). The Na$^+$-stimulated uptake of both inosine and uridine was saturable (K_m =28.1 ± 7.1 μM and 20.6 ± 5.6 μM, respectively) in the transfected cells and was significantly inhibited by the naturally occurring nucleosides (1 mM) inosine and uridine and to a lesser extent by thymidine. The nucleoside analogs ddI (IC$_{50}$ = 46 μM) and 2CdA (IC$_{50}$ = 13 μM) also significantly inhibited the Na$^+$-stimulated uptake of ^{3}H-inosine. A Na$^+$-stimulated uptake of ^{3}H-2CdA was observed suggesting that 2CdA is also a permeant of SPNT$_{int}$. In summary, HeLa cells transiently transfected with SPNT$_{int}$ represent a useful tool to study the kinetics and interactions of drugs with SPNT$_{int}$.

Chapter 4

Nucleosides and nucleoside analogs are actively transported by the human kidney. With the recent cloning of a purine-selective nucleoside transporter (hSPNT1) from human kidney (1), it is now possible to study the interaction of nucleosides and nucleoside analogs with this transport protein and gain a more detailed knowledge of the underlying mechanisms of nucleoside transport in the human kidney. In this study we examined the substrate selectivity of hSPNT1 by determining the uptake of various [^{3}H]nucleosides and [^{3}H]nucleoside analogs in HeLa cells transfected with the cDNA of hSPNT1. We determined that the naturally-occurring nucleosides, adenosine, inosine, and uridine, are substrates for this carrier, whereas thymidine is not. The nucleoside analogs, 2', 3'-dideoxyadenosine (ddA), 2', 3'-dideoxycytidine (ddC), 2',3'-dideoxyinosine (ddI), and 2-chloro-2'deoxyadenosine (2CdA), significantly inhibited the uptake of [^{3}H]inosine in HeLa cells transiently transfected with hSPNT1 cDNA. However, there was no significant Na$^+$-

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dependent uptake of [^{3}H]ddI or [^{3}H]2CdA in the transfected cells suggesting that these nucleoside analogs are not permeants of hSPNT1. Interestingly, 2CdA was considerably less potent in inhibiting [^{3}H]inosine uptake in HeLa cells expressing hSPNT1 than in cells expressing rSPNT (IC_{50}= 479 μM vs. 13 μM) suggesting that there may be notable species differences in the interaction of some nucleoside analogs with purine selective nucleoside transporters.

Chapter 5

In 1994, a Na$^+$-dependent, pyrimidine-selective nucleoside transporter (cNT1) that may play a role in drug absorption was cloned from rat intestine (28). Although the function of the cloned cNT1 can be studied, there is currently no method to detect the expressed transport protein. The aim of this study was to develop an antibody (Ab) detection system for cNT1 by incorporating an epitope tag on the N-terminus of this transporter. The cNT1 transporter cDNA was cloned by RT-PCR and then ligated into pGEM-T. A sense primer encoding EQKLISEEDL (c-myc epitope) and part of the N-terminal sequence of cNT1 and an antisense primer directed to a segment of pGEM-T were synthesized. PCR with these primers was carried out and the products were ligated into the appropriate vector and analyzed by restriction digestion. To characterize the functional activity of the epitope-tagged transporter, cNT1.tag, and to compare this activity to that of the wild-type, cNT1, the cDNA encoding cNT1.tag or cNT1 was transiently transfected in HeLa cells. Both cNT1 and cNT1.tag displayed Na$^+$-dependent ^{3}H-thymidine uptake with similar kinetic parameters. Inhibition of cNT1 or cNT1.tag expressed in HeLa cells was examined. Significant inhibition was observed with the nucleoside analogs AZT and ddC, but not with ddA, ddI or AraC. Na$^+$-dependent ^{3}H-thymidine uptake (at 0.5 hr) in *Xenopus laevis* oocytes injected with the cRNA of the c-*myc* tagged transporter was enhanced (0.025 ± 0.006 pmol/ oocyte) when compared to its uptake in uninjected oocytes (0.007 ± 0.003 pmol/ oocyte) (P < 0.05). Oocytes injected with the tagged cRNA

expressed a protein which was detected (the untagged transporter was not detected) by immunoblotting with the anti-c-*myc* mAb. These data represent the first demonstration of a tagged nucleoside transporter that is functional when expressed in *Xenopus laevis* oocytes. Abs can be used to detect and study the tagged transporter to obtain further information on its structure, function, and intracellular trafficking.

Chapter 6

The expression of the pyrimidine- selective nucleoside transporter, cNT1, was examined in HeLa cells transiently transfected with the cNT1 cDNA. cNT1 displayed Na^+-dependent ^3H-thymidine uptake. Inhibition of the expressed pyrimidine-selective carrier was also examined. Significant inhibition was observed with the nucleoside analogs AZT, 5-fluorouridine, and 2-chloroadenosine (1 mM). Of the endogenous nucleosides examined, significant inhibition was observed with the pyrimidine nucleoside thymidine. However, no significant decrease in the uptake of ^3H-thymidine was observed with the endogenous purine nucleoside inosine (1 mM). Further characterization of the protein expressed following transfection of cNT1 cDNA in HeLa cells was carried out using immunofluorescence confocal microscopy. Specific staining was observed with a cNT1-specific anti-peptide antibody.

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CHAPTER 2

CHARACTERIZATION OF A HIGH AFFINITY NUCLEOSIDE TRANSPORTER IN A HUMAN CHORIOCARCINOMA CELL LINE (JAR)

Nucleosides and nucleoside analogs are currently used in pregnant women for the treatment of viral infections, cardiac arrhythmias and for cancer chemotherapy (1-8). A knowledge of the mechanisms of nucleoside transport across the placenta is critical in understanding the delivery and toxicity of these compounds to the fetus. In addition, mechanisms involved in nucleoside transport across the placenta are important in the disposition and targeting of endogenous nucleosides to the placenta and developing fetus (7, 9, 10). Because nucleosides are polar molecules, transporters located in the plasma membrane are essential in the transcellular or transepithelial flux of these compounds.

To date, there have been a limited number of functional studies of nucleoside transport mechanisms in the human placenta (4, 11-15). These studies, which have been carried out in isolated brush border and basolateral membrane vesicles and in isolated perfused placenta, suggest that nucleosides are transported in the human placenta by an equilibrative nucleoside transporter which is sensitive to NBMPR (*es*) but does not interact with the nucleoside analogs, 2', 3'-dideoxyinosine (ddI) and azidothymidine (AZT). There is also some evidence that an equilibrative nucleoside transporter which is insensitive to NBMPR (*ei*) may also play a role in transplacental nucleoside flux.

During the last year, exciting advances have occurred in the understanding of placental transport of nucleosides. Two subtypes (*es* and *ei*) of the human equilibrative nucleoside transporters were cloned from human placenta (16,17). The *es* subtype (hENT1) is a 456- residue protein, with a predicted membrane topology of 11 transmembrane domains. The *ei* subtype (hENT2) is 46% identical to hENT1 at the amino acid level and also encodes a protein of 456 amino acids. Functional studies carried out in *Xenopus laevis* oocytes indicate that both proteins mediate the uptake of adenosine and

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uridine, with K_m values in the high micromolar range. In addition, the transport of uridine by hENT2 was not inhibited by low (nM) concentrations of NBMPR, consistent with characteristics of the *ei* subtype; however, hENT1 was inhibited by nanomolar concentrations of NBMPR, consistent with *es*. hENT1 and hENT2 do not share significant sequence homology with the cDNAs encoding Na^+- coupled nucleoside transport proteins. However, both hENT1 and hENT2 share significant sequence homology with other proteins of unknown function.

Although functional data together with the recent cloning of both hENT1 and hENT2 (16,17) confirm the existence of equilibrative nucleoside transporters in human placenta, other relevant nucleoside transport mechanisms may not have been identified because of limitations in the experimental methods. First, transport processes may be obscured in intact perfused placenta because of possible breaks in the membrane. In addition, adenosine, the primary substrate used in these studies, is extensively metabolized in the intact perfused placenta and to some extent, in isolated membrane vesicles. Metabolism confounds the interpretation of uptake data. Finally, a primary active transporter (ATP-driven) would not have been detected in the studies in isolated membrane vesicles, which are devoid of ATP-regeneration mechanisms.

In this study, we used JAR cells as a model system to determine the mechanisms of nucleoside transport. JAR cells are derived from a trophoblastic tumor of the human placenta (18), which mimics normal placenta functions such as hormone secretion and cellular metabolism (18-24). Several transporters have been characterized in JAR cells including the serotonin transporter and the thyroid hormone transporter. These transporters have been identified in term placenta as well.

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Materials

JAR cells were purchased from the American Type Culture Collection (ATCC). RPMI-1640, fetal bovine serum, 1% amphotericin B and 1% penicillin-streptomycin were obtained from the University of California, Cell Culture Facility. Cells from passage 725 to 745 were used in all studies. All chemicals, including nucleosides, nucleoside analogs, and nucleobases were obtained from Sigma. Trizol was purchased from Gibco/ BRL. Human placental tissue was obtained from the University of California, San Francisco. Corning tissue culture plates were purchased from Fisher. Thin layer chromatography plates were purchased from EM Reagents. Butanol and acetic acid were purchased from Fisher Scientific.

Methods

Cell Culture

JAR cells were maintained in a humidified, 5% CO_2, 95% air atmosphere in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 1% amphotericin B and 1% penicillin-streptomycin. Cells were seeded at a density of 0.5 x 10^6 cells per well. For the days in culture study, medium was changed every 24 hours. For other studies, medium was changed 24 hours after plating and uptake studies were carried out 48 hours after the initial seeding.

Uptake Studies

JAR cells

Uptake studies were carried out in JAR cells on day 2 after plating at 25°C. Following an initial time course, a 10 second time point chosen from the linear phase of the curve was used in further studies. To determine the uptake of the model nucleoside thymidine, cells were incubated in Na^+ buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 25 mM HEPES/ Tris, pH 7.4) or Na^+- free

buffer, in which Na^+ was replaced by 140 mM choline chloride. Uptake was terminated by the removal of the uptake buffer (^{3}H-thymidine (28 nM) in either Na^+ or Na^+-free buffer), followed by three washes in ice- cold Na^+- free buffer. Cells were then solubilized in 0.5% Triton- X 100 and radioactivity was determined by scintillation counting (Beckman, Palo Alto, CA).

Because thymidine uptake was not Na^+-dependent (Fig. 2), Na^+-free buffer was used for the subsequent uptake studies. Initial studies, to determine thymidine uptake at different days following plating, were carried out. The uptake of ^{3}H-thymidine (28 nM) at 10 seconds in the presence or absence of unlabeled thymidine (2 µM) was examined in JAR cells over a period of six days (48, 72, 96, 120 and 144 hours) after plating. The effect of temperature on thymidine uptake was determined by carrying out uptake studies at 25°C and at 4°C. Inhibition studies were carried out in the presence and absence of a number of nucleosides, nucleoside analogs, and nucleobases (2 µM, Table 1).

Primary cultured cells isolated from human placenta

The primary cultured cells were prepared from normal term placenta using standard methods (25, 26). Cells were harvested from human placenta at term and seeded on 12-well Falcon tissue culture plates. For uptake studies, the cells were incubated with ^{3}H-thymidine for 15 minutes in the presence and absence of unlabeled thymidine (5 µM). Briefly, cells were incubated with ^{3}H-thymidine (28 nM) in Na^+-free buffer for 15 min. Uptake was carried out as described above for JAR cells. These cells were the kind gift of Dr. Nicholas P. Illsley.

Xenopus laevis **oocytes**

The method of expression in *Xenopus laevis* oocytes was employed to determine whether it is possible to express the high affinity nucleoside transporter identified in JAR cells in *Xenopus laevis* oocytes. First, total RNA was isolated from JAR cells followed by purification of poly (A)$^+$ RNA (mRNA). The mRNA was injected into *Xenopus laevis* oocytes and the uptake of ^{3}H-thymidine (128 nM) determined in the presence and absence

of unlabeled thymidine (0.5 μM) as a functional assay for transport. Uptake was carried out in Na^+-free buffer using methods previously described (27). Briefly, oocytes were incubated in Na^+-free buffer (100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM / Tris, pH 7.4) at 25° C containing ^3H-thymidine with or without unlabeled thymidine. Oocytes were incubated in the uptake mixture for 30 min and then uptake was terminated by the addition of 3 ml ice-cold choline buffer, followed by three washes in the same buffer. Oocytes were solubilized in 10% SDS and then resuspended scintillation fluid. Radioactivity was determined by scintillation counting.

Energy Dependence

The effect of the metabolic inhibitor, 2, 4-dinitrophenol (DNP; 250 μM) on ^3H-thymidine uptake in JAR cells was determined at 10 sec, 30 sec, and 1 min as described in 'Uptake Studies'. Briefly, cells were incubated with ^3H-thymidine in the presence and absence of DNP. To account for nonspecific uptake, uptake in the presence and absence of unlabeled thymidine (2 μM) was also measured.

Thin Layer Chromatography

Metabolism of ^3H-thymidine was measured by thin layer chromatography (TLC). ^3H-thymidine (28 nM) was incubated with JAR cells for 10 seconds. Uptake was stopped as described for JAR cells. Cells were then solubilized with 200 μl of 0.5% Triton X-100. A sample from each well was assayed by TLC to determine the extent of thymidine metabolism. (Similar results were obtained at both 5 and 15 minutes, data not shown). A mobile phase of 600 ml butanol: 100 ml acetic acid: 100 ml H_2O was used to separate nucleosides from nucleotides.

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Protein Assay

For each plate used in an uptake study, 2-3 wells were reserved for protein analysis. Cells were washed twice with Na^+-free buffer and then solubilized with 0.5 ml of 1 N NaOH. The solution was neutralized by addition of 0.5 ml of 1 N HCl. A sample was taken for each protein assay. The Bradford method was used to carry out the assay using the Bio-Rad reagent. Bovine serum albumin (BSA) was used to generate the standard curve. Absorbance was read at 595 nm and the amount of protein/well was calculated from the standard curve.

Data Analysis

Unless otherwise specified, data are expressed as mean ± standard deviation (SD) of uptake values obtained in at least 3 wells. For Michaelis-Menten studies, rate of uptake was expressed as pmol/mg protein/5 min. Data were fit to the equation V= $V_{max}[S]/(K_m+[S])+ k_{ns}[S]$ where V is the rate of thymidine uptake, [S] is the thymidine concentration, k_{ns} is a constant which represents non-specific uptake. The Kaleidagraph® fitting program was used to fit the data and parameter estimates are expressed as a mean ± SE. For determination of statistical significance, an unpaired Student's *t*-test was used and $p < 0.05$ (one tail) was considered significant.

Results

Initial Characterization of Nucleoside Uptake in JAR Cells.

To ascertain the expression of nucleoside transport over time in JAR cells, the uptake of ^{3}H-thymidine was measured as a function of days in culture. Briefly, JAR cells were seeded on 12-well plates and then uptake was measured 48, 72, 96, 120 and 144 hours after plating in the presence and absence of unlabeled thymidine (2 μM). ^{3}H-thymidine uptake was most pronounced on day two (48 hours after plating) and slowly decreased through day six (144 hours post-plating) indicating that inhibitable thymidine uptake was present early in culture and decreased with time (Fig. 1).

Uptake in the presence and absence of Na$^+$ in JAR cells was examined on day 2 and no significant difference was observed between uptake in the presence or absence of Na$^+$ (Fig. 2). Since metabolism of thymidine would interfere with the interpretation of uptake data, studies were carried out to determine the extent of thymidine metabolism at 10 seconds. At this time point, no significant metabolism of thymidine was observed.

Temperature Dependence and Energy Dependence

Temperature dependence is an important characteristic of carrier-mediated processes. The uptake of thymidine appears to be temperature dependent and is significantly reduced at 4°C ($p<0.05$) (Fig. 3). Furthermore, when JAR cells were incubated with DNP at 10 sec, 30 sec, and 1 min in the presence and absence of 2 μM unlabeled thymidine, a significant decrease in uptake was observed (Fig. 4). This experiment suggests that thymidine uptake may be energy dependent in JAR cells.
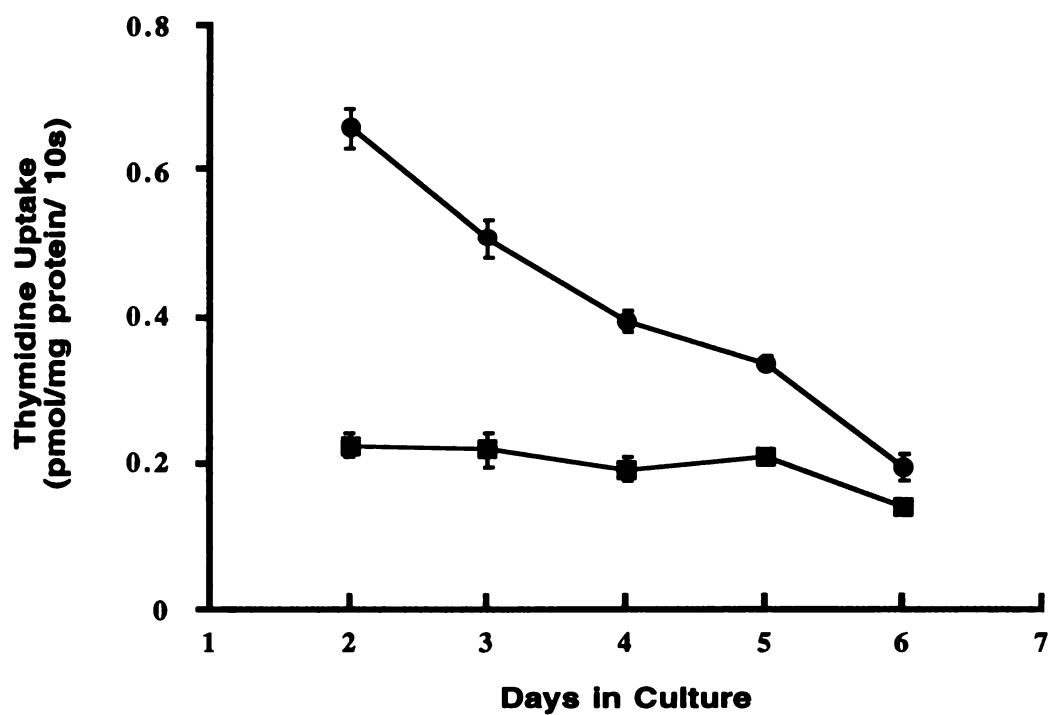
Figure 1. Thymidine transport in JAR cells as a function of days in culture. The uptake of ^{3}H-thymidine (28 nM) at 10 seconds in the presence (closed squares) and absence (closed circles) of unlabeled thymidine (2 μM) was examined in JAR cells over a period of six days after plating. Data are presented from a single experiment (mean ± SD, n=3).

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³H-Thymidine Uptake (pmol/mg protein)

10

5

0

0   **10**   **20**   **30**

Time (min)
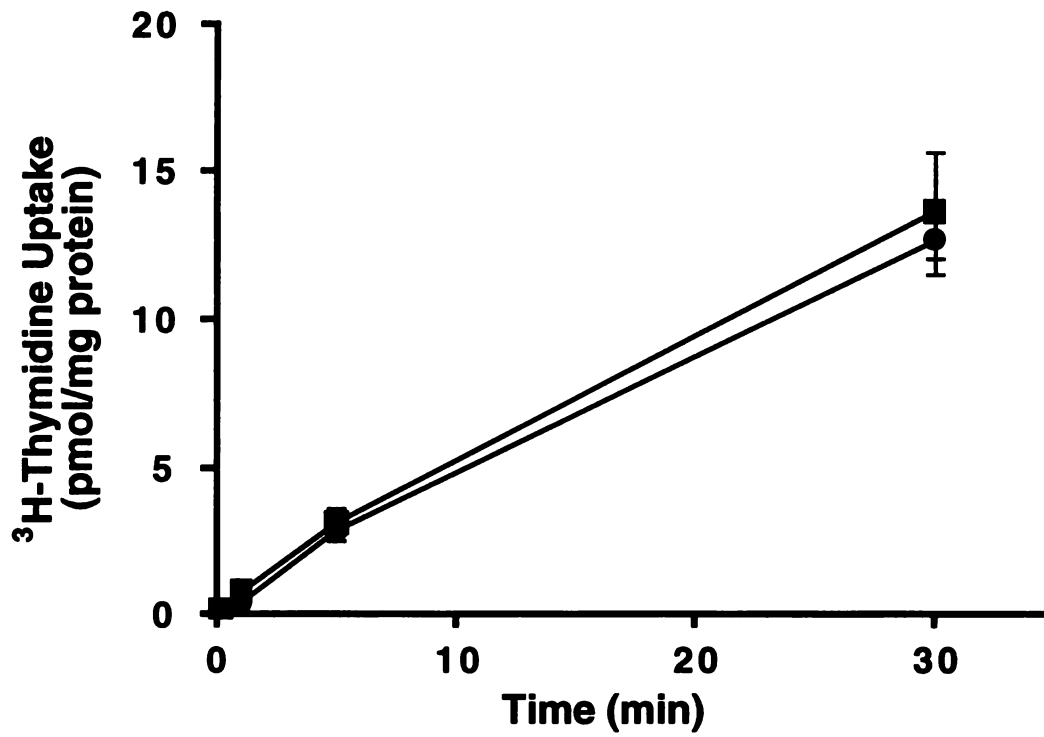
Figure 2. The effect of Na⁺ on ³H-thymidine uptake in JAR cells. The uptake of ³H-thymidine was measured in the presence (closed squares) and absence (closed circles) of Na⁺ over time. Data are presented as mean ± SD from at least three wells.
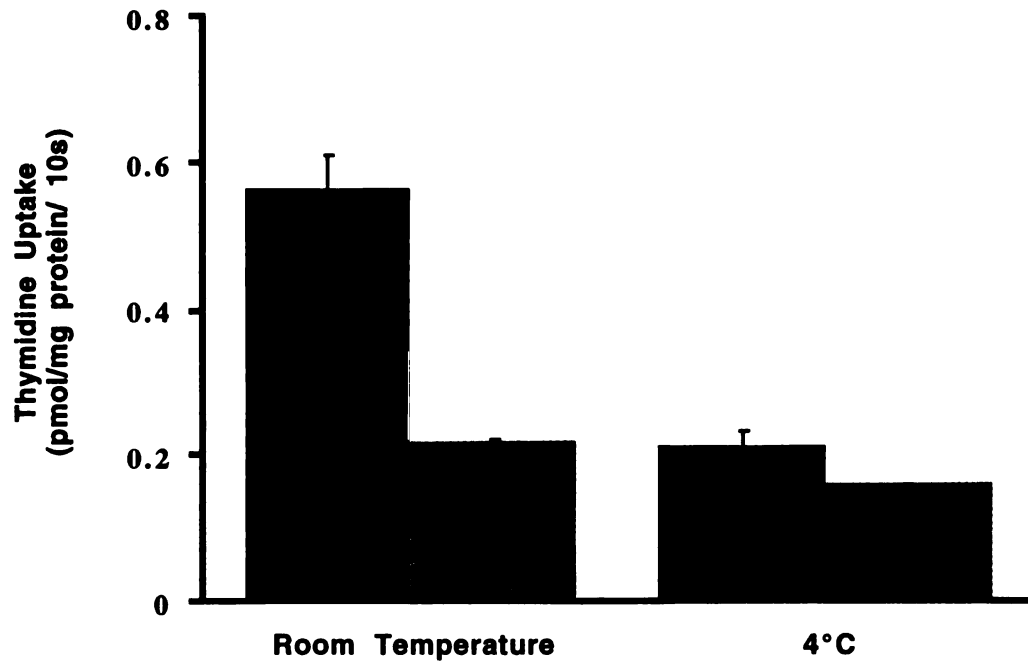
Figure 3. Effect of temperature on thymidine uptake in JAR cells. Uptake of ^{3}H-
thymidine (28 nM) was measured at $4°C$ and at $25°C$ in the presence (gray bars) and
absence (black bars) of 2 μM unlabeled thymidine. The uptake of thymidine is
significantly reduced at $4°C$ ($p<0.05$). Data are presented as mean ± SD, n=3.
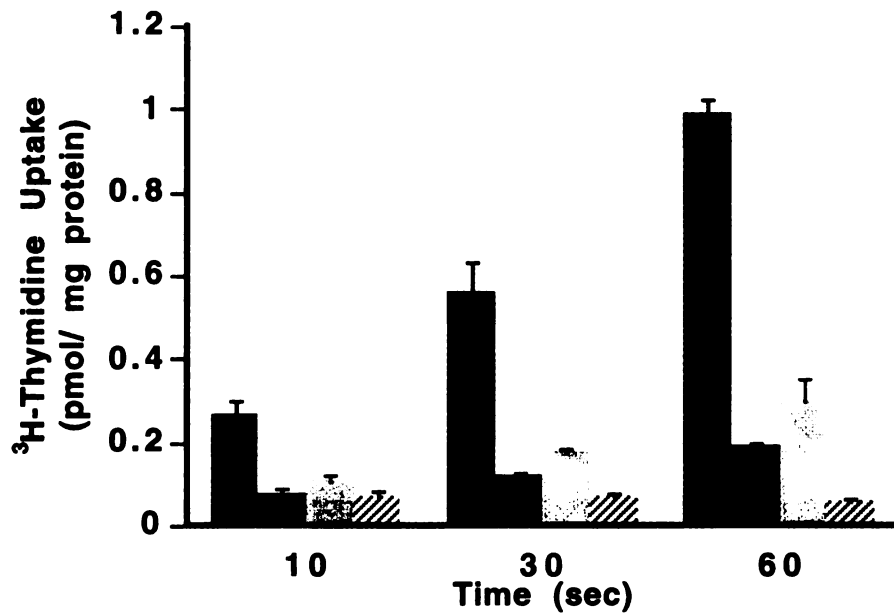
Figure 4. Effect of the metabolic inhibitor 2, 4-dinitrophenol on thymidine uptake in JAR cells. The uptake of ^{3}H- thymidine (two bars on left for each time point) was measured in the presence (black bar) and absence of DNP (250 µM) (gray bar). ^{3}H- thymidine uptake was also measured in the presence of unlabeled thymidine (2 µM) (two bars on right for each time point) in the presence (hatched bar) and absence (light gray bar) of DNP. Uptake was measured at 10 sec, 30 sec and 1 min. Data are presented as mean ± SD (n=3).

Saturability of Transport

To determine whether thymidine uptake into JAR cells is via a saturable, carrier-mediated process, ^{3}H-thymidine uptake was measured in the presence of increasing concentrations of thymidine (Fig. 5). Data were fit to the equation $V_{max}[S]/(K_m+[S])$ + $k_{ns}[S]$ representing both a saturable and a linear component. (K_m=169 nM ; V_{max}=2.06 pmol/mg protein/10 sec). This experiment indicates that thymidine uptake into JAR cells is a saturable, high affinity process.

Inhibition Studies

^{3}H-Thymidine uptake (28 nM) in JAR cells was measured in the presence and absence of nucleosides, nucleobases and nucleoside analogs at a concentration of 2 μM (Table 1). The inhibition profile for thymidine uptake in JAR cells was very broad, with significant ($p< 0.05$) inhibition of thymidine uptake in the presence of 2 μM of the endogenous nucleosides thymidine, adenosine, cytidine and uridine. The dideoxy-nucleoside analogs, ddI, ddA and ddC and the nucleoside analog, AZT also displayed significant inhibition of thymidine uptake. At low concentrations (10 nM) nitrobenzylthioinosine (NBMPR) displayed slight inhibition of thymidine uptake at 10 sec; however at higher concentrations (10 μM) both NBMPR and dipyridamole displayed significant inhibition of ^{3}H-thymidine uptake (data not shown). Interestingly, the nucleobases, adenine, thymine, guanine and uracil, as well as the nucleobase analog, acyclovir, significantly inhibited thymidine uptake in JAR. Hypoxanthine and formycin B, inhibitors of equilibrative nucleoside transport, did not significantly inhibit thymidine uptake. This experiment further distinguishes this transporter from other previously characterized equilibrative transporters in human placenta. This inhibition was broadly selective, and unique from previously described concentrative human nucleoside transporters, in that this transporter is inhibited by both nucleosides and nucleobases.

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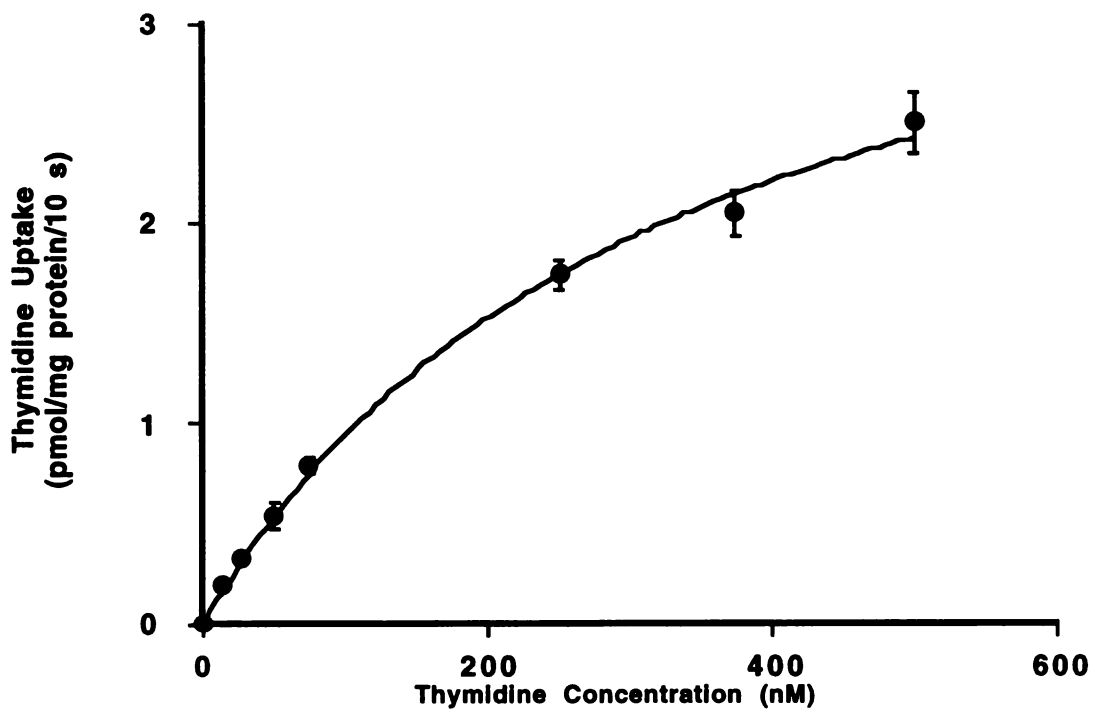
Figure 5. Concentration dependence of ^{3}H-thymidine uptake in JAR cells. The uptake of

[3]H-thymidine was determined at 10 seconds as a function of thymidine concentration. Data

were fit to the equation $[V_{max}[S]/(K_m+[S]) + k_{ns}[S]]$ representing both a saturable and a

linear component (K_m= 169 nM; V_{max}= 2.06 pmol/mg protein/10 sec).

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Table 1. Inhibition of ^{3}H-Thymidine Uptake in JAR Cells

Compound	pmol/mg protein/ 10sec	Std. dev.
Control	0.382	0.061
Thymidine	0.155*	0.028
Formycin B	0.322	0.032
Guanosine	0.257	0.040
Adenosine	0.231*	0.031
Uridine	0.175*	0.042
ddC	0.182*	0.006
ddA	0.021*	0.008
ddI	0.162*	0.012
AZT	0.113*	0.009
Adenine	0.207*	0.008
Acyclovir	0.267	0.071
Thymine	0.166*	0.030
Uracil	0.174*	0.017
Cytidine	0.204*	0.022
Hypoxanthine	0.307	0.024
Guanine	0.241*	0.022

^{3}H-thymidine uptake in JAR cells in the presence and absence of a number of nucleosides, nucleoside analogs, and nucleobases ($2\mu M$). (* Indicates significantly different from control, $p<0.05$ (n=4))

Thymidine Uptake in Primary Cultured Cells Isolated from Human Placenta at Term

In initial sudies, uptake activity of ^{3}H-thymidine was examined in primary cultured cells isolated from human placenta at term. This experiment was carried out to determine whether a counterpart to the transporter identified in JAR cells may be present in the human placenta at term. Primary cultured cells were isolated from human term placenta and were incubated with ^{3}H-thymidine (28 nM) for 15 minutes in the presence and absence of unlabeled thymidine (5 μM). Significant inhibition of thymidine uptake was observed in these cells (Fig. 6), indicating that a counterpart to the transporter characterized in JAR may be present in human placenta.

Expression of Nucleoside Transport Activity in Xenopus Laevis Oocytes

Thymidine uptake was examined in *Xenopus laevis* oocytes following injection of the mRNA isolated from JAR cells into oocytes (see Methods). Four days following injection, the uptake of ^{3}H-thymidine (180 nM) in the presence and absence of unlabeled thymidine (15 μM) was determined (Fig. 7). This experiment demonstrates that high affinity inhibitable thymidine uptake is detected in oocytes injected with mRNA from JAR cells. Further studies are needed to determine whether this method can be used as a functional assay for the high affinity nucleoside transporter in JAR cells.

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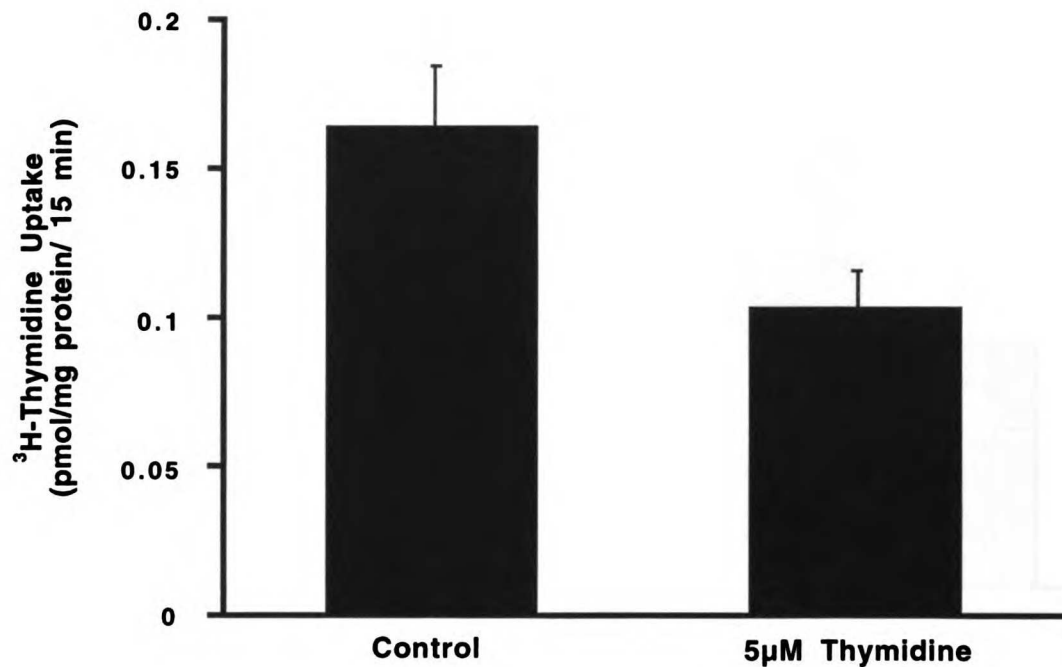
Figure 6. ^{3}H-Thymidine uptake and inhibition in primary cultured cells from human term placenta. Cells were harvested from human placenta at term as described in "Methods" and seeded on 12-well Falcon tissue culture plates. The cells were incubated with ^{3}H-thymidine for 15 minutes in the presence and absence of unlabeled thymidine (5 μM). Data are presented as mean ± SD, n = 3, from a single experiment.
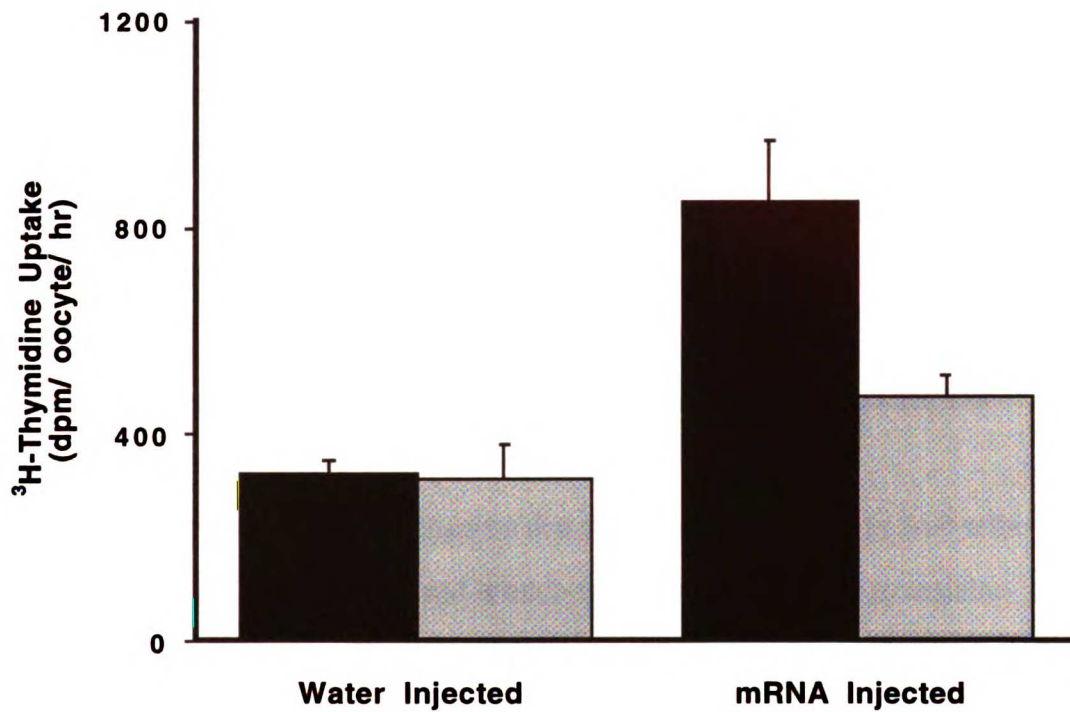
Figure 7. Thymidine uptake in *Xenopus laevis* oocytes injected with JAR mRNA. mRNA was isolated from JAR cells and injected into oocytes. ^{3}H-Thymidine (128 nM) uptake in the presence (black bars) and absence (gray bars) of thymidine (5 μM) was measured on day 4 following injection (see Methods). Data are presented as the mean ± SD (n= 9)

Discussion

Nucleoside analogs, such as AZT, are currently being given to pregnant women who are infected with HIV to prevent the transplacental transfer of virus (9). With the administration of such compounds, there is great concern for long term effects of exposure of the developing fetus to these nucleoside analogs (9, 27, 28). A recent study examining both human trophoblast tissue and the trophoblast-derived cell line, JAR, concluded that AZT is not metabolized to 3'-amino-3'-deoxythymidine (AMT), a toxic catabolite of AZT in either cells or tissue (7). However, there was significant accumulation of AZT in the cells. AZT is fairly hydrophobic, and therefore may cross the placenta primarily via simple diffusion. There may also be facilitated nucleoside processes responsible for this phenomenon.

The nucleoside transport mechanism that we have characterized in JAR cells transports thymidine (K_m= 169 nM) and is inhibited by both purine and pyrimidine nucleosides and nucleobases. Thymidine transport was not directly coupled to sodium; however it was inhibited by the metabolic inhibitor 2,4-dinitrophenol, consistent with an energy dependent process (Fig. 4). Nucleoside analogs including AZT, ddC and ddI as well as nucleobases interacted with this transport mechanism. Preliminary data suggest that a high affinity thymidine transport mechanism is also present in primary cultured cells from human term placenta (Fig. 6).

Because this transport mechanism identified in JAR has such a high affinity for nucleosides (nanomolar range), it may be important in the scavenging of nucleosides when concentrations are very low. In fact, physiological concentrations of nucleosides are in the high nanomolar range. Although speculative, it is possible that when concentrations fall below this level, a high affinity nucleoside transporter may be critical for the salvage of nucleosides. With the recent cloning of the equilibrative nucleoside transporters (16, 17), a greater understanding of the transplacental transport of nucleosides is now possible. The nucleoside transporter we have characterized in JAR cells appears to be distinct from

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previously described equilibrative transporters located in the placenta. Notably, hypoxanthine and formycin B, which are inhibitors of equilibrative transport, do not interact with this transporter. The affinity of this transporter for thymidine (K_m 169 nM) is in the range of affinity constants for nupC, but is considerably higher than the affinity of thymidine for previously characterized equilibrative nucleoside transporters in the placenta (μM range). Furthermore, unlike the equilibrative transporters, data suggest that this transporter is dependent on metabolic energy. Initial studies suggest that this transport mechanism is expressed in *Xenopus laevis* oocytes injected with the mRNA derived from JAR and in primary cultured cells from human placenta at term. Such a transporter in the placenta may provide one explanation for the accumulation of AZT inside JAR and trophoblast cells.

It is also important to distinguish the uptake process characterized in JAR cells from a possible high affinity binding process. The significant decrease in uptake with a decrease in temperature (Fig. 3) suggests that thymidine uptake is via a carrier mediated process. Furthermore, the addition of the metabolic inhibitor, DNP, to the uptake medium resulted in a significant decrease in thymidine uptake in JAR cells (Fig. 4). These data, which suggest that thymidine uptake in JAR cells may be energy-dependent, are not consistent with a binding process. In conclusion, we have characterized an uptake mechanism for thymidine in JAR which differs from the previously characterized equilibrative transport mechanism in terms of its affinity, inhibition profile and energy dependence.

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CHAPTER 3

TRANSIENT EXPRESSION OF A PURINE-SELECTIVE NUCLEOSIDE TRANSPORTER (SPNT$_{int}$) IN A HUMAN CELL LINE (HELA)[1]

Nucleosides and nucleoside analogs are used clinically in the treatment of a wide array of disease states including viral infections, cardiac arrhythmias and various neoplasms. Many nucleoside analogs are poorly absorbed, polar molecules and it is becoming increasingly clear that transporters located in the intestinal brush border membrane may function in the absorption of these analogs (1-4). Two major classes of nucleoside transport processes have been described, equilibrative (5, 6) and concentrative (7-10). Equilibrative nucleoside transporters are Na^+-independent, facilitative carriers and are broadly-selective for nucleosides, accepting both purines and pyrimidines. In contrast, concentrative nucleoside transporters are secondarily active, Na^+-dependent carriers and differ in their substrate selectivity. Four major concentrative processes have been described, one purine-selective (N1) (7, 9, 11), one pyrimidine-selective (N2) (10, 12), and two broadly selective processes (N3 and N4) (8, 9, 13-16).

Recently, a Na^+-dependent, purine-selective transporter, SPNT, was cloned from a rat liver cDNA library by expression cloning in *Xenopus laevis* oocytes (7). Northern analysis suggested that the transporter was expressed in high levels in rat intestine. Subsequently, using RT-PCR and rat intestinal mRNA as a template, SPNT$_{int}$ was cloned (3, 17). The cloning of the transporter from rat intestine together with the high level of expression of the mRNA transcript of SPNT in rat intestine suggests that this transporter may play a role in the intestinal absorption of nucleosides and nucleoside analogs.

The functional characteristics of SPNT have been studied in *Xenopus laevis* oocytes (3, 7, 17). However, because of seasonal variability and the complex micro-

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injection techniques required for protein expression, oocytes are poorly suited for routine screening of compounds for potential interactions with $SPNT_{int}$. Furthermore, for oocyte expression, cDNA has to be transcribed and capped and the resultant cRNA injected into the oocyte. This cRNA is subject to degradation by nucleases during storage (19). Therefore, the development of a mammalian heterologous expression system would be a useful model to study the interaction of drugs with $SPNT_{int}$ as well as the regulation and processing of the transport protein.

In this study, we chose HeLa cells to transfect the cDNA of $SPNT_{int}$. HeLa have been well-characterized for the equilibrative nucleoside transporter, but do not exhibit a Na^+-dependent nucleoside transport process (18). This cell line has been used previously for the expression of transport proteins (19, 20). We developed an expression system for the purine-selective nucleoside transporter, $SPNT_{int}$, using HeLa cells and a lipid transfection method. This method is reproducible and allows for high levels of expression within 36 hours following transfection. HeLa cells transiently transfected with the cDNA of $SPNT_{int}$ represent a useful model in the study of the kinetics and interactions of compounds with $SPNT_{int}$.

Materials

Zidovudine (AZT), 2',3'-dideoxyadenosine (ddA), 2',3'-dideoxycytidine (ddC), ddI, uridine, thymidine, inosine, 2CdA (2-chloro, 2'-deoxyadenosine), 2-chloroadenosine, and acyclovir were purchased from Sigma. ^{3}H-Inosine (sp. act. 28 Ci/mmole), ^{3}H-ddI (sp. act. 38 Ci/mmole), ^{3}H-2CdA (sp. act. 3.8 Ci/mmole), and ^{3}H-uridine (sp. act. 43.8 Ci/mmole) were purchased from Moravek. The Bradford reagent was obtained from Bio-rad. DNA isolation kits were from Qiagen. LIPOFECTAMINE™, Opti-MEM™ (serum-free media), the Superscript Preamplification System, and the Trizol™ Reagent were purchased from Gibco/BRL. DME H21 medium, MEM, trypsin, phosphate buffered saline-calcium, magnesium free (PBS), and fetal bovine serum (FBS) were purchased from

the UCSF Cell Culture Facility. Corning 12-well plates were used for cell plating and Nunc flasks for cell maintenance. The pcDNA3 vector was supplied by Invitrogen and enzymes used for subcloning were from Gibco/BRL, New England Biolabs (NEB) or Boehringer Mannheim Corporation. Nu-serum was from Collaborative Biomedical Products. The pGEM-T vector was from Promega. The Maxi-Oligo(dT) Cellulose Spin Column was purchased from 5′ and 3′, Boulder, CO. IEC-6 cells and HeLa cells were obtained at the UCSF Cell Culture Facility, original stocks were from ATCC.

Methods

RT-PCR Cloning of SPNT$_{int}$

The cDNA of SPNT$_{int}$ was isolated from a rat intestinal epithelial cell line, IEC-6, by reverse transcriptase polymerase chain reaction (RT-PCR). Briefly, IEC-6 cells were cultured in DME H21 medium containing 10% Nu-serum. When the cells formed a confluent monolayer, total cellular RNA was isolated using the Trizol™ Reagent. Poly (A$^+$) RNA was isolated using the Maxi-Oligo(dT) Cellulose Spin Column. First strand cDNA was synthesized from 0.5 µg mRNA using the Superscript Preamplication System. Selective PCR amplification was performed using two primers (sense: 5'-CCTCCACTT CCTGCTTGTGAGAGA-3', and antisense: 5'-CACTTTATTACAGAAAGCT TTTTAGTAATG-3') derived from the 5' and 3' flanking regions of the published rat SPNT sequence. PCR was performed under the following conditions: 94°C for 1 min, 50°C for 1.5 min, 72°C for 2 min , 30 cycles followed by a final 15 min incubation at 72°C. The resulting 2.8 kb PCR product was subcloned into the pGEM-T vector to form the pGEM.SPNT$_{int}$ construct. pGEM.SPNT$_{int}$ was subjected to restriction analysis and a T7 promoter-oriented construct was selected. The sequence of the T7-oriented pGEM.SPNT$_{int}$ was determined by automated DNA sequencing (Applied Biosystems Inc.). The resulting sequence predicted a protein nearly identical to the published SPNT

except the 419 alanine residue was substituted by a glycine residue. Two PCR products were sequenced and both showed the same glycine substitution at position 419.

Generation of the Expression Construct

To express rat $SPNT_{int}$ in HeLa cells, the 2.8 kb cDNA of $SPNT_{int}$ was removed from the pGEM-T vector and subcloned into the mammalian expression vector (pcDNA3). Briefly, $pGEM.SPNT_{int}$ was linearized with *SstII* and blunt-ended with T4 DNA polymerase. Subsequently the 2.8 kb insert was removed by a *NotI* cut and ligated into pcDNA3 at the *EcoRV/ NotI* sites, resulting in the $pcDNA3.SPNT_{int}$ construct. This construct was confirmed by restriction enzyme analysis.

DNA Isolation

DNA for the transfection studies was isolated with the Qiagen Endo-free DNA isolation kit. The yield from each isolation was approximately 300-700 µg DNA. Several different DNA preparations were used for this study at concentrations of 2-5 mg/ml of DNA. The DNA was resuspended in endotoxin-free TE buffer (Qiagen) and the concentration determined by spectroscopy.

Transfection

HeLa cells were grown in a 5% CO_2/ 95% air, humidified environment. Cells were seeded at a density of 3.6×10^5 cells/well in 12 well plates 24 hours prior to transfection. Lipid (LIPOFECTAMINE™) was used as the vehicle to deliver DNA to the cells following a modified protocol from Gibco/ BRL. For each well 0.1 ml of Opti-MEM media was incubated with 2 µg of DNA and 0.1 ml of media was incubated with 6 µg of lipid. The two solutions were mixed together and then incubated for 20 minutes at room temperature. Following incubation, 0.8 ml Opti-MEM per well was added to the previous mixture and 0.1 ml of this mixture was applied to the cells in each well. Transfection time was 16

hours and this was stopped by aspiration of the transfection medium and addition of fresh MEM containing 10% FBS.

Uptake Measurements

In general, uptake studies were carried out as follows. Cells were washed twice with Na^+-free buffer. This wash solution was aspirated and the reaction mixture of either ^3H-inosine or ^3H-uridine was added to the well and incubated for the given time. Uptake of 1 μM ^3H-inosine (70 nM of radiolabeled inosine plus 1 μM unlabeled inosine) or 5 μM ^3H-uridine (50 nM of radiolabeled uridine plus 5 μM unlabeled uridine) was measured over time. All uptake assays were carried out in the presence of 10 μM nitrobenzylthioinosine (NBMPR). Uptake was measured in the presence of Na^+(128 mM NaCl, 4.73 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 5 mM HEPES, pH 7.4) or absence of Na^+ in which Na^+ is replaced by choline (128 mM). The uptake was stopped by aspirating the reaction mixture and washing 3 times with ice-cold Na^+-free buffer. Cells were then solubilized with 1 ml 0.5% Triton-X 100 and 0.5 ml was sampled for scintillation counting.

Inhibition Studies

Inhibition experiments were carried out in duplicate or triplicate for 5 min. Data are presented as the mean ± SD. Uptake assays were stopped as stated above. All inhibition studies were carried out in the presence and absence of Na^+. Initial studies were also carried out with transfection of empty vector. The concentrations of the inhibitors are specified in the figure and table legends.

Protein Assay

For each plate used in an uptake study, 2-3 wells were reserved for protein analysis. Cells were washed twice with Na^+-free buffer and then solubilized with 0.5 ml

of 1 N NaOH. The solution was neutralized by addition of 0.5 ml of 1 N HCl. A sample

was taken for each protein assay. The Bradford method was used to carry out the assay

using the Bio-Rad reagent. Bovine serum albumin (BSA) was used to generate the

standard curve. Absorbance was read at 595 nm and the amount of protein/well was

calculated from the standard curve.

Data Analysis

Unless otherwise specified, data are expressed as mean ± standard deviation (SD)

of uptake values obtained in 2 to 3 wells. For Michaelis-Menten studies, rate of uptake

was expressed as pmol/mg protein/5 min. Data were fit to the equation $V = V_{max}$

$[S]/(K_m+[S]) + (k_{ns}[S])$ where V is the rate of inosine or uridine uptake, [S] is the inosine

or uridine concentration, k_{ns} is a constant which represents non-specific uptake expressed

in pmol/mg protein/ 5 min. The k_{ns} was the slope of the plot of rate of uptake versus

inosine or uridine concentration in the presence of Na^+ in cells transfected with empty

vector. The Kaleidagraph fitting program was used to fit the data and parameter estimates

are expressed as a mean ± SE. For IC_{50} studies, data were fit to the equation $V = V_0 /[1+$

$(I/IC_{50})^n + 3.69]$ where V is the uptake of inosine in the presence of inhibitor, V_0 is

inosine uptake in the absence of inhibitor, I is the inhibitor concentration, n is the slope,

and the constant 3.69, determined experimentally, represents uptake (expressed as

pmol/mg protein/ 5 min) in the absence of Na^+. For determination of statistical

significance, Student's unpaired t-test was used and $p < 0.05$ (one tail) was considered

significant.

Results

Initial Characterization of SPNT$_{int}$ Expression in Transfected HeLa Cells.

Before transfection studies, we examined the endogenous uptake of ^{3}H-uridine in confluent HeLa cell monolayers. Our results confirmed that there was no detectable Na$^+$-dependent uptake of ^{3}H-uridine suggesting that HeLa would be an appropriate model for SPNT$_{int}$ transfection (data not shown). Endogenous nucleoside uptake was inhibited by NBMPR at a concentration of 10 μM; therefore, NBMPR was included in uptake studies.

Preliminary characterization of the transporter transfected in HeLa cells was necessary to determine an initial time, in the linear range, for further kinetic studies. Following incubation with ^{3}H-inosine, uptake in the presence of Na$^+$ increased with time in the transfected cells, while that in the absence of Na$^+$ did not (Fig.1). Na$^+$-stimulated ^{3}H-inosine uptake in HeLa cells transfected with pcDNA3 vector which did not contain the insert (empty vector) was similar to uptake in the absence of Na$^+$ in cells transfected with SPNT$_{int}$ (Fig. 1). Na$^+$-stimulated ^{3}H-inosine uptake in the transfected HeLa cells was linear at early times (1, 3 and 5 min). At later times, i.e., 60 min (data not shown), uptake began to equilibrate. Five minutes was selected for kinetic studies because it was in the linear range and allowed maximal differentiation between Na$^+$-dependent and independent uptake. Thin layer chromatography (TLC) demonstrated that at 5 min less than 10% of ^{3}H-inosine is metabolized in the transfected HeLa cells (data not shown).

The expression level of SPNT$_{int}$ at several times following transfection was determined. At 36 h post-transfection, Na$^+$-dependent ^{3}H-inosine uptake was significantly greater than uptake in transfected cells in the absence of Na$^+$ (46.2 ± 1.37 vs 26.2 ± 0.61, p < 0.05 ; Fig. 2). Na$^+$-dependent ^{3}H-inosine uptake continued to increase and was enhanced approximately threefold in comparison to Na$^+$- independent uptake at 66 hours following transfection. Expression began to decline at 90 hours (Fig.2) and was not
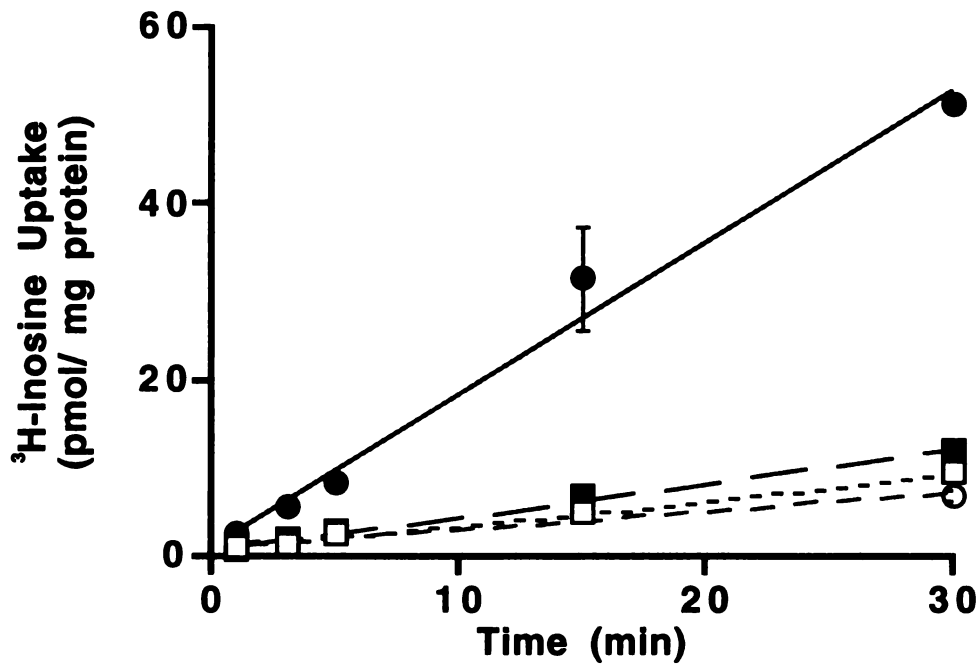
Figure 1. Time course of [^{3}H]inosine (1 μM) uptake in the presence and absence of Na$^+$.

The uptake of [^{3}H]inosine was measured over time in the presence ($\bullet$) and absence($\blacksquare$) of

Na$^+$ in HeLa cells transfected with SPNT$_{int}$. Control uptake of [^{3}H]inosine was carried out

in cells transfected with pcDNA3 vector without the SPNT$_{int}$ insert (empty vector) in the

presence (O) and absence ($\square$) of Na$^+$.
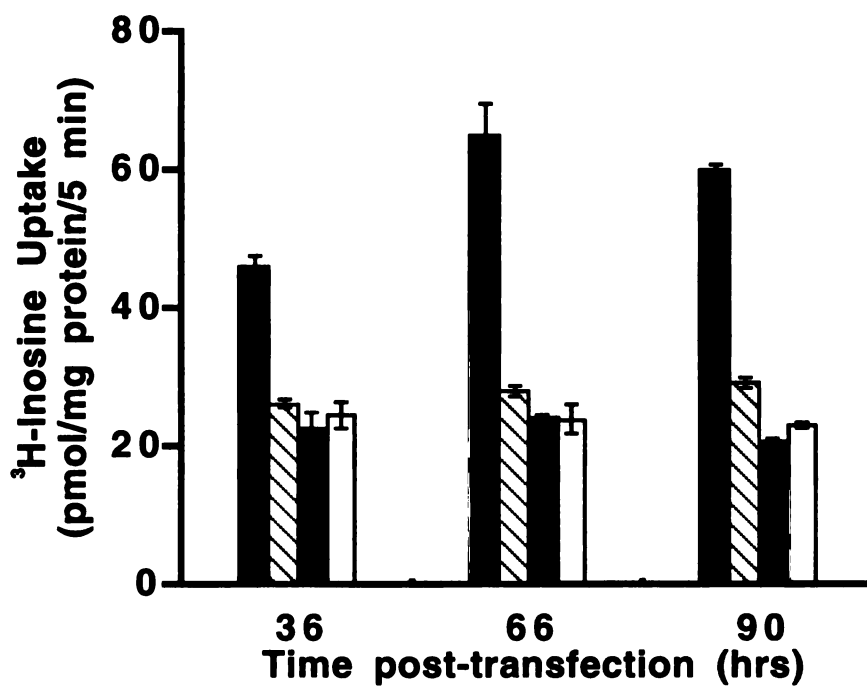
Figure 2. Expression of transfected SPNT$_{int}$ in HeLa cells over time. Following transfection of SPNT$_{int}$ in HeLa cells, the 5 min uptake of [^{3}H]inosine was measured at 36, 66, and 90 hours post-transfection in the presence (solid bars) and absence (hatched bars) of Na$^+$. Control [^{3}H]inosine uptake in cells transfected with empty vector was also measured in the presence (shaded bars) and absence (open bars) of Na$^+$ at 5 min.

present 120 hours after the initial transfection (data not shown). For further studies, uptake of the model compounds was measured between 36 and 90 h after transfection.

Michaelis-Menten Studies of ^{3}H-inosine and ^{3}H-uridine

The rates of uptake of the nucleosides, ^{3}H-inosine (Fig. 3A) and ^{3}H-uridine (Fig. 3B), were studied as a function of concentration in the range of 1 μM to 500 μM. Because the rate of uptake was linear at the higher concentrations, an equation consisting of both a saturable and a linear term was used in the fits. Data at the lower concentrations are presented in the Fig. 3 and data at all concentrations are presented as insets. Both compounds exhibited saturation kinetics. The K_m and V_{max} (mean $\pm$ SE) values for inosine were 28.1 $\pm$ 7.1 μM and 343 $\pm$ 42 pmol/mg protein/5 min. For uridine, the respective values were 20.6 $\pm$ 5.6 μM and 543 $\pm$ 51.7 pmol/mg protein/5 min. The k_{ns} for inosine and uridine were 1.9 and 4.3 pmol/mg protein/5 min, respectively.

Inhibition Studies

We examined the uptake of ^{3}H-inosine in the presence of several endogenous nucleosides and nucleoside analogs each at a concentration of 1 mM. Significant inhibition of ^{3}H-inosine uptake (Fig. 4A) ($p < 0.05$) was observed in the presence of the endogenous nucleosides uridine and inosine. Thymidine produced less inhibition of ^{3}H-inosine uptake. Significant inhibition ($p < 0.05$) of ^{3}H-inosine uptake was observed in the presence of ddI, ddA, acyclovir, and 2CdA (Fig. 4B). Similar results were obtained when ^{3}H-uridine was used as the model compound (Table 1). Further characterization of the inhibition by ddI and 2CdA on ^{3}H-inosine uptake was carried out due to the clinical importance of these two compounds. The IC_{50}'s of ddI and 2CdA were 46 μM (Fig. 5A) and 13 μM (Fig. 5B), respectively.
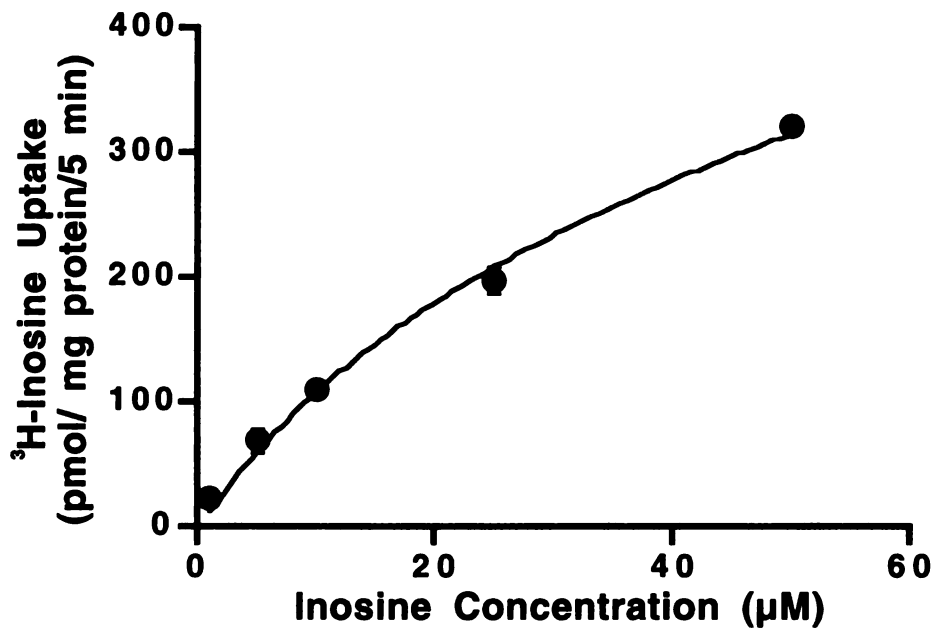
Figure 3A. Saturability of [³H]inosine uptake. Uptake of [³H]inosine was measured in the presence of increasing concentrations of inosine. Uptake was measured at 5 min and data were fit to the equation $V = V_{max} *[S]/(K_m+[S])+k_{ns}*[S]$). Data from Na^+-dependent uptake, adjusted for non-specific uptake (see Methods), are presented as mean ± SE (n=3) of a representative experiment.
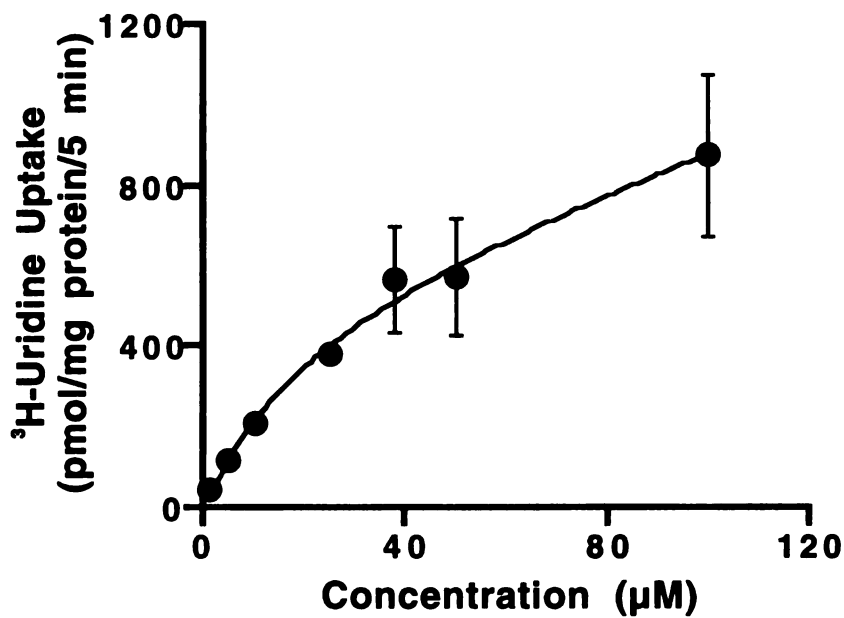
Figure 3B. Saturability of [³H]uridine uptake. Uptake of [³H]uridine was measured in the

presence of increasing concentrations of uridine in the presence and absence of Na^+.

Uptake was measured at 5 min and data were fit to the equation $V=$

$V_{max}[S]/(K_m+[S])+k_{ns}[S]$. Data from Na^+-dependent uptake (see Methods) are presented

as mean ± SE (n=3) of a representative experiment.
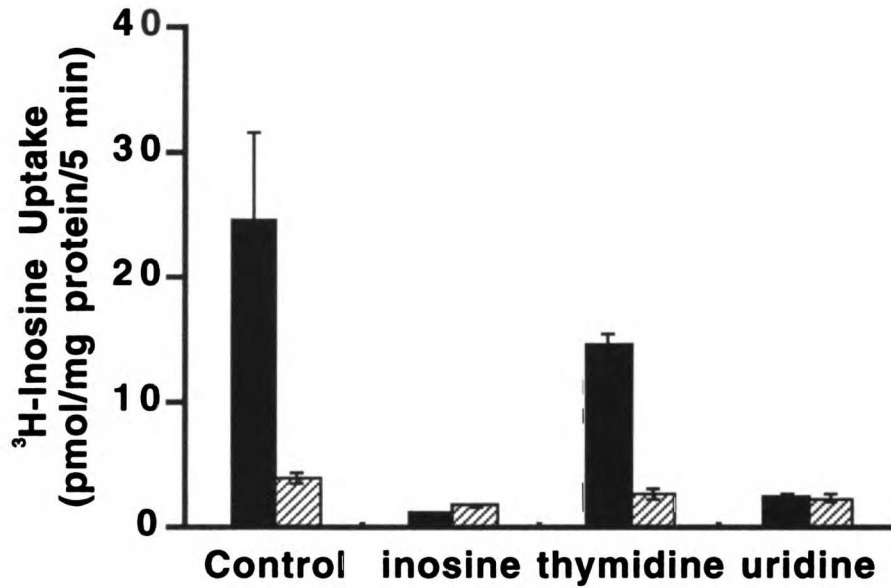
Figure 4A. Inhibition of [³H]inosine uptake. Uptake of [³H]inosine (1 μM) was measured in the presence or absence (Control) of 1 mM of the given compound in the presence (solid bars) and absence (hatched bars) of Na^+ in HeLa cell transfected with $SPNT_{int}$. Uptake was carried out for 5 min and is presented mean ± SD.
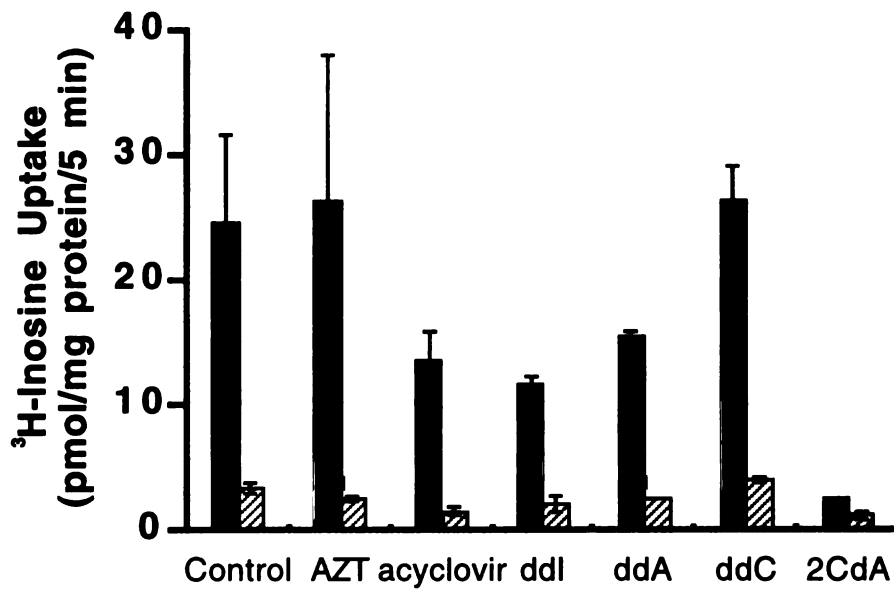
Figure 4B. Inhibition of [^{3}H]inosine uptake. Uptake of [^{3}H]inosine (1 μM) was measured in the presence or absence (Control) of 1 mM of the given compound in the presence (solid bars) and absence (hatched bars) of Na^+ in HeLa cell transfected with $SPNT_{int}$. Uptake was carried out for 5 min and is presented as mean $\pm$ SD.

Table 1. Inhibition of ^{3}H-Uridine Uptake. Uptake of 5 μM ^{3}H-uridine was measured in the presence of 1 mM of the given compound. Uptake was for 5 min and is presented as Na^+-dependent uptake in cells transfected with $SPNT_{int}$ or with empty vector. Control cells represent Na^+-dependent uptake without inhibitor. Data represent the mean ± SD of results from 2 to 3 wells each.

	HeLa cells transfected with $SPNT_{int}$ (pmol/mg protein/5 min)	HeLa cells transfected with empty vector (pmol/mg protein/5 min)
Control	61.6 ± 0.47	17.3 ± 1.51
Inosine	19.5 ± 0.28	18.1 ± 1.40
Thymidine	41.9 ± 2.43	16.9 ±1.87
Uridine	20.9 ± 2.49	17.3 ± 2.51
AZT	53.9 ± 1.18	18.7 ± 0.93
2-Chloroadenosine	17.9 ± 1.03	13.9 ± 0.99
[a]without Na^+	18.7 ± 0.60	17.4 ± 0.25

[a]Uptake of ^{3}H-uridine in the absence of Na^+.

[b]All values are significantly different from control ($p<0.05$).
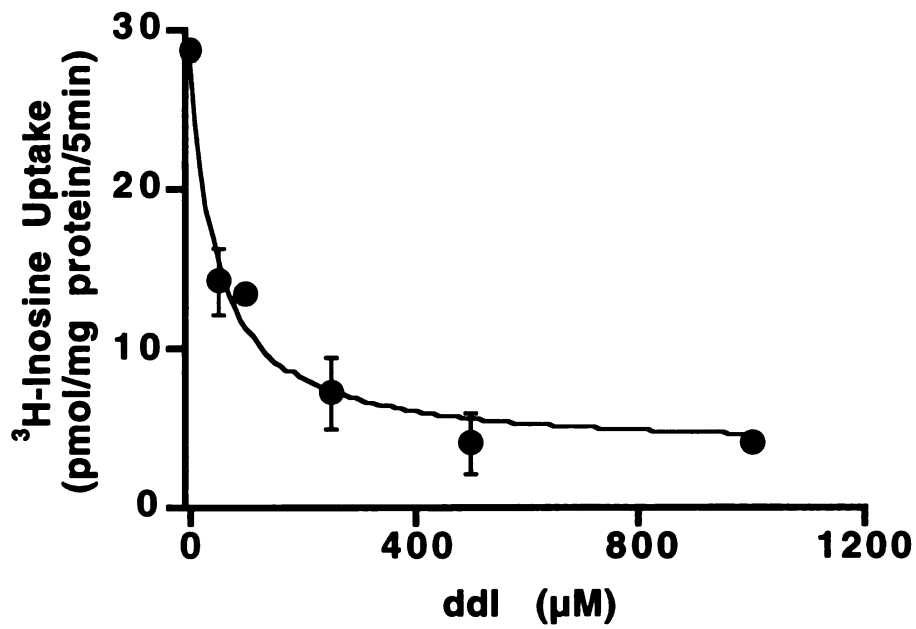
Figure 5A. IC_{50} of ddI: Uptake of [^{3}H]inosine was measured in the presence of increasing

concentrations of unlabeled ddI in the presence of Na^+. Data were fit to the equation $V = V_o$

$/[1 + (I/IC_{50})^n + 3.69]$ (see Methods). Data are presented as mean $\pm$ SE.
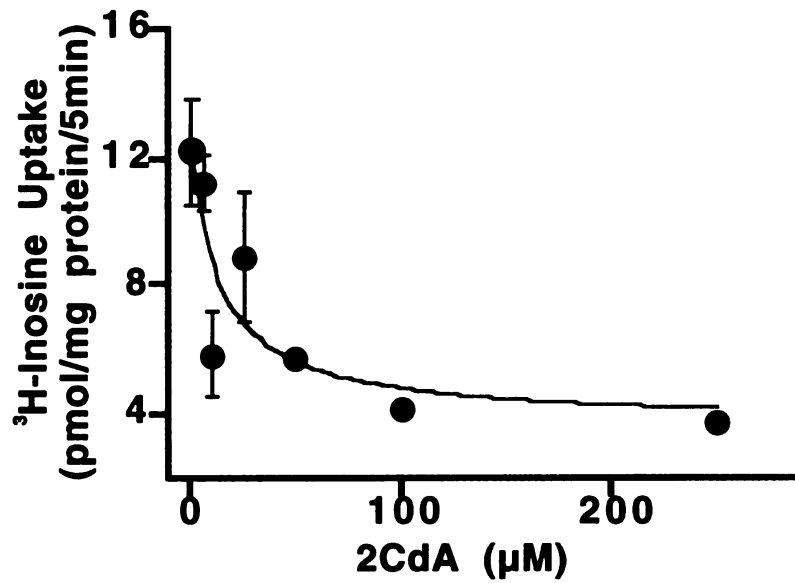
Figure 5B. IC_{50} of 2CdA. Uptake of [^{3}H]inosine was measured at 5 min in the presence

of increasing concentrations of 2CdA in a Na^+-containing buffer. Data were fit to the

equation $V = V_0 / [1 + (I/IC_{50})^n + 3.69]$ (see Methods). Data are presented as mean ± SE.

Permeant Studies

To determine whether 2CdA and ddI were also permeants of SPNT, uptake studies were performed with radiolabeled compounds. Na^+-dependent ^3H-2CdA uptake (at 5 min) was enhanced approximately 3-fold over uptake in the absence of Na^+ (Table 2) whereas the Na^+-dependent uptake of ^3H-ddI was not enhanced, even at later times (data not shown). These data suggest that 2CdA is a permeant of $SPNT_{int}$ whereas ddI is not.

Significant inhibition of ^3H-2CdA uptake was observed in the presence of both unlabeled 2CdA (1 mM) and unlabeled inosine (1 mM) (Table 2).

Table 2. ^{3}H-2CdA uptake in HeLa cells transfected with SPNT$_{int}$. ^{3}H-2CdA uptake was measured at 5 min at 25°C in the presence of Na$^+$ (control) and 1 mM 2CdA, inosine or thymidine. Data represent the mean ± SD of results from 2 to 3 wells each and are presented as pmol/mg protein/5 min.

Control	6.85 ± 0.60
Inosine	[a]2.91 ± 0.23
Thymidine	4.69 ± 1.58
2CdA	[a]2.23 ± 0.06
[b]without Na$^+$	[a]2.99 ± 0.18

[a]Data are statistically different from control (p<0.05).

[b]The control for this study was 8.87 ± 0.42 pmol/mg protein/5 min.

Discussion

Nucleoside analogs are currently being used in the treatment of infections of viruses such as cytomegalovirus, hepatitis B virus, HIV (21, 22), and herpes simplex virus. Furthermore, antineoplastic agents such as 2CdA are important in cancer chemotherapy (23). The endogenous nucleoside, adenosine, is used in the treatment of arrhythmias (24). Understanding the interaction of these analogs with the purine-selective nucleoside transporter cloned from intestine may lead to a better understanding of the oral absorption of such compounds (25). Interactions of these compounds with nucleoside transporters may therefore be clinically important.

HeLa cells have been used for the expression of several transporters, including the human placental folate transporter (19) and a Na^+-dependent L-proline transporter cloned from rat brain (20). HeLa cells possess equilibrative nucleoside processes which have been well characterized (26) but do not exhibit Na^+-dependent nucleoside uptake. In this study, we demonstrated the functional expression, following transfection, of a saturable, Na^+-dependent, purine-selective nucleoside transporter ($SPNT_{int}$) in HeLa cells. Both uridine and inosine, known permeants of the purine-selective transporter were found to exhibit saturable and inhibitable uptake. The K_m of inosine in HeLa (28 μM) is slightly greater than that in *Xenopus laevis* oocytes (14.6 μM) (unpublished data from this laboratory). The reason for this difference is unknown, but may be due to differences in experimental conditions, membrane effects on protein tertiary structure or to differences in processing of the protein between oocytes and HeLa. Comparative data for uridine kinetics are not available.

Inhibition studies confirmed the expected substrate selectivity, based on previous functional studies of SPNT in *Xenopus laevis* oocytes (3, 7, 17). Studies on the interaction of dideoxy- nucleoside analogs with SPNT suggest that ddI and ddA, but not AZT and ddC, significantly inhibited the uptake of inosine by $SPNT_{int}$. Because AZT and ddC are pyrimidine analogs whereas ddI and ddA are purine analogs, the data are

consistent with the known purine selectivity of SPNT. Both AZT and ddC have been shown to interact with the pyrimidine-selective nucleoside transporter, cNT1 in transfected COS-1 cells (27). Since ddI is administered orally and has a low bioavailability, we further characterized its interaction with $SPNT_{int}$. Although ddI is a reasonably potent inhibitor of inosine transport by $SPNT_{int}$, it does not appear to be a permeant. These data suggest that $SPNT_{int}$ does not play a role in the intestinal absorption of ddI.

In contrast, 2CdA (2-chloro, 2'-deoxyadenosine) appears to function as both an inhibitor (IC_{50} = 13.8 µM) and a permeant of $SPNT_{int}$ (Fig. 5B and Table II). 2CdA has been shown to be effective in the treatment of hairy cell leukemia (28, 29). Efficacy of 2CdA has also been demonstrated in chronic lymphocytic leukemia (30), non-Hodgkin's lymphoma, and cutaneous T-cell lymphoma (28). Furthermore, although marketed as an injectable formulation, clinical studies have shown that 2CdA is orally available and well tolerated by patients (28, 29). Our data suggest that $SPNT_{int}$ may play a role in the oral absorption of 2CdA. The system described here may serve as a model in which to further study the interaction of 2CdA as well as other nucleoside analogs with $SPNT_{int}$.

In summary, we have described the first transient expression system for the purine-selective nucleoside transporter, $SPNT_{int}$, in HeLa cells. We have also demonstrated for the first time that the antineoplastic agent, 2CdA, is a substrate for $SPNT_{int}$. This expression system is simple, reliable and reproducible and may be used to study drug interactions with this carrier as well as to address questions related to the function and intracellular processing of the protein.

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CHAPTER 4

FUNCTIONAL CHARACTERIZATION OF A HUMAN Na⁺-DEPENDENT, PURINE-SELECTIVE, NUCLEOSIDE TRANSPORTER (hSPNT1) IN A MAMMALIAN EXPRESSION SYSTEM

By selective reabsorption and secretion, transporters in the kidney play a role in maintaining total body and tissue specific homeostasis of naturally-occurring nucleosides (1-5). These transporters may be particularly important in regulating the local concentrations of nucleosides such as adenosine which have pronounced effects on renal function (6, 7). There have been limited studies investigating the disposition of endogenous nucleosides in the human kidney. Kuttesch and co-workers observed that adenosine was reabsorbed whereas 2'-deoxyadenosine was actively secreted in patients lacking adenosine deaminase or treated with the deaminase inhibitor, deoxycoformycin (7, 8). In addition, the 2'-deoxyadenosine analog, 2-chloro-2'-deoxyadenosine (2CdA or cladribine) is actively secreted by the human kidney (7). The transporters responsible for the reabsorption of adenosine and the secretion of 2'-deoxyadenosine and 2CdA are clearly distinct, but have not been identified to date.

With the cloning of several Na⁺-dependent nucleoside transporters (1, 2, 9, 10) the understanding of these carrier proteins at a molecular level is increasing rapidly. Recently, the cDNA encoding a purine-selective Na⁺-dependent nucleoside transporter was cloned from human kidney in this laboratory (1). Northern blot analysis revealed a broad distribution of multiple hSPNT1 transcripts, with expression in liver, intestine, pancreas, heart, skeletal muscle and in human kidney (1). In contrast, mRNA transcripts of the rat homolog, rSPNT, were not detected in rat kidney by Northern blotting methods (10) and only a weak partial transcript was detected in rat kidney by RT-PCR (11). These data suggest that there may be species differences in the renal handling of purine nucleosides.

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Furthermore, it is not known whether there are intrinsic differences in the functional characteristics of the cloned human and rat purine-selective transporters.

Mammalian expression systems have been used previously in this and other laboratories to characterize the function of the cloned purine and pyrimidine selective nucleoside transporters from rat (12, 13). The goal of this study was to develop a transiently transfected mammalian expression system for hSPNT1 and to elucidate its functional characteristics and interactions with nucleosides and nucleoside analogs. In particular, the role of hSPNT1 in the disposition of adenosine and its 2'-deoxy analogs, 2CdA and 2'-deoxyadenosine, was examined.

Materials

HeLa cells were purchased from the American Type Culture Collection (ATCC). Minimum Essential Media (MEM), fetal bovine serum, penicillin-streptomycin, and fungizone were obtained from the University of California, San Francisco Cell Culture Facility. ddA, ddC, ddI, uridine, thymidine, inosine, adenosine, guanosine, nitrobenzylthioinosine, 2CdA, 2'-deoxyadenosine, and acyclovir were purchased from Sigma. The DNA isolation kit was obtained from Qiagen. The radioisotopes [^{3}H]inosine (S.A. 27.8 Ci/mmol), [^{3}H]uridine (S.A. 43.8 Ci/mmol), [^{3}H]adenosine (S.A. 45.8 Ci/mmol), [^{3}H]2'-deoxyadenosine (27.8 Ci/mmol), [^{3}H]2CdA (S.A. 3.8 Ci/mmol), and [^{3}H]ddI (S.A. 38 Ci/mmole) were from Moravek. Lipofectamine and Opti-MEM media were supplied by Gibco/ BRL. Cell culture plates from Nunc and 12-well plates from Costar were used to maintain the cells and for uptake studies, respectively. The Bradford reagent for protein studies was purchased from Bio-Rad. Nitrobenzylthioinisine was dissolved in DMSO and was used at a concentration of 10 μM in all uptake studies to inhibit the endogenous equilibrative nucleoside transporter, which is expressed in HeLa cells (14).

Methods

Transfection

The cDNA encoding hSPNT1 was subcloned into the pcDNA3 vector. Plasmid DNA was prepared using the Qiagen Maxi-prep kit as previously described (13). An initial titering of the lipid demonstrated that significant expression of the transporter was observed with DNA to lipid ratios between 1:4 and 1:6. Maximal expression was observed at a ratio of 1:6 for the cDNA encoding hSPNT1; therefore this ratio was used for the study. HeLa cells were seeded at a density of 2 x10^5 cells/ well 24 hours prior to transfection. Transfection was carried out as previously described (13).

Permeant Studies

Uptake studies were carried out 36 to 72 hours after transfection. Uptake was measured in the presence of Na^+ (128 mM NaCl, 4.73 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 5 mM HEPES, pH 7.4) or absence of Na^+ (Na^+ is replaced by 128 mM choline). The uptake was stopped by aspirating the reaction mixture and washing 3 times with ice-cold Na^+-free buffer. Cells were then solubilized with 1 ml 0.5% Triton-X 100 and 0.5 ml was sampled for liquid scintillation counting (Beckman, Palo Alto, CA).

For uptake studies with [^{3}H]nucleosides a tracer amount of labeled compound was used plus 1 µM of unlabeled compound. Protein concentrations were determined using the Bradford reagent, as described previously (13). Nitrobenzylthioinisine was added to uptake medium at a concentration of 10 µM in all uptake studies to inhibit the endogenous equilibrative nucleoside transporter, which is expressed in HeLa cells (14, 15).

Inhibition Studies

For inhibition studies, [^{3}H]inosine was used as the permeant and the amount of unlabeled compound used is indicated in the table or figure legends. In general, this concentration was 0.5 mM. These experiments were carried out in duplicate or triplicate for 5 min. Data are presented as the mean ± SD. Uptake assays were stopped as stated above. Inhibition studies were carried out in the presence and absence of Na^+. As a control, Na^+-dependent uptake was analyzed in cells transfected with empty vector.

Data Analysis

In general, data are expressed as mean ± standard deviation (SD) of uptake values obtained in at least 2-3 wells. For Michaelis-Menten studies, rate of uptake was expressed as pmol/mg protein/5 min. Data were fit to the equation $V = V_{max}[S] / (K_m + [S])$ where V is the rate of inosine or uridine uptake and [S] represents the concentration of inosine or uridine. The Kaleidagraph® fitting program was used to fit the data and parameter estimates are

expressed as a mean $\pm$ SE. For IC_{50} studies, data were fit to the equation V= V_o /[1+

$(I/IC_{50})^n$] where V is the uptake of inosine in the presence of inhibitor, V_o is inosine uptake

in the absence of inhibitor, I is the inhibitor concentration, and n is the Hill coefficient. An

unpaired Student's t-test from Primer of Biostatistics software (Version 3, by Stanton A.

Glantz, McGraw-Hill, 1991) was used for determination of statistical significance, and P <

0.05 was considered statistically significant.

Results

Uptake and Inhibition Studies

The uptake of ^{3}H-inosine in the presence and absence of Na$^+$ was linear up to 30

min (data not shown) in HeLa cells transiently transfected with hSPNT1 cDNA. An initial

time point of 5 min was used for further kinetic studies. At 0.5 mM, the purine

nucleosides, adenosine, guanosine and inosine, significantly inhibited Na$^+$-dependent

[^{3}H]inosine uptake whereas the pyrimidine nucleosides, thymidine and cytidine did not

(Fig. 1). The pyrimidine nucleoside, uridine, which is a substrate (3, 17-19) of all known

mammalian nucleoside transporters, also significantly inhibited [^{3}H]inosine uptake

(P<0.05).

The interaction of various therapeutic nucleoside analogs with the transporter was

studied. The purine nucleoside analogs, ddI and ddA (0.5 mM) significantly inhibited the

Na$^+$-dependent uptake of [^{3}H]inosine, whereas the pyrimidine nucleoside analog, ddC, did

not significantly inhibit [^{3}H]inosine uptake at concentrations up to 1 mM (Table 1). The

IC_{50} of ddI in interacting with hSPNT1 was 19 μM which is similar to that obtained for

rSPNT (Fig. 2) (13). In contrast, the IC_{50} for 2CdA in interacting with hSPNT1 is

significantly different from that obtained previously for the rat homolog, rSPNT, in

transfected HeLa cells (479 μM versus 13 μM) (Fig. 3).
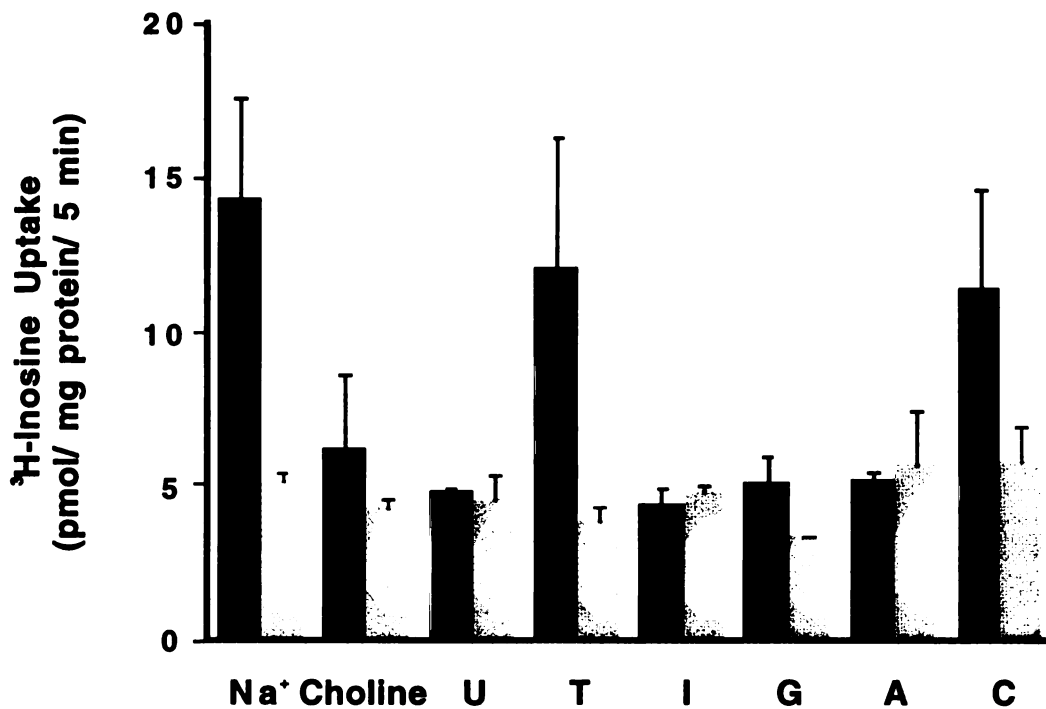
Figure 1. The effect of nucleosides on the Na^+-dependent uptake of [^{3}H]inosine in HeLa cells transiently transfected with the cDNA encoding hSPNT1. Solid bars indicate data obtained in cells transfected with hSPNT1 cDNA and gray bars indicate data obtained in cells transfected with empty vector. (A: adenosine, I: inosine, T: thymidine, U: uridine, G: guanosine, C: cytidine). Bars represent mean (± S.D.) of data obtained from 2 to 3 wells in a representative of two experiments.

Table 1. Inhibition of [³H]Inosine Uptake by Nucleoside Analogs in HeLa Cells Transfected with the cDNA of hSPNT1. Uptake of [³H]inosine was measured in the presence and absence ddA, ddC, and ddI (0.5 mM) in HeLa cells transiently transfected with hSPNT1 cDNA. Control represents Na⁺-dependent uptake of [³H]inosine in HeLa cells expressing hSPNT1. [³H]Inosine uptake in the absence of Na⁺, in which Na⁺ is replaced by choline, was also measured.

Compound	Uptake (pmol/ mg protein/ 5 min) (Mean ± SD)
Control	77.7± 9.71
Absence of Na⁺	9.77± 0.13*
ddA	18.4± 0.50*
ddC	54.3± 1.37
ddI	5.17± 0.37*

* Indicates significantly different from control (P< 0.05)
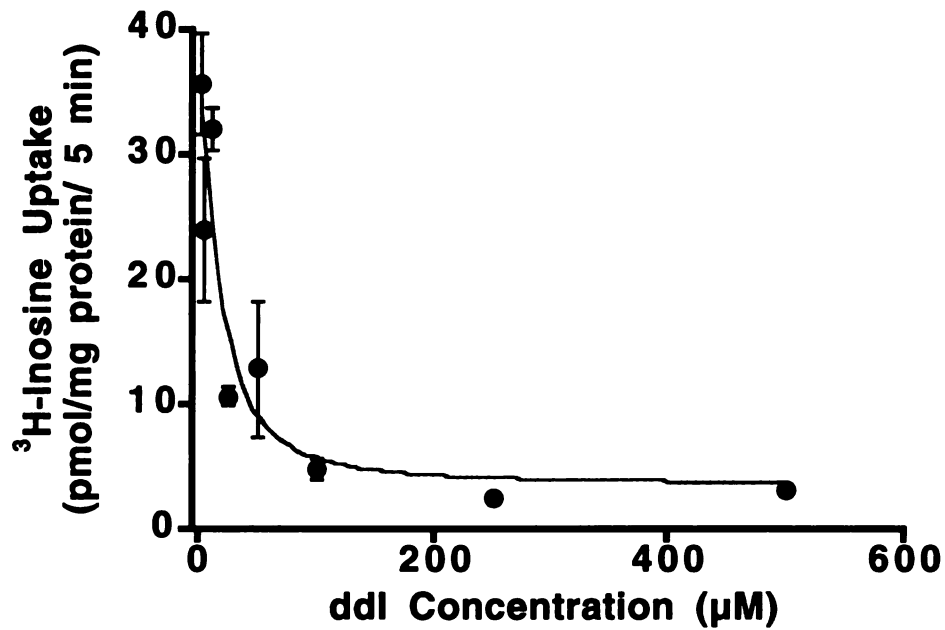
Figure 2. The effect of ddI on the Na^+-dependent uptake of [^{3}H]inosine was determined in HeLa cells transfected with the cDNA encoding hSPNT1. [^{3}H]Inosine uptake was measured in the presence of Na^+, and of increasing concentrations of ddI. Data were fit to the equation: $V = V_0 /[1 + (I/IC_{50})^n]$. Points represent the mean ($\pm$ S.D.) of data obtained from 2 to 3 wells in a single experiment. The IC_{50} value for this experiment was 19 μM.
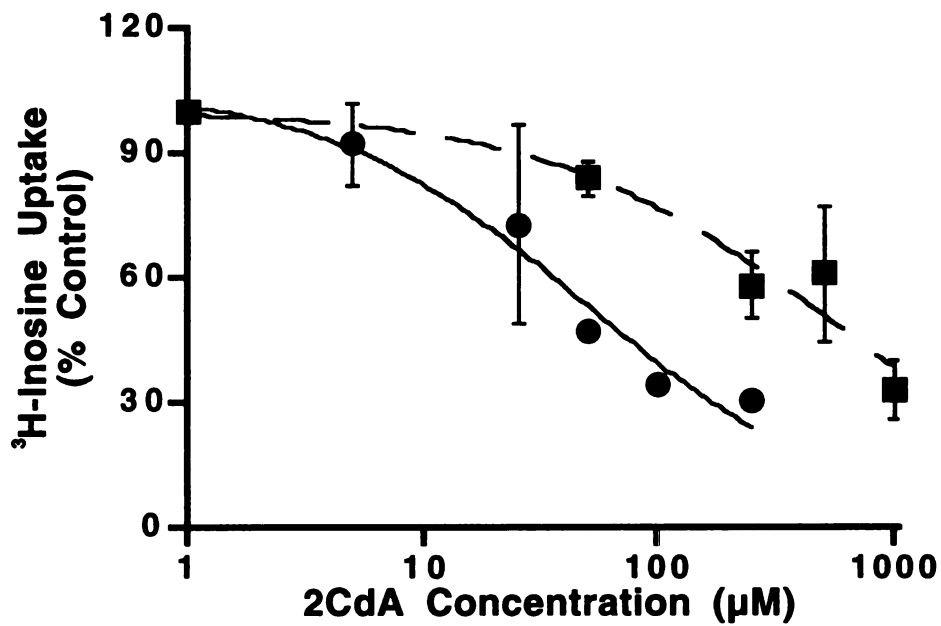
Figure 3. Comparison of the potencies of interaction of 2CdA with hSPNT1 (squares, this study) and rSPNT (circles, (13)). Uptake of [^{3}H]inosine was measured at 5 min in the presence of various concentrations of 2CdA in cells transfected with either the cDNA encoding hSPNT1 or rSPNT. Points represent mean (± S.D.) of data obtained from 2 to 3 wells in a representative of two experiments.

Permeant Studies

While inhibition studies do show interaction with the carrier, such studies do not necessarily identify substrates (or permeants) of the transporter. To identify other substrates of hSPNT1, the uptake of several [³H]-labeled nucleosides and nucleoside analogs was determined in HeLa cells transiently transfected with the cDNA of hSPNT. Uptake of [³H]inosine, [³H]uridine, [³H]thymidine, [³H]adenosine, [³H]2'-deoxyadenosine, [³H]2CdA, and [³H]ddI was measured for 5 min in the presence and absence of Na^+ in HeLa cells transiently transfected with hSPNT1 cDNA. [³H]Inosine and [³H]uridine demonstrated significant ($P<0.05$) uptake by this carrier whereas [³H]ddI and [³H]thymidine did not (Fig. 4). [³H]Adenosine showed significant uptake in the cells expressing hSPNT1 whereas [³H]2'-deoxyadenosine and [³H]2CdA did not (Fig. 5). The kinetics of uridine and inosine uptake were further characterized. Both compounds demonstrated saturable uptake in the transfected cells (Table 2); however, in comparison to the interaction of inosine (K_m= 13.7 ± 8.09 µM; V_{max}= 182 ± 40 pmol/ mg protein/ 5 min), the interaction of uridine was characterized by a lower affinity and higher capacity (K_m= 114 ± 44 µM; V_{max}= 728 ± 98 pmol/ mg protein/ 5 min). The Na^+-dependent uptake of [³H]adenosine was significantly higher in HeLa cells transfected with the cDNA encoding rSPNT versus cells transfected with the cDNA encoding hSPNT1 (Fig. 6).
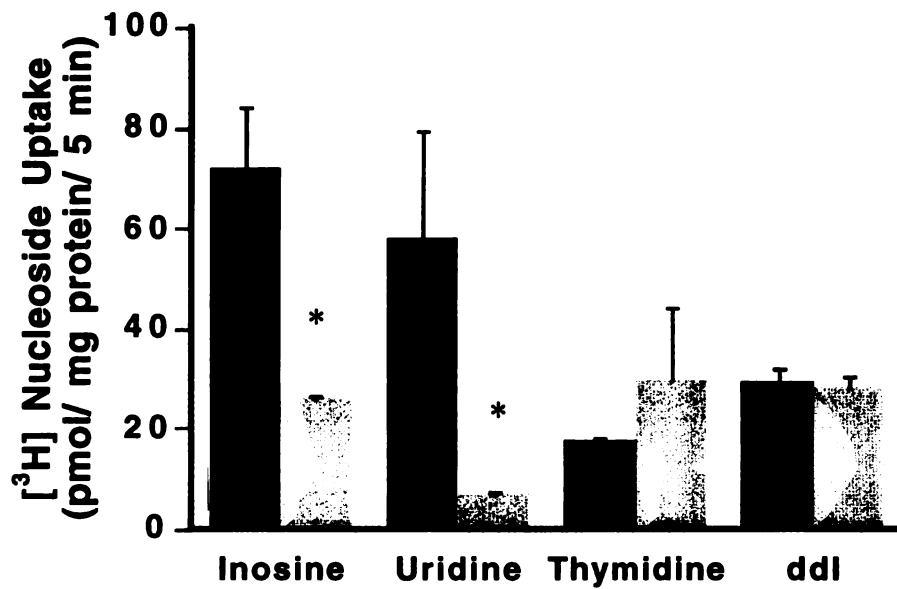
Figure 4. Uptake of [³H]nucleosides and [³H]nucleoside analogs in HeLa cells transiently

transfected with the cDNA encoding hSPNT1. Uptake was carried out for 5 min in the

presence (black bars) and absence (gray bars) of Na^+. Compounds which demonstrated

significant Na^+-dependent uptake ($p < 0.05$) are indicated by *.
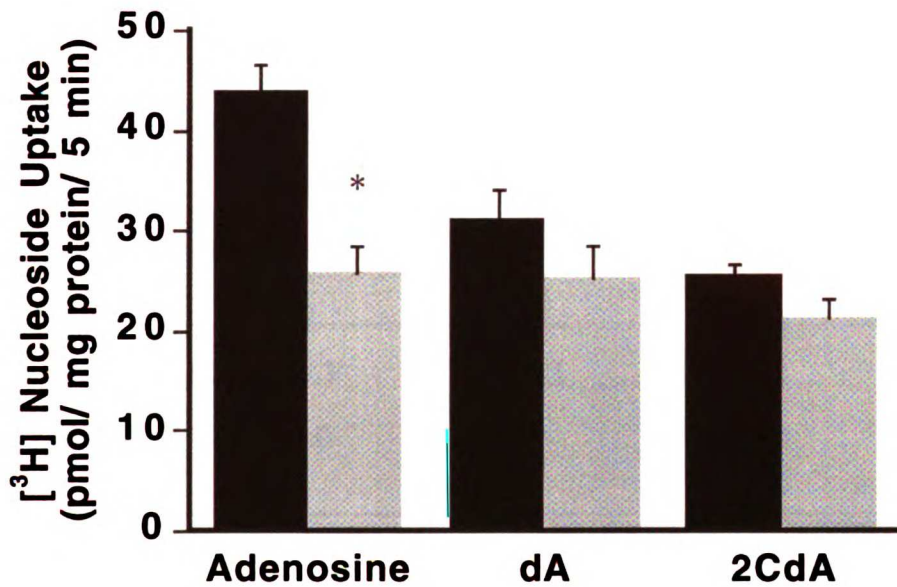
Figure 5. Uptake of [^{3}H]adenosine, [^{3}H]2'-deoxyadenosine , and [^{3}H]2CdA (1 μM) in HeLa cells transiently transfected with the cDNA encoding hSPNT1. Uptake was carried out for 5 min in the presence (solid bars) and absence (gray bars) of Na$^+$. (* indicates substrate with significant Na$^+$-dependent uptake, P< 0.05).

Table 2. Michaelis-Menten Parameters for Nucleoside Uptake in HeLa Cells Transfected with either the cDNA of rSPNT (13) or hSPNT1.

COMPOUND	hSPNT1		rSPNT	
	V_{max} (pmol/mg protein/5 min)	K_m (μM)	V_{max} (pmol/mg protein/5 min)	K_m (μM)
Inosine	182 ± 40.3	13.7 ± 8.1	343 ± 42	28.1 ± 7.1
Uridine	728 ± 98.0	114 ± 44	543 ± 52	20.6 ± 5.6
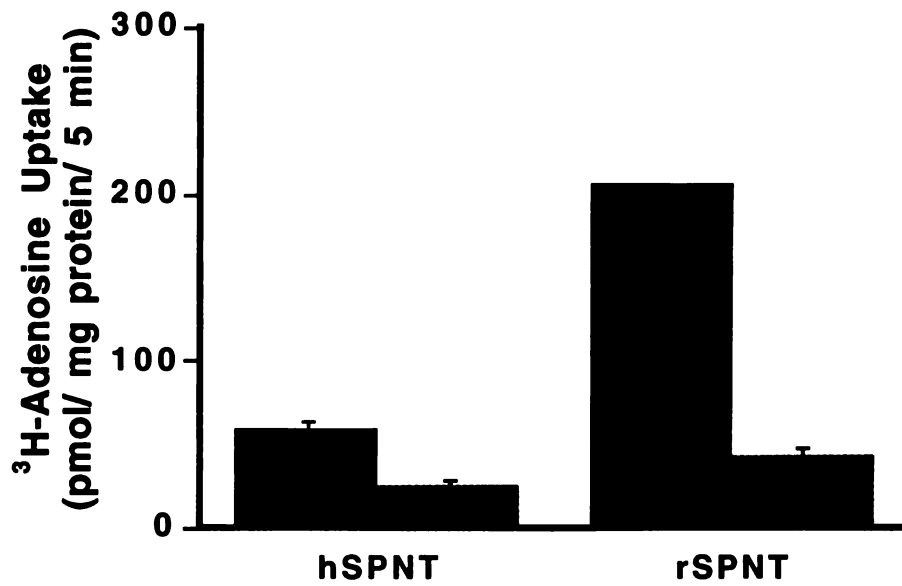
Figure 6. Adenosine uptake in HeLa cells transfected with rSPNT or hSPNT1. Uptake of [³H]adenosine (1 μM) was measured at 5 min in HeLa cells expressing either rSPNT or hSPNT1 in the presence and absence of Na⁺.

Discussion

In this study we demonstrated that hSPNT1 can be transiently expressed in HeLa cells using the technique of lipofection. Similar techniques have been used previously in this laboratory to transiently transfect HeLa with the cDNA of the rat homolog, rSPNT (13). The experimental conditions required for optimum expression of hSPNT1 were essentially identical to those described for rSPNT except that higher lipid to DNA ratios were used in this study (6:1 in comparison to 3:1 used previously).

The kidney plays a vital role in maintaining total body and local purine homeostasis. Limited data suggest that 2'-deoxyribonucleosides are handled differently from ribonucleosides by the human kidney. Namely, 2'-deoxyadenosine and 2CdA are both actively secreted whereas adenosine is actively reabsorbed. Previously, we demonstrated that both adenosine and 2'-deoxyadenosine inhibit [^{3}H]inosine uptake in *X. laevis* oocytes expressing hSPNT1; however, the IC_{50} of adenosine was lower than that of 2'-deoxyadenosine (23 μM vs. 110 μM). In this study, we addressed the question of whether adenosine and 2'-deoxyadenosine are permeants of hSPNT1. Our data demonstrate that adenosine is a permeant of hSPNT1 whereas neither 2'-deoxyadenosine nor 2CdA showed significant uptake by the transporter. However, both compounds showed a slight, albeit not significant, Na^+-dependent uptake suggesting that these compounds may be poor permeants of hSPNT1. Collectively, the data suggest that hSPNT1 may play a role in the reabsorption of adenosine in the human kidney, but does not appear to play a role in the secretion of either 2'-deoxyadenosine or 2CdA.

In this study, the K_m of adenosine for hSPNT1 was 17 μM and was somewhat lower than that obtained previously (50 μM) by Ritzel *et al.* for hCNT1, a human pyrimidine selective transporter in the same gene family (2). The reverse was true for uridine. That is, the K_m of uridine for hSPNT1 was 114 μM and was 45 μM for hCNT1 (2). These data suggest that while both hSPNT and hCNT1 transport adenosine and uridine, the purine -selective transporter has a higher affinity for adenosine and the

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pyrimidine-selective transporter has a higher affinity for uridine. Both transporters may play a role in the reabsorption of adenosine and uridine in the human kidney.

Similar to 2'-deoxyadenosine and 2CdA, the antiviral nucleoside analog, ddI, is actively secreted in the human kidney. Our data demonstrate that ddI was a potent inhibitor (IC_{50} of 19 μM) of ^{3}H-inosine uptake in HeLa cells expressing hSPNT1; however, ddI was not a permeant. These data suggest that hSPNT1 does not contribute to the secretory clearance of either dideoxy or 2'-deoxy-nucleosides

Our results demonstrate possible species differences between the human transporter and that cloned from rat (Table 3). The IC_{50} of 2CdA (479 μM) in inhibiting [^{3}H]inosine uptake in cells expressing hSPNT1 was markedly different from the IC_{50} of 2CdA (13 μM) in cells expressing rSPNT. Furthermore, consistent with previous studies in *Xenopus laevis* oocytes, the uptake of [^{3}H]2CdA in hSPNT1 cDNA transfected cells was only slightly enhanced. In contrast, in HeLa cells transfected with the cDNA of rSPNT (13) Na^+-dependent uptake of [^{3}H]2CdA was enhanced more than two-fold over that in the absence of Na^+. Because 2CdA is an antineoplastic agent with numerous clinical applications (20-24), a knowledge of species differences in the interaction of this compound and other 2'-deoxynucleoside analogs with the purine-selective transporters may be significant for future evaluation of these compounds in animal models.

Adenosine (1 μM) exhibits a higher rate of uptake in cells expressing rSPNT in comparison to those expressing hSPNT1. This higher uptake rate may be due to differences in the K_m of adenosine in interacting with the rat and human transporters (6 μM vs. 17 μM, Table 3). However, further studies are needed to clarify the mechanisms responsible for the species differences in the kinetics of adenosine uptake by rSPNT and hSPNT1.

The species differences observed with 2CdA and adenosine in interacting with hSPNT1 and rSPNT may be due to differences in the molecular structures of the two clones. A recent study from this laboratory (25) examined the molecular basis for substrate

Table 3. Comparison of Kinetic Parameters of Nucleosides and Nucleoside Analogs in Interacting with rSPNT and hSPNT1 in Transfected HeLa Cells.

Compound	rSPNT K_m (μM)	hSPNT1 K_m (μM)	References
2CdA[a]	13	479	(13)
Uridine	20.6	114	(10, 13)
	[b] 14	[b] 80	
Adenosine	[b] 6	17	(10)
Inosine	28.1	13.7	(1, 11, 13)
	[b] 14.6	[b] 4.5	
ddI[a]	46	19	(11, 13)

[a] Value reported as IC_{50} instead of K_m.

[b] Data were obtained from uptake studies in *Xenopus laevis* oocytes.

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selectivity using the purine and pyrimidine-selective rat clones. Similar studies between hSPNT1 and rSPNT may elucidate important species differences. Interestingly, rSPNT is not expressed (to a significant extent) in rat kidney (10, 11). This may suggest an important difference in the renal handling of purine nucleosides between species, which must be considered when evaluating such compounds in animal models.

In summary, we have expressed the human purine-selective transporter (hSPNT1) in HeLa cells. The characteristics of hSPNT1 in interacting with a number of nucleosides and nucleoside analogs were determined. Notable species differences in the function of hSPNT1 when compared with the rat homolog, rSPNT, were observed. This suggests caution should be taken when evaluating purine nucleosides and nucleoside analogs in the rat. The underlying molecular mechanisms which contribute to such species differences remain to be elucidated. Expression of the cDNA encoding hSPNT in HeLa cells represents a useful model to further characterize interactions of nucleosides and nucleoside analogs with the transporter.

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CHAPTER 5

EPITOPE-TAGGING OF A PYRIMIDINE-SELECTIVE NUCLEOSIDE TRANSPORTER (cNT1) AND FUNCTIONAL EXPRESSION IN *XENOPUS LAEVIS* OOCYTES AND HELA CELLS

Since the original cloning manuscript was published, numerous studies in heterologous expression systems have been carried out to characterize the functional properties of the cloned Na^+-dependent, pyrimidine-selective, nucleoside transporter, cNT1 (1-4). In contrast, relatively little work has been done directly addressing questions about the structural properties of the expressed cNT1 protein. For example, the predicted topology of the transporter has not been confirmed. cNT1 is a protein of 648 amino acids with 14 predicted transmembrane domains (4). Additional predicted properties include 3 *N*-linked glycosylation sites and four potential protein kinase C- dependent phosphorylation sites (4). Of the three predicted glycosylation sites two are intracellular, and one resides within the membrane (4). However, this prediction has not been confirmed directly by employing antibodies specific for the cNT1 transporter or by detection with an antibody specific for an epitope tag which has been engineered into the cDNA encoding the transporter.

The mRNA transcripts of cNT1 are present in epithelia including both the proximal tubule and intestine. However, to date the distribution of cNT1 in polarized epithelial cells has not been ascertained. The localization of cNT1 to either the basolateral or brush border membrane is important in the understanding of the physiological role of this transporter. A complication for determining the protein sorting of cNT1 is that Na^+-coupled transporters are expressed in well- differentiated cell lines such as MDCK (unpublished data, this laboratory), OK (5) and CaCo-2 cells (6). These cell lines are commonly employed in the study of intracellular sorting of cloned transporters (7). Thus, specific detection of cNT1 would be confounded by the presence of highly homologous Na^+-dependent nucleoside

transporters. The use of epitope-tagging, which incorporates a unique sequence recognized by a specific antibody which does not cross-react with endogenously expressed proteins would enable the study of cNT1 in differentiated cell lines.

The goal of this study was to develop a functional, epitope-tagged cNT1 that could be used to elucidate the structural properties or sorting of the transporter. Functional studies were carried out in both *Xenopus laevis* oocytes and transfected HeLa cells.

Materials

Primers were synthesized by the University of California, San Francisco, Biomolecular Research Center (UCSF BRC) and sequencing was carried out by the UCSF BRC. The anti-*c-myc* monoclonal antibody was the kind gift of Dr. J. Michael Bishop, UCSF. ^{3}H-Thymidine was purchased from Moravek. Materials for Western blotting (loading buffer, acrylamide, running buffer, and transfer buffer) were supplied by Bio-Rad. Restriction enzymes were from Gibco/ BRL. Secondary antibody, goat anti-mouse, was purchased from the UCSF Cell Culture Facility.

Methods

Incorporation of the Epitope-Tag into cNT1 cDNA

The sequence encoding the epitope tag was incorporated into the cNT1 cDNA by reverse-transcription polymerase chain reaction (RT-PCR). Two PCR reactions were carried out, a control reaction using the primers to the 3' and 5'- ends of cNT1 and, for the tag construct, the same 3' (antisense)-primer was used, however the 5' (sense)-primer was replaced by an additional sequence which incorporated the sequence encoding the *c-myc* epitope tag (EQKLISEEDL).

Transfection and Functional Studies in HeLa Cells

Transfection of HeLa cells with the cDNA of cNT1 or the tagged transporter termed cNT1.tag was carried out as described previously ((8), Chapter 3). An initial lipid titering determined that a ratio of 1:3 (DNA: lipid) was optimal for expression of cNT1 (see Chapter 6). Uptake of ^{3}H-thymidine was measured in transfected and untransfected cells in the presence and absence of Na$^+$. Uptake was stopped by the addition of ice cold choline buffer followed by three washes. Cells were solubilized with 0.5% Triton X-100 and radioactivity was determined by liquid scintillation counting. Protein determinations were carried out as described previously (Chapter 3). For inhibition studies, cells were

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incubated with ^{3}H-thymidine in the presence of Na$^+$, and in the presence or absence of the given compound (1 mM).

Functional Studies in Xenopus laevis oocytes

The cRNA encoding either cNT1 or cNT1.tag was injected into Xenopus laevis oocytes and uptake of ^{3}H-thymidine was carried out on day 2 following injection. Uptake was carried out in the presence or absence of Na$^+$, using methods previously described (9). Briefly, oocytes were incubated in either Na$^+$ buffer (100 mM NaCl, 2 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM / Tris, pH 7.4) at 25°C or Na$^+$-free buffer, in which Na$^+$ was replaced by choline (100 mM) containing 5.5 µM thymidine (0.5 µM ^{3}H-thymidine + 5 µM unlabeled thymidine). Oocytes were incubated in the uptake mixture (30 min or 1 hr) and then uptake was terminated by the addition of 3 ml ice-cold choline buffer, followed by three washes in the same buffer. Inhibition studies were carried out as stated above. In addition Na$^+$-dependent uptake was measured in the presence of unlabeled nucleosides (1 mM).

Isolation of Membrane Proteins and Detection of the cNT1.tag Expressed Transporter by Western Blotting

Membrane proteins were isolated from *Xenopus laevis* oocytes injected with the cRNA encoding either cNT1 or cNT1.tag on day four following injection. Briefly, approximately 40 oocytes were homogenized, using a dounce homogenizer, in ice cold homogenization buffer (83 mM NaCl, 1 mM MgCl$_2$, 10 mM Hepes, pH 7.9, 5 mM EDTA, 5 mM EGTA, and 1 mM PMSF). Following homogenization, the mixture was centrifuged two times at 2,500 rpm, using a Beckman JA14 rotor (Beckman Instruments, Palo Alto, CA). Following these spins, the supernatant was collected and layered onto a 15% sucrose gradient and then centrifuged at 160,000 g for 1.5 hours. The pellet was then collected and resuspended in buffer (100 mM NaCl, 10 mM MOPS, 1 µl/ oocyte). (10).

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Immunodetection of the isolated membrane proteins was carried out by

resuspending the membrane pellet solution in SDS sample buffer and running the samples

on an 8% SDS/ PAGE gel which was then transferred to nitrocellulose paper. Detection of

the epitope-tagged transporter was carried out by initially blocking the nitrocellulose with

nonfat milk solution (2.5%), followed by incubating the nitrocellulose with anti-c-myc

supernatant, followed by three washes and then detection with a goat-anti mouse secondary

antibody. Following incubation with the secondary antibody, the nitrocellulose was

washed three times and then protein was detected using the BCIP/ NBT detection kit (Bio-

Rad). The secondary antibody used in this study is conjugated to alkaline phosphatase.

This anti-mouse antibody binds specifically to the proteins labeled with c-*myc* Ab. The

BCIP/ NBT reagent is specific for alkaline phosphatase, and therefore the proteins labeled

with the secondary antibody are detected.

Data Analysis

In general, data are expressed as mean ± standard deviation (SD). For Michaelis-

Menten studies, the rate of uptake was expressed as pmol/mg protein/ min. Data were fit to

the equation $V = V_{max} [S] /(K_m + [S])$ where V is the rate of thymidine uptake and [S]

represents the concentration of thymidine. The Kaleidagraph® fitting program was used to

fit the data and parameter estimates are expressed as a mean ± SE. An unpaired Student's

t-test from Primer of Biostatistics software (Version 3, by Stanton A. Glantz, McGraw-

Hill, 1991) was used for determination of statistical significance, and $P < 0.05$ was

considered statistically significant.

Results

Sequencing and Restriction Analysis of cNT1.tag

To confirm the addition of the DNA sequence encoding the c-myc epitope tag to the 5'-end of cNT1 cDNA, the PCR products of both the control and the tagged construct were analyzed by DNA gel electrophoresis (Fig. 1). As shown, a slightly larger molecular size was obtained for the cDNA of the tagged construct in comparison to the wild type cNT1. Restriction digests of the PCR products, following ligation into pCR2.1 also confirmed the addition of the sequence encoding c-myc (data not shown). Direct sequencing was carried out from the T7 promoter of the pCR2.1 vector to check the sequence of the 5' end of the clone (University of California, Biomolecular Resource Center). Direct sequencing confirmed the addition of the epitope tag sequence to the cNT1 construct, termed cNT1.tag (Table 1).

Kinetic Properties of the Epitope-Tagged cNT1

To compare the functional properties of the tagged transporter with those of the native clone, we chose to transfect the cDNA encoding both cNT1 and cNT1.tag into HeLa cells and compare kinetic parameters. The Na^+-dependent uptake of ^3H-thymidine in Hela cells expressing either cNT1 or cNT1.tag, or in untransfected cells (Fig. 2) was determined. Significant thymidine uptake was observed in HeLa cells expressing either the tagged or native cNT1. No significant thymidine uptake was observed in untransfected cells. The kinetic properties of thymidine uptake in HeLa cells expressing cNT1.tag were similar to those in cells expressing cNT1 (Table 2).

Inhibition of ^3H-Thymidine Uptake by Nucleoside Analogs in HeLa Cells Expressing cNT1.tag

The effect of the nucleoside analogs (1 mM) ddI, ddA, ddC, AraC and AZT on the Na^+-dependent thymidine uptake in HeLa cells expressing cNT1.tag or cNT1 was

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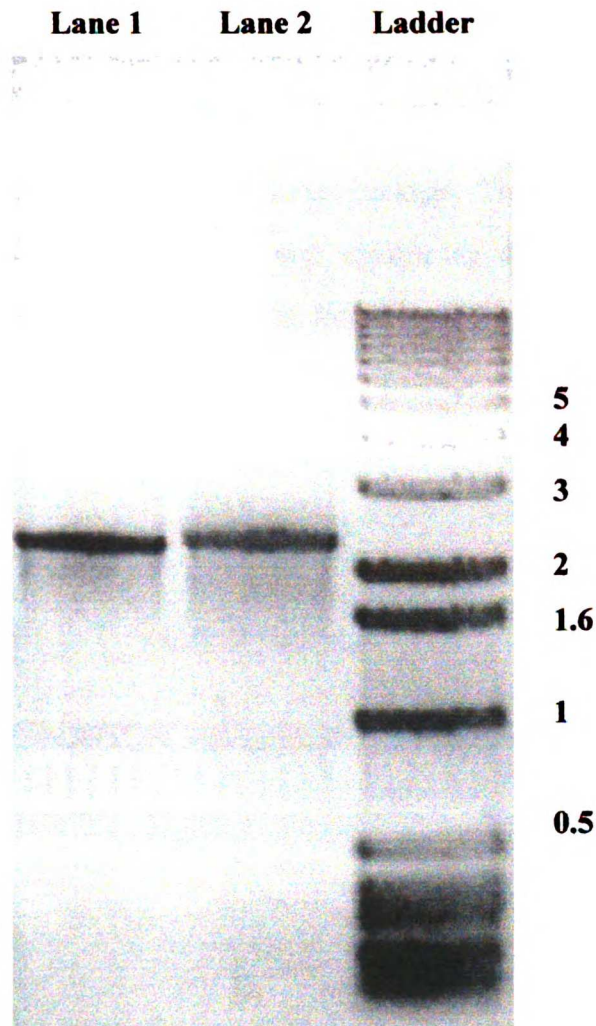
Figure 1. Polyacrylamide DNA gel of RT-PCR products cNT1 and cNT1.tag. RT-PCR

was carried out using the pcR2.1 vector as a template. The vector contained the cDNA

encoding cNT1 in the proper orientation. Primers were designed against the 3' and 5'

ends of the cNT1 DNA sequence (see Methods). Lane 1 shows the PCR product

following amplification with gene specific primers. Lane 2 shows the PCR product

following amplification with the same 3' (antisense) primer as that used in Lane 1, but

with a different sense primer, which incorporated the sequence encoding the c-*myc*

epitope tag (see Methods).

Table 1. Alignment of N-terminal regions of cNT1 and cNT1.tag following DNA sequencing and translation. The 5' ends of both cNT1 and cNT1.tag were sequenced (see Methods). Both sequences were then translated to the corresponding protein sequence and the two proteins were aligned using the GCG software package. The area highlighted in gray corresponds to the sequence encoding the c-*myc* epitope tag, confirming that this sequence was properly incorporated into the cDNA following RT-PCR.

```
cNT1.tag   1  MEQKLISEEDLADNTQRQRESISLTPMAHGLENMGAEFL  39
              :||||||||||||||||||||||||||||||
cNT1       1  ..........MADNTQRQRESISLTPMAHGLENMGAEFL  29
```
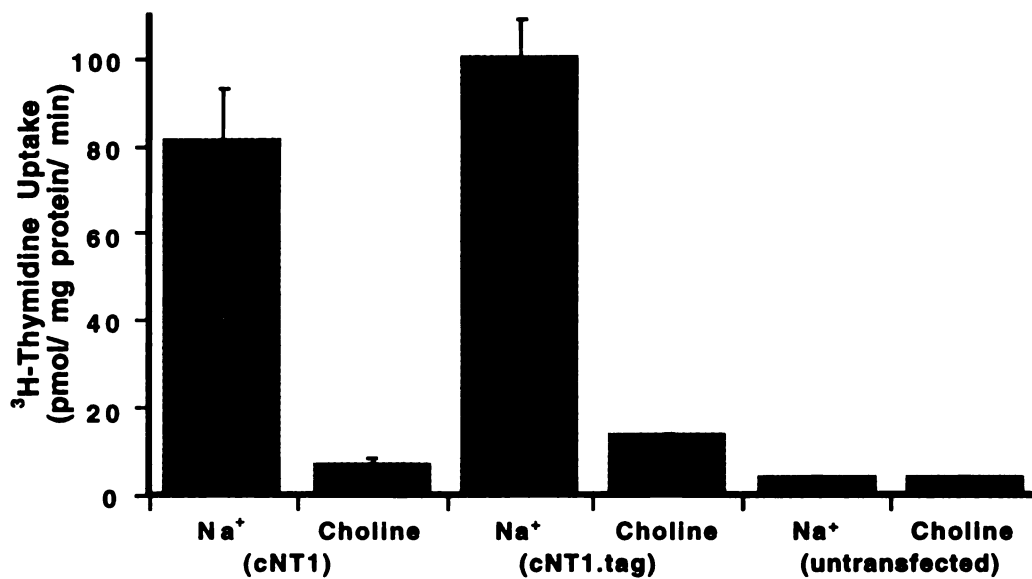
Figure 2. Expression of cNT1 and cNT1.tag in the presence and absence of Na^+. Uptake of ^{3}H-thymidine was measured in the presence and absence of Na^+ in HeLa cells transfected with either cNT1 or cNT1.tag. Untransfected cells served as the negative control. Data are presented as mean± SD (n= 2). This is a representative experiment from three separate experiments.

Table 2. Kinetic Parameters of cNT1 and cNT1.tag. The saturability of thymidine uptake in HeLa cells was determined over a range of concentrations. Data were fit to the equation: rate of uptake = $V_{max}[S]/(K_m + [S])$. Results are presented as mean ± SE.

	K_m (µM)	V_{max} (pmol/mg protein/ min)
cNT1	9.0 ± 7.3	370 ± 81
cNT1.tag	11 ± 5.6	373 ± 55

examined (Fig. 3). Significant inhibition of Na^+-dependent ^3H-thymidine uptake was observed in the presence of AZT and ddC in transfected cells (P< 0.05). The nucleoside analogs ddA and ddI did not inhibit ^3H-thymidine uptake. Again, the inhibition profile confirmed that the addition of the tag sequence to the 5'-end of cNT1 does not result in a functional change.

Expression of cNT1.tag in Xenopus Laevis Oocytes

    Xenopus laevis oocytes were injected with 50 nl of the cRNA encoding the tagged, pyrimidine-selective nucleoside transporter; uninjected oocytes were used as the negative control. Uptake studies were carried out two days post-injection in the presence or absence of Na^+ (see Methods). $Na^+$-dependent ^3H-thymidine uptake (at 0.5 hr) in *Xenopus laevis* oocytes injected with the cRNA of the *c-myc* tagged transporter was enhanced (0.025 ± 0.006 pmol/oocyte) when compared to its uptake in uninjected oocytes (0.007± 0.003 pmol/ oocyte) (P<0.05) (Fig. 4A). Significant inhibition of Na^+- dependent ^3H-thymidine uptake (1 hr) was observed in the presence of thymidine (P< 0.05), but not in the presence of inosine in the oocytes injected with the cRNA of cNT1.tag (Fig. 4B).

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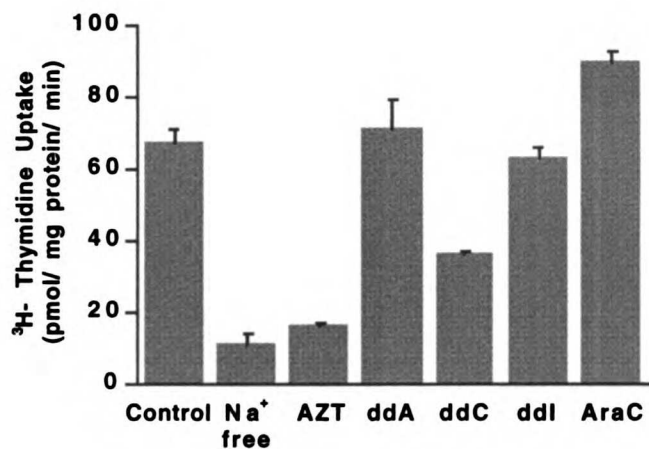
A
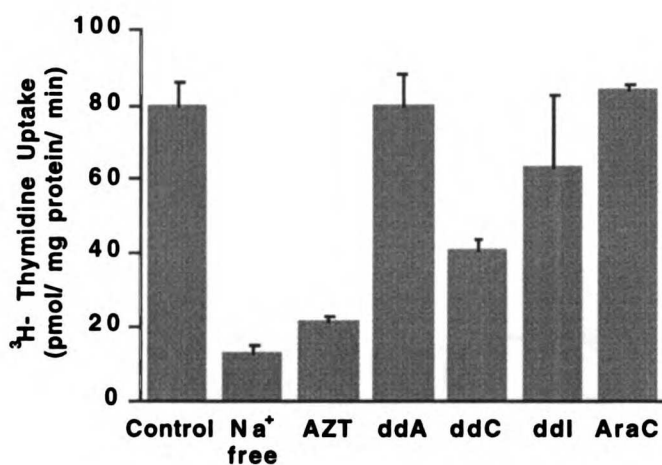
B

Figure 3. Uptake of ^{3}H-thymidine was measured in the presence and absence of a number of nucleoside analogs. Control represents Na$^+$-dependent uptake in the absence of inhibitor. Na$^+$-free represents thymidine uptake in which Na$^+$ was replaced by choline (see Methods). Uptake in HeLa cells transfected with cNT1 is shown in Panel A and uptake in cNT1.tag transfected cells in presented in panel B. Data are presented as mean ± SD (n= 2).
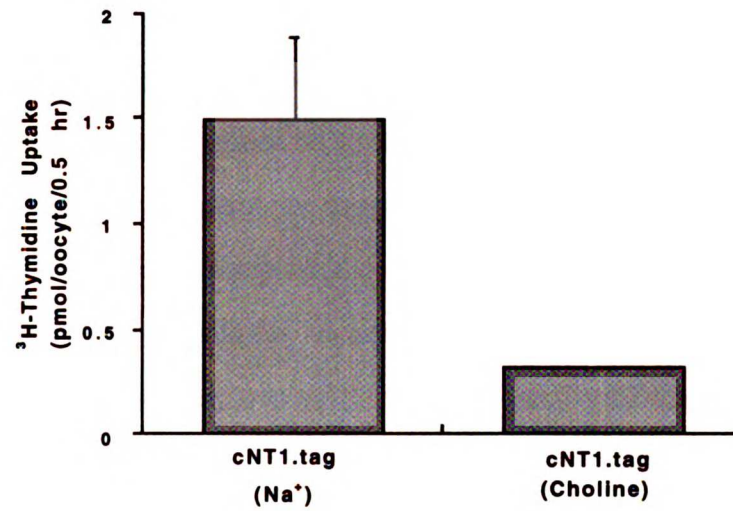
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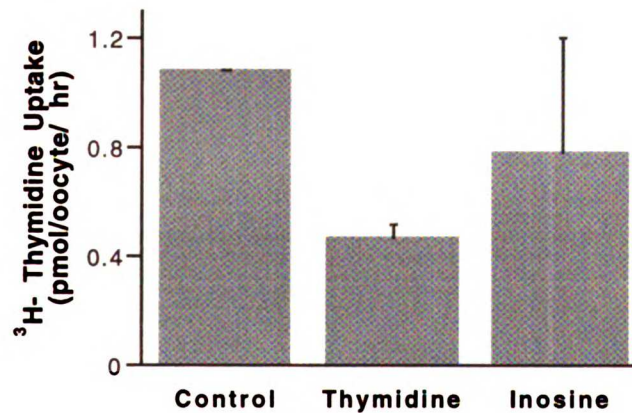
B

Figure 4. Functional activity of cNT1.tag in *Xenopus laevis* oocytes. The uptake of ^{3}H-thymdine was measured in the presence and absence of Na^{+} (A). Significant Na^{+}-dependent uptake was observed ($P < 0.05$). Na^{+}- dependent thymidine uptake (B) was measured in the presence and absence of thymidine or inosine (1 mM).

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Immunoblotting of cNT1.tag

To confirm that the injected cRNA encoding the epitope-tagged transporter

(cNT1.tag) was properly translated and expressed, the membrane fraction was isolated

from *Xenopus laevis* oocytes injected with the tagged cRNA (see Methods). This

membrane fraction was then separated on an SDS/PAGE gel and transferred to

nitrocellulose. As a negative control, the membrane fraction isolated from oocytes injected

with the cRNA of cNT1 was also separated on an SDS/PAGE gel. The membrane fraction

isolated from oocytes injected with the 'tagged' cRNA expressed a protein which was

detected (the untagged transporter was not detected) by immunoblotting (see Methods) with

the anti-*c-myc* mAb. Two bands were detected by Western blotting. One band was

approximately 40,000Da and the other was 42,000 Da (Fig. 5).

Figure 2. Immunoblotting of protein extracted from *Xenopus laevis* oocytes injected with the cRNA encoding the epitope-tagged nucleoside transporter, cNT1.tag. The membrane fraction was isolated 48 hours after injection (see Methods).

Discussion

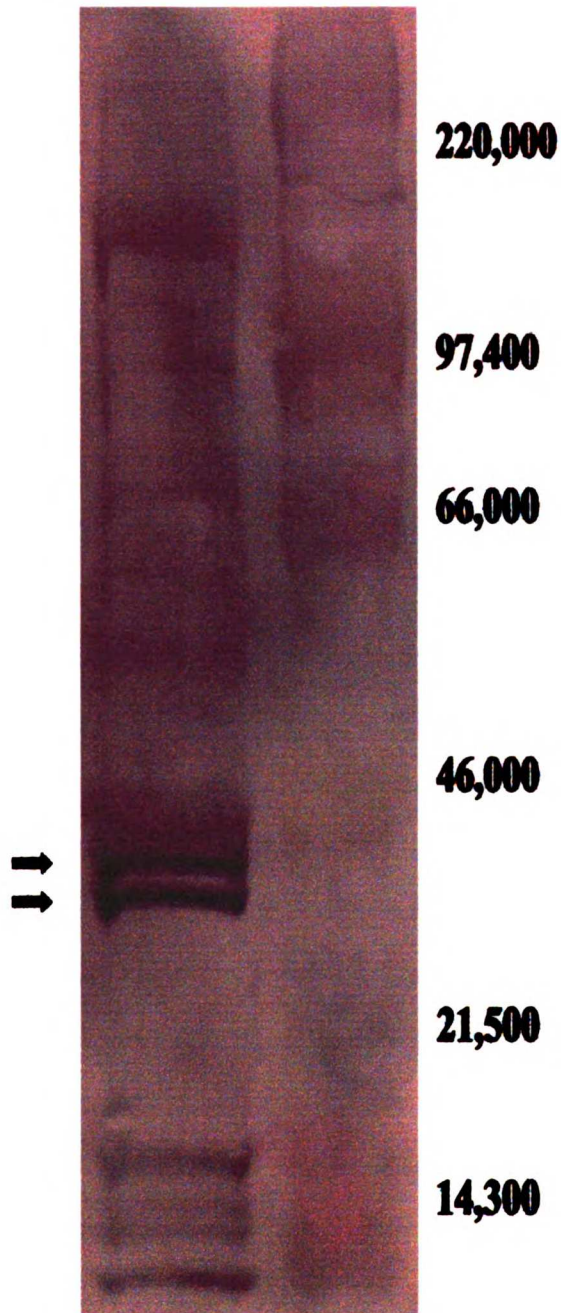As the number of isolated cDNAs encoding nucleoside transporters continues to increase (4, 11-13), a system which can be readily applied to study the properties of the expressed proteins is very appealing. An epitope tag offers an approach to study protein properties while awaiting the development of protein specific antibodies. This technique has been successfully applied to the study of a number of membrane transporters (14, 15) and receptors (16, 17). Furthermore, an epitope tag enables the expression and study of nucleoside transporters in differentiated cell lines, regardless of endogenous levels.

Epitope insertion has been applied to determine the membrane topology of the Multidrug Resistance-associated Protein (MRP). In a study by Kast and Gros (18) epitopes were incorporated at predicted hydrophilic segments of MRP and the cDNAs encoding these epitope-tagged proteins were then transfected and analyzed in HeLa cells. Heterologous expression system, together with epitope-tagging may also be used to address the question of membrane topology.

The functional Na^+-dependent transport of thymidine in HeLa cells transfected with cNT1 or cNT1.tag cDNA together with functional studies in *Xenopus laevis* oocytes injected with the cRNA of the cNT1.tag construct confirm that the transporter remains functional with the addition of the epitope tag to the N-terminus of the expressed protein. These results are in good agreement with data obtained by Fang *et al.* demonstrating that a C- terminus *c-myc-* tagged cNT1 is functional in COS cells (3). In addition, the inhibition profile and kinetic parameters are similar when Na^+-dependent thymidine uptake in cNT1 cDNA transfected cells vs. cNT1.tag transfected cells are compared (Figures 3, 4 and Table 2).

The detection of two bands on the immunoblot may indicate that the protein is glycosylated, with one band representing the glycosylated form of the protein and the other that which is not glycosylated. A recent abstract presented by Hamilton *et al.* also identified two bands of the expressed, untagged, cNT1 when injected into oocytes (19). In

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that study, treatment with endoglycosidase F resulted in a faster migration of the bands, which suggests that the protein may be glycosylated. This is in contrast to a single band detected by immunoblotting when the C-terminus, c-*myc* epitope-tagged cNT1 was expressed in COS-1 cells (3). Differences in glycosylation of the protein may be attributed to different expression systems. The two bands, which potentially represent glycosylated forms of the protein, were only detected in the oocyte expression system. In contrast, a single band was detected when plasma membranes were isolated from COS-1 cells and analyzed by immunoblotting.

In this study the cNT1.tag band had molecular sizes of 40,000 Da and 42,000 Da which is in good agreement with the size of the single band detected in the COS-1 cells. One explanation for the smaller size bands observed in two studies is that membrane proteins often migrate faster on Western blots than other proteins. Therefore the apparent size determined based on the protein ladder, may actually be an underestimation of the actual size of the protein (3).

Many polarized cell lines such as MDCK(unpublished data, this laboratory), OK (5), and CaCo-2 (6) express Na^+-dependent nucleoside transporters. For this reason, an epitope-tagged nucleoside transporter may serve as an important tool to distinguish between cloned and the endogenously expressed transporters. This method has been successfully applied to the study of other membrane transporters (14, 15).

In summary, a sequence encoding the *c-myc* epitope was incorporated into the cDNA encoding cNT1 by RT-PCR. The addition of this sequence was confirmed by restriction analysis and direct sequencing. Studies in HeLa cells transfected with cNT1.tag cDNA demonstrated that the expressed protein retained the functional characteristics of the native cNT1. Injection of the cRNA encoding cNT1.tag into *Xenopus laevis* oocytes resulted in a functional protein which displayed Na^+-dependent uptake and was inhibited by the pyrimidine nucleoside thymidine, but not the purine nucleoside inosine. This epitope-tagged transporter represents a useful tool for the study of protein sorting and the

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CHAPTER 6

FUNCTIONAL EXPRESSION OF THE PYRIMIDINE-SELECTIVE

NUCLEOSIDE TRANSPORTER (cNT1) IN

HELA CELLS

The Na^+-dependent pyrimidine-selective nucleoside transporter, cNT1, is expressed in both the rat kidney and intestine (1, 2). The cDNA encoding this nucleoside transporter was first described in 1994 (3). Northern blotting studies and RT-PCR studies identified cNT1 transcripts in the rat kidney, intestine, and in lower abundance in brain (4). The functional characteristics of this cloned nucleoside transporter have been examined in both *Xenopus laevis* oocytes and transfected COS-1 cells (5-7). However, detailed studies of the expressed protein using specific antibodies directed against the cloned transport protein have not been described. Such studies are important for addressing questions regarding tissue distribution and membrane trafficking of the expressed protein.

In preliminary studies in this laboratory, we have developed an anti-peptide antibody specific for the pyrimidine-selective nucleoside transporter, cNT1. The goals of this study were to determine if transfected HeLa cells would serve as a reliable model to study the pyrimidine-selective nucleoside transporter, cNT1. Both functional studies and studies using a peptide antibody against the N-terminus of the protein were performed. In this chapter the details of antibody development and characterization of the expression system used to study cNT1 are presented.

Materials

^{3}H-Thymidine was purchased from Moravek. ddI, ddA, ddC, 2-chloroadenosine, 5-fluorouridine, Ara-C, and nitrobenzylthioinosine, and fish gelatin (for blocking) were from Sigma. The peptide used to generate the antibody was synthesized by the Stanford Biomolecular Resource Group. FITC-labeled secondary antibody and all media and media supplements were purchased from the UCSF Cell Culture Facility. Anti-cNT1 antibody was generated at BabCo (Berkeley, CA). Transwell slides and T-175 flask, which were used to maintain the cells, were supplied by Nunc. HeLa cells were purchased from the American Type Culture Collection (ATCC), all media and supplies were obtained from the UCSF Cell Culture Facility.

Methods

Transfection Studies

Transfection of HeLa cells with the cDNA of cNT1 was carried out as described previously (10, Chapter 3). An initial lipid titering determined that a ratio of 1:3 (DNA: lipid) was optimal for expression of cNT1 (Fig. 1).

Uptake Studies

Uptake of ^{3}H-thymidine was measured for 5 minutes in transfected and untransfected cells in the presence and absence of Na$^+$. Uptake was stopped by the addition of ice cold choline buffer followed by three washes. Cells were solubilized with 0.5% Triton X-100 and radioactivity was determined by liquid scintillation counting. Protein determinations were as described previously (10, Chapter 3). For inhibition studies, cells were incubated with ^{3}H-thymidine in the presence of Na$^+$, and in the presence or absence of the given compound (1 mM).

Antibody Preparation

To determine an appropriate peptide region of the pyrimidine-selective protein to synthesize for antibody production, sequence alignments were carried out and calculation of the antigenic index (AI) was undertaken by the method of Jameson and Wolf (11, 12) using the Genetics Computer Group (GCG) Sequence Analysis Software Package. The development of an antibody specific for cNT1 (pyrimidine-selective nucleoside transporter) but not SPNT (purine-selective nucleoside transporter) required the use of a peptide region lacking homology between cNT1 and SPNT. A region was chosen which was unique for cNT1 and displayed a high AI. The AI is determined by taking a number of weighted measures of secondary structure including hydrophilicity and surface probability into consideration. A peptide corresponding to the N-terminus of cNT1 (PEGEGGLNKAER) was synthesized by the Stanford University Biomolecular Resource Center. An additional cysteine residue was added to the C-terminus of the peptide for conjugation to keyhole limpet hemocyanin (KLH). An antibody against the conjugated peptide was then produced in rabbit (BabCO, Berkeley, CA).

Confocal Microscopy

Cells were fixed with 3.7% formamide for 10 min. Cells were then blocked and permeabilized simultaneously as described by Tolbert and Lameh (13). Briefly, cells were blocked and permeabilized simultaneously with 0.25% fish gelatin and 0.04% saponin. Incubation with the anti-peptide antibody (1:100) was for 1 hour, followed by 4 washes and incubation with an FITC(fluoroscein-isothyocyanate)-conjugated Ab at a dilution of 1: 500. The slides were mounted using Fluoromount G. Visualization of the labeled protein was carried out using a Bio-Rad MRC-600 confocal head which was attached to an Optiphot II Nikon microscope with a Plan Apo 60 X objective lens, 1.4 numeric aperture. A blue high sensitivity filter block was used to detect FITC emission.

Data Analysis

In general, data are expressed as mean ± standard deviation (SD). An unpaired Student's *t*-test from Primer of Biostatistics software (Version 3, by Stanton A. Glantz, McGraw-Hill, 1991) was used for determination of statistical significance, and $P < 0.05$ was considered statistically significant.

Results

Initial Characterization of cNT1 in HeLa Cells

The expression of HeLa cells transfected with the cDNA encoding cNT1 was examined in the presence of increasing concentrations of lipid. At a concentration of 2 mg/ ml of lipid per 2 µg DNA, very little Na^+-dependent thymidine uptake was observed. Uptake increased at 4 mg/ ml lipid, and was most pronounced at 6 mg/ ml lipid and 2 µg DNA (i.e. a 1:3 DNA: lipid ratio). For subsequent studies, the 1:3 ratio was used (Fig. 1). Initial characterization was carried out to determine the expression of Na^+-dependent thymidine uptake over time. Next, uptake of ^3H-thymidine was measured in the presence or absence of Na^+ in cells transfected with cNT1 cDNA and in HeLa cells transfected with empty vector (pcDNA3 without the cNT1 insert). Significant Na^+-dependent uptake (4-fold higher than Na^+-independent uptake) was observed in cells expressing cNT1; however, there was no significant Na^+-dependent thymidine uptake in HeLa cells transfected with empty vector (Fig. 2). ^3H-thymidine was incubated with transfected cells at a number of time points in the presence and absence of Na^+. Significant Na^+-dependent uptake was observed over time (Fig. 3).

Inhibition of ^3H-Thymidine Uptake by Nucleosides and Nucleoside Analogs in HeLa Cells Expressing cNT1

We examined the substrate selectivity of cNT1 expressed in HeLa cells. To confirm the pyrimidine-selectivity of the expressed protein in this system, ^3H-thymidine
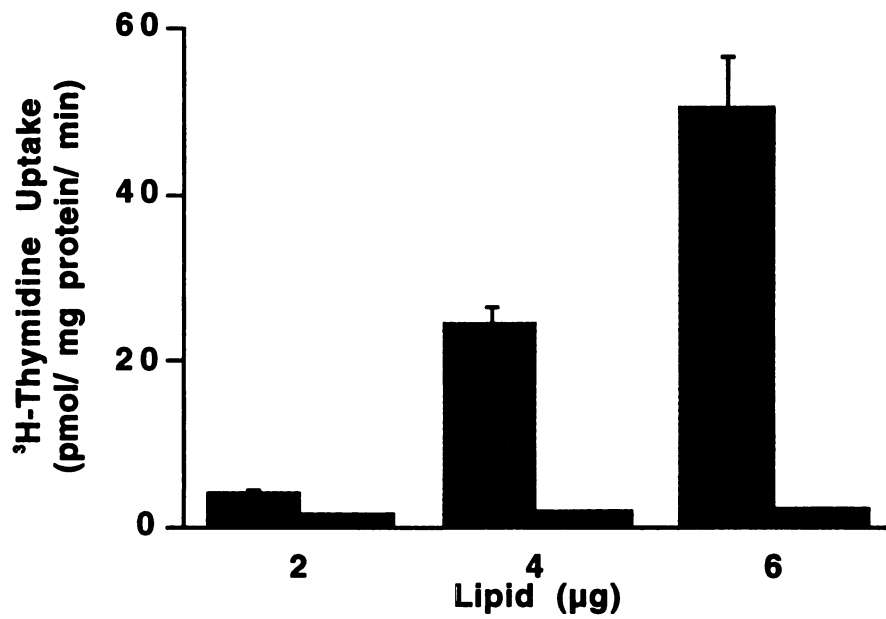
Figure 1. Titration of lipid in HeLa cells transfected with cNT1 cDNA. HeLa cells were transfected with 2 µg plasmid DNA containing the cNT1 cDNA insert/ well. DNA concentrations remained constant while lipid concentrations were from 2 to 6 µg lipid per well. Uptake of ^{3}H-thymidine was carried out in the presence (gray bars) or absence (black bars) of Na$^+$. Data are presented as mean ± SD (n= 2).

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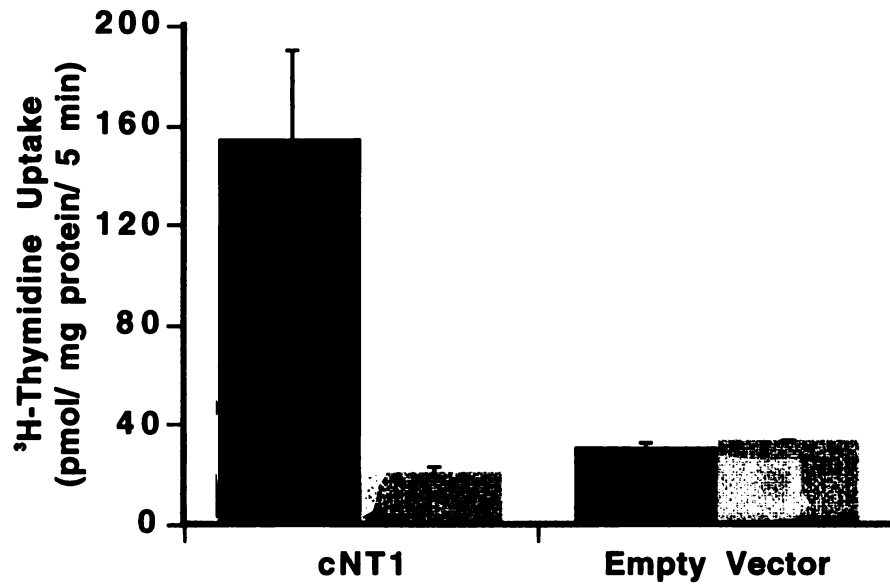
Figure 2. Na⁺- dependent uptake in HeLa cells transfected with cNT1 cDNA or empty vector. Uptake of ³H-thymidine was measured in the presence (black bars) and absence (gray bars) of Na⁺ in cells transfected with cNT1 or empty vector (pcDNA3). Data are presented as mean ± SD (n= 4).

Figure 3. Time course of thymidine uptake in HeLa cells transfected with cNT1 cDNA.

Uptake of ^{3}H- thymidine was measured in transfected HeLa cells over time in the presence

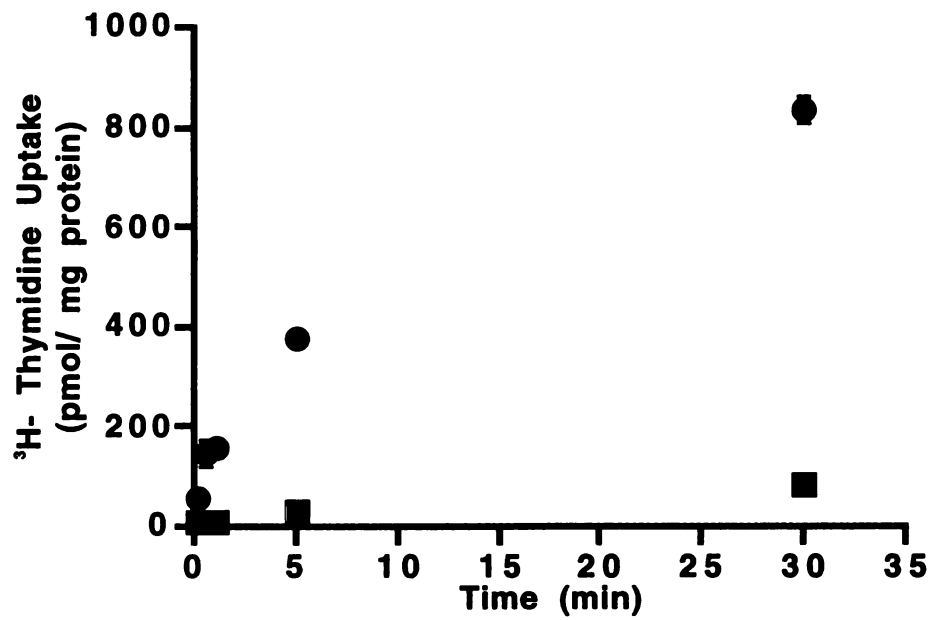(closed circles) and absence (closed squares) of Na$^+$. Data are presented as mean ± SD (n=

2).

uptake was measured in the presence and absence of inosine or thymidine (1 mM). As expected, significant inhibition was observed by thymidine, but not by inosine, a purine nucleoside (Fig. 4). Finally, the interaction of the nucleoside analogs AZT, 5-fluorouridine and 2-chloroadenosine with cNT1 was examined in HeLa cells transfected with the cDNA encoding cNT1 (Fig. 5). Significant inhibition of Na^+-dependent, ^3H-thymidine uptake was observed in the presence of AZT, 2-chloroadenosine and 5-fluorouridine in transfected cells (1 mM)(P< 0.05).

Confocal Microscopy of cNT1 transfected cells

HeLa cells were seeded on Transwell, glass, coated slides, from Nunc. Transfection procedures and seeding densities were the same as those used in uptake studies. However, on day 2 following transfection, cells were fixed and stained, in place of functional studies. Visualization using the confocal microscope displayed a significant region of staining in cNT1 transfected HeLa cells (panel A, Fig. 6) when compared to staining of transfected cells treated with pre-immune sera (panel B, Fig. 6) or staining of untransfected cells treated with Ab (data not shown).
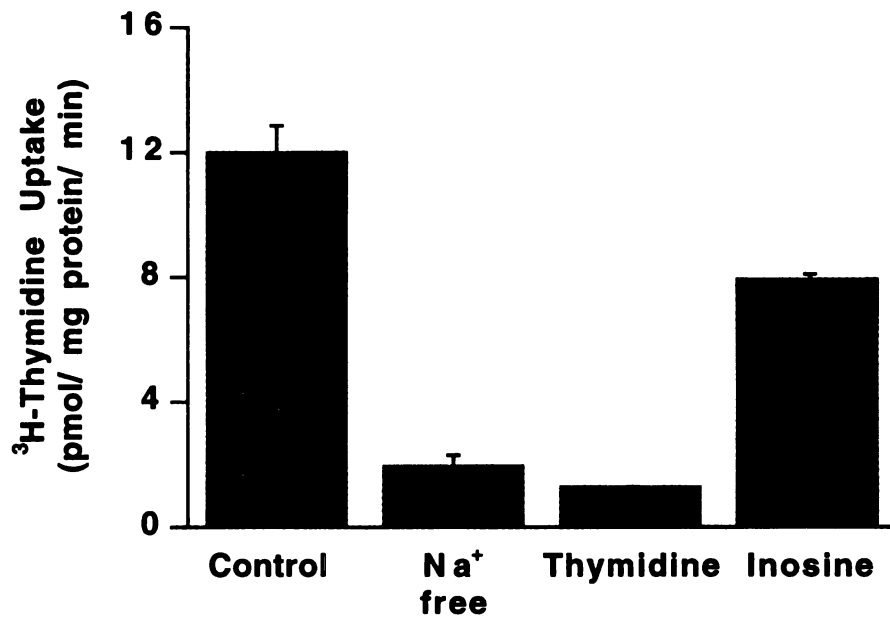
Figure 4. Inhibition of Na⁺-dependent uptake of ³H-thymidine with the unlabeled

nucleosides thymidine and inosine (1 mM). Na⁺-dependent thymidine uptake was carried

out in the presence and absence of thymidine or inosine. Na⁺-free uptake represents

thymidine uptake in Na⁺-free buffer (see Methods).
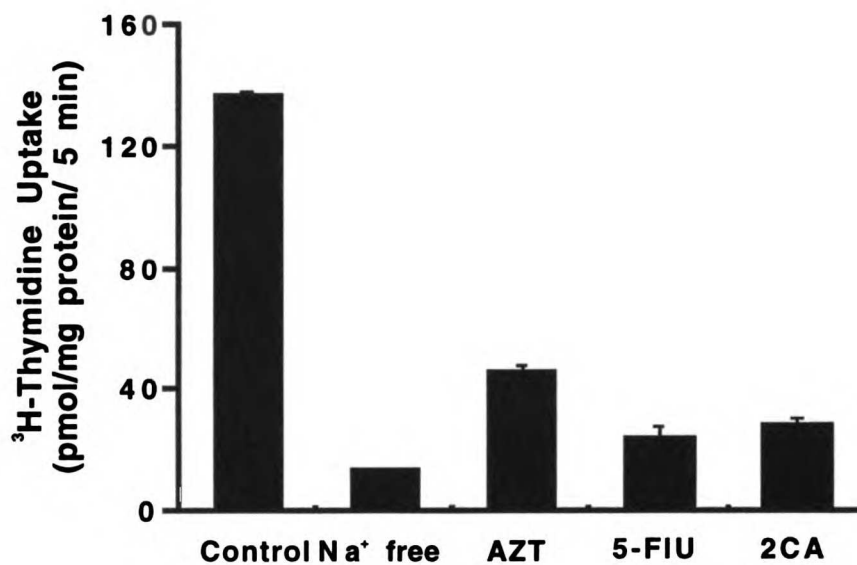
Figure 5. Inhibition of thymidine uptake in cNT1 transfected cells. ^{3}H-Thymidine uptake was determined in HeLa cells transfected with cNT1 cDNA. Na$^+$-dependent uptake was carried out in the absence (Control) of unlabeled nucleoside analogs and in the presence of AZT (azidothymidine), 5-FlU (5-fluorouridine) and 2CA (2-chloroadenosine) (1 mM). Na$^+$-free uptake represents uptake in the absence of Na$^+$ (see Methods). Data are presented as mean ± SD (n= 2).
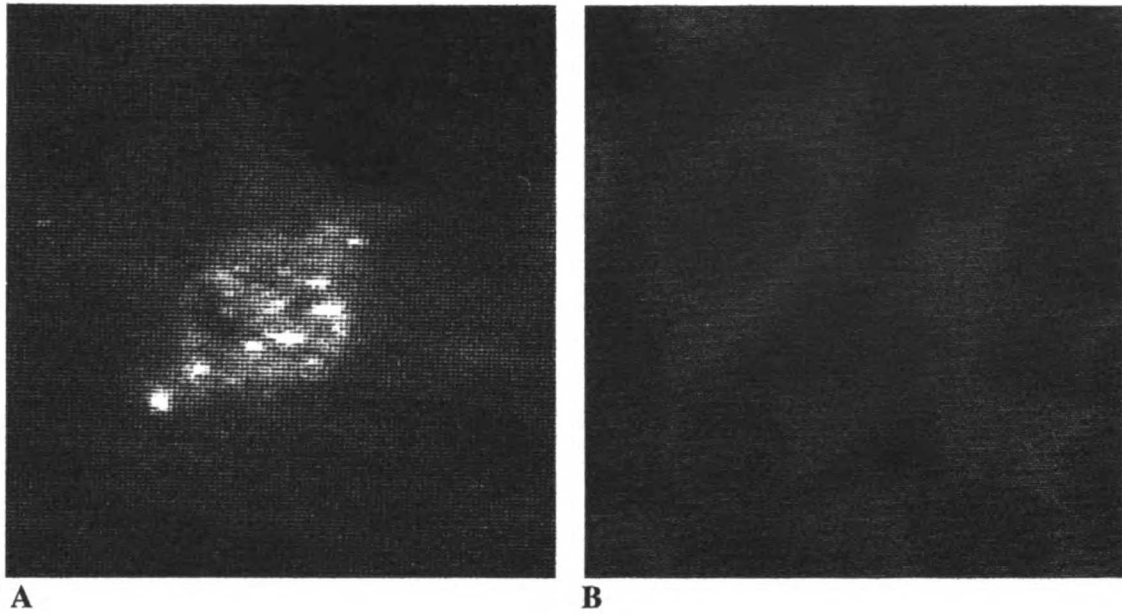
Figure 6. Confocal microscopy of the pyrimidine-selective nucleoside transporter, cNT1, transiently transfected in HeLa cells. Cells were transfected with the cDNA encoding cNT1 or mock-transfected with empty vector, and then stained with either a cNT1-specific antibody or pre-immune sera. Panel A represents staining of cNT1-transfected cells with cNT1 anti-peptide antibody. Cells expressing cNT1 stained with preimmune sera are shown in panel B.

Discussion

In this study we determined that functional characteristics of the cNT1 transporter may be studied in HeLa cells transfected with cNT1 cDNA. The interaction of a number of nucleoside analogs with cNT1 expressed in HeLa was examined. The pyrimidine nucleoside analog, AZT, displayed significant inhibition of Na^+-dependent thymidine uptake in the cells transfected with cNT1 cDNA, consistent with functional studies carried out in *Xenopus laevis* oocytes (7). However, the purine nucleoside analogs, ddI and ddA did not inhibit Na^+-dependent uptake of thymidine in the transfected cells. The application of this system may aid in the rapid screening of nucleosides and nucleoside analogs with the cloned pyrimidine-selective nucleoside transporter as well as with the cloned purine-selective transporters (see Chapters 3 and 4).

To construct an antibody specific for cNT1, protein sequences of cNT1 and SPNT were aligned and regions, which did not share significant sequence homology, were identified for peptide synthesis and subsequent antibody production. The unique peptide regions identified by sequence alignment were then further analyzed to determine the antigenic index (AI) using the methods described by Jameson and Wolf (see Methods) (11, 12) and the GCG software package. Briefly, a high AI is associated with high hydrophilicity and surface probability. Both the N- and C- termini lack significant homology based on sequence alignments of cNT1 and SPNT. However, the peptide regions identified in the N-terminus also displayed appropriate AI values, indicating that this region would be more appropriate for antibody production. Therefore, a peptide was synthesized and an antibody produced to the N-terminal region of the transporter.

In this study we used a specific Ab and confocal microscopy to detect the protein expressed following transfection of cNT1 cDNA in HeLa cells (Fig. 6). This study represents the first use of a specific Ab and confocal microscopy to detect cNT1. Clearly, the study has implications for future studies addressing questions concerning the membrane topology of cNT1 (14). Original predictions indicated that both the N- and C-termini were

intracellular. However, preliminary studies by Hamilton *et al.* (15) suggest that these predictions may be erroneous. Cells transfected with cNT1 cDNA may be used to address this question by using permeabilized vs. non-permeabilized cells together with the cNT1-specific Ab used in Fig. 6.

In addition, the development of an antibody specific for the N2-selective nucleoside transporter in rat may be used to localize distribution of the transporter in different tissues, such as kidney, intestine, and brain. The use of antibodies for this purpose has been applied to a number of other membrane transporters (8, 16, 17). Localization of the cNT1 nucleoside transporter to different regions of the intestine may be another application for the cNT1-specific Ab. Previously, the distribution of cNT1 mRNA transcripts was determined along different regions of the rat intestine (18) using RT-PCR. PCR may amplify message which is present in low abundance. It may therefore be useful to determine protein expression of cNT1 using the specific anti-peptide Ab. This Ab is unique to cNT1 and, as noted above, would not be expected to cross react with the purine selective nucleoside transporter, SPNT. Finally, localization of cNT1 to either the brush border or basolateral membrane in kidney, intestine, and choroid plexus would greatly enhance the understanding of the physiological role of this transporter in the respective tissues.

In summary, we have described the development of a functional expression system for the pyrimidine- selective nucleoside transporter, cNT1. This provides further confirmation that function is not altered by the addition of c-*myc* at the N-terminus of the protein encoded by cNT1. The protein expressed in this system was detected using confocal microscopy. This work has important implications for future studies.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

Studies in this dissertation contributed to the establishment of mammalian expression systems for cloned nucleoside transporters, the development of antibodies to detect nucleoside transporters and a greater understanding of kinetics and functional characteristics of cloned nucleoside transporters. The development and application of a transient expression system for the purine-selective nucleoside transporter, SPNT, in HeLa cells is described in detail (Chapter 3). This expression system was modified and applied to study kinetics and drug interactions of the cloned human homolog of SPNT, hSPNT1 (Chapter 4). The development of an epitope-tagged nucleoside transporter, cNT1.tag is described (Chapter 5) that may have potential applications in the study of the trafficking and membrane topology of this transporter. Finally, antibodies specific for the cloned nucleoside transporters, SPNT and cNT1 were developed and the initial characterization of the cNT1 anti- peptide antibody is presented (Chapter 6).

At the beginning of this dissertation research, very little molecular information was available on either equilibrative or concentrative nucleoside transporters. However, the field of nucleoside transport has undergone significant growth in recent years. Knowledge of molecular characteristics of the cDNAs encoding nucleoside transport proteins has benefited from a vast increase in the number of cloned equilibrative and concentrative nucleoside transporters (1-6). Kinetic characteristics determined from previous functional studies in membrane vesicles isolated from specific tissues (i.e., kidney, intestine) as well as those determined from tissue slices (choroid plexus) have been reexamined with the isolated cDNAs expressed in heterologous expression systems such as *Xenopus laevis* oocytes or mammalian cells. The development of mammalian expression systems (Chapter 3, (7)) for the study of cloned Na^+-dependent nucleoside transporters has provided an alternative to the *Xenopus laevis* oocyte expression system, which is subject to seasonal

variability, and provides a method for the rapid screening of nucleosides and nucleoside analogs with the expressed proteins. Cloning of the human homologs of N1 and N2 (hSPNT1 and hCNT1, respectively) has provided an opportunity to study species differences which exist between the rat and human homologs (Chapter 4). Finally, critical regions for nucleoside recognition for the Na^+-independent nucleoside transporters have been localized (8) to within one transmembrane region, leading to a more complete picture of how nucleosides interact with their respective transport proteins.

With the recent cloning of the cDNAs encoding the human homologs of both the purine (N1) and pyrimidine (N2) nucleoside transporters, it is now possible to critically examine species differences observed between the expressed proteins. Significant species differences have been observed for the adenosine receptors (9). We determined that there are also species differences in the functional properties of the rat and human homologs of the N1 transporters (SPNT and hSPNT1) (Chapter 4). The human clones also offer the opportunity to directly examine drug interactions with a specific nucleoside transporter. The species differences observed between rSPNT and hSPNT1 underscore the importance of determining kinetic parameters and examining drug interactions with the human clones.

Data in HeLa cells transfected with the cDNA of hSPNT1 demonstrate that inosine is transported at a faster rate than adenosine, in comparative studies. This is in contrast to data obtained in HeLa cells transfected with rSPNT cDNA. One hypothesis for this phenomenon was introduced in 1990 by Centelles *et al.* (10). The suggestion was that adenosine is first cleaved by ecto-adenosine deaminase to inosine, and then inosine is taken up by the cell. Further evidence of a functional link between ectonucleotidase activity, responsible for the breakdown of nucleotides, and nucleoside transport was presented by Che *et al.* in a study on Na^+-nucleoside transport in canicular membrane vesicles from the rat liver (11, 12). This mechanism may be a possible explanation for preference for inosine rather than adenosine for hSPNT1; however more extensive kinetic studies are necessary to

determine the actual mechanism and confirm that the V_{max} values are considerably different for the two compounds.

An understanding of the regulation and protein targeting of nucleoside transporters is also critical in gaining a more detailed picture of the overall role of nucleoside transporters in the body. Well-differentiated cell lines, which are commonly used to address such questions, such as MDCK, OK and CaCo-2, are not appropriate for such studies of the Na^+-dependent nucleoside transporters due to high levels of endogenous expression in these cell lines (13, 14). We have therefore developed an epitope-tagged nucleoside transporter, which retains function, but may be detected by an antibody which does not cross-react with endogenous nucleoside transporter. As discussed in Chapter 5, an epitope tag allows for the study of a number of different proteins with a single antibody. In addition this antibody recognizes a unique sequence, which does not cross-react with the protein(s) of interest.

While much progress has been made in obtaining and functionally expressing the cDNAs encoding nucleoside transport proteins, very little advancement has taken place in understanding the role and tissue specific expression (distribution) of these cloned transporters. The orientation of these proteins in the membrane has yet to be determined. In addition, posttranslational modifications such as glycosylation and phosphorylation, as well as regulation of the nucleoside transporters, have not been critically examined. Antibodies developed and characterized in this laboratory (Chapter 6) may be instrumental in addressing questions concerning tissue distribution and localization to either the brush border or basolateral membrane in tissues such as the choroid plexus, kidney, and intestine. In Chapter 6, an anti-peptide antibody which detects a unique region of the pyrimidine nucleoside transporter N2 (cNT1) was described and detection of expressed protein, following transfection of cNT1 cDNA, in HeLa cells was visualized using fluorescence confocal microscopy. We also have developed an anti-peptide antibody against the N1 nucleoside transporter (SPNT). Localization and distribution of the

pyrimidine and purine nucleoside transporters will also contribute to the understanding of the physiological roles that these nucleoside transporters play *in vivo*.

The physiological role of both equilibrative and concentrative nucleoside transporters can now be more carefully and completely dissected with the individual cloned cDNAs encoding the nucleoside transporters now available. The clinically used nucleoside, adenosine, has major physiological effects, including a reduction in glomerular filtration in the kidney and a reduction in heart rate by decreasing the pacemaking rate of sinoatrial heart cells (15). Adenosine is also a catabolite of adenine nucleotides and nucleoside transporters may play major roles in both the termination of action of adenosine, as well as in the salvage of this compound in the tissues that are unable to carry out de novo synthesis of nucleosides.

In the work presented in this dissertation, functional studies of both endogenous and cloned nucleoside transporters were carried out. The cloning of various nucleoside transporters has enabled the teasing apart of overlapping substrate selectivities, by enabling an opportunity to study the individual transporters in isolation. Some complications that will need to be addressed in the future, however, are in the area of membrane targeting. Because nucleoside transporters are present and expressed in a number of differentiated cell lines (i.e. MDCK, OK and CaCo-2) these cell lines which are routinely applied to address specific questions regarding membrane trafficking may not be appropriate for the study of Na^+-coupled nucleoside transporters. This may be one area where an epitope-tag that is either engineered into the cDNA encoding the transporter to be studied or is part of the vector sequence may be very appropriate.

While much *in vitro* data have been obtained for the cloned nucleoside transporters, very little *in vivo* data are available. The development of a knockout mouse for any of the cloned nucleoside transporters would provide novel information concerning the physiological role and importance of the different transporters. The N1 and N2 subtypes appear to have very different tissue distribution and an *in vivo* model, such as a knockout

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