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Functional Expression of Human Intestinal Na^+-Dependent and Na^+-Independent Nucleoside Transporters in *Xenopus laevis* Oocytes

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ABSTRACT. We have shown previously that the human jejunal brush border membrane expresses both the N1 *(cif)* and the N2 *(cit)* Na^+-dependent (concentrative) nucleoside transporters but not the Na^+-independent (facilitative) nitrobenzylmercaptopurineriboside (NBMPR)-sensitive *(es)* transporter (Patil SD and Unadkat JD, *Am J Physiol*, **272:** 1314–1320, 1997). In the present study, we have demonstrated that when *Xenopus laevis* oocytes are microinjected with human jejunal mRNA, four nucleoside transporters are expressed simultaneously, namely the N1 and N2 Na^+-dependent nucleoside transporters and the *es* and the NBMPR-insensitive *(ei)* Na^+-independent transporters. The expressed Na^+-dependent nucleoside transporters showed substrate specificity identical to that previously described by us using jejunal brush border membrane vesicles (Patil SD and Unadkat JD, *Am J Physiol*, **272:** 1314–1320, 1997). The expressed *es* and *ei* Na^+-independent transporters demonstrated broad substrate selectivity with both purines and pyrimidines capable of inhibiting the uptake of guanosine and thymidine mediated by this transporter. The expressed Na^+-dependent nucleoside transporters mediated the transport of their respective nucleoside substrates with a high affinity and a low capacity, whereas the *es* and the *ei* transporters mediated the transport of nucleosides with a low affinity and a high capacity. Collectively, these observations suggest that the Na^+-independent nucleoside transporters are expressed in the basolateral membrane of the human jejunal epithelium. Based on these data, we hypothesize that the concentrative transporters in the brush border membrane and equilibrative transporters in the basolateral membrane are arranged in series in the human jejunal epithelium to allow efficient vectorial transport of nucleosides from the lumen to the blood. The simultaneous expression of four nucleoside transporters in *X. laevis* oocytes establishes a basis for molecular cloning of these four human nucleoside transporters. BIOCHEM PHARMACOL 53;12:1909–1918, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. nucleoside transporters; human intestine; mRNA; Na^+-dependent; Na^+-independent, *es* and *ei*; oocyte expression

Nucleoside permeation across mammalian cell membranes is mediated by multiple transporters that fall into two broad categories. The first consists of Na^+-dependent concentrative transporters that mediate only the influx of nucleosides. The second consists of Na^+-independent equilibrative (facilitative) transporters that mediate both influx and efflux of nucleosides. Based on substrate selectivity, five Na^+-dependent nucleoside transporter subtypes have been identified to date. N1 *(cif)* is purine specific, and guanosine serves as a model substrate. N2 *(cit)* is pyrimidine specific, and thymidine serves as a model substrate. Uridine and adenosine are substrates for both the N1 and N2 transporters [1–3]. N3 *(cib)* transports both purines and pyrimidines [1, 4–7]. N4, found in the human kidney, is identical to N2 in its substrate specificity, except that it also transports guanosine [8]. Although the substrate specificity of the N5 *(cs)* transporter found in human leukemic cells has yet to be established, it differs from the N1–N4 transporters in that it is highly sensitive to inhibition by less than a 10 nM concentration of NBMPR[§] or dipyridamole [9]. N1–N4 nucleoside transporters are not sensitive to either NBMPR or dipyridamole.

In contrast to the Na^+-dependent concentrative transporters, the Na^+-independent equilibrative nucleoside transporters have broad substrate specificity, low affinity and high capacity, and are categorized by their differential sensitivity to NBMPR [2, 3]. The NBMPR-insensitive equilibrative transporter *(ei)* is not inhibited by NBMPR concentrations up to 1 μM, whereas the NBMPR-sensitive transporter *(es)* is inhibited by NBMPR concentrations as

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§ *Abbreviations:* NBMPR, nitrobenzylmercaptopurineriboside; *es*, NBMPR-sensitive; *ei*, NBMPR-insensitive; and BBMV, brush border membrane vesicles.

low as 0.1 nM with maximal inhibition occurring at 1–10 μM [10–14].

Intestinal cells are poor in their capacity to synthesize nucleosides *de novo*, and they rely on salvage pathways for their nucleoside requirements [15]. Therefore, intestinal epithelial cells have to synthesize and target nucleoside transporters to their brush border membranes to salvage dietary nucleosides and catabolized nucleotides found in the intestinal lumen. However, no data are currently available on the identity of the nucleoside transporters present in the human intestine, their distribution on the brush border and the basolateral membrane, or the mechanism(s) by which they transport nucleosides and their analogs. Although the nucleoside transporters of several animal species have been identified and studied in various tissues [1, 4, 5, 16–21], due to tissue-specific and species-specific expression of nucleoside transporters, these data cannot be extrapolated to the human intestine. Indeed, data obtained in our laboratory using human jejunal BBMV indicate that unlike the rat intestine where N1, N2, and N3 transporters are present [4], only the N1 and N2 Na^+-dependent nucleoside transporters are expressed in the human jejunum [22]. We report below our studies on expression of the human jejunal nucleoside transporters in the widely used heterologous expression cloning system, the *Xenopus laevis* oocytes. Interestingly, our results indicate that oocytes injected with human jejunal mRNA not only express the N1 and the N2 Na^+-dependent nucleoside transporters, but also express the facilitative *es* and *ei* Na^+-independent nucleoside transporters. This is in contrast to our studies with the jejunal BBMV, where no NBMPR-sensitive uptake of nucleosides was observed [22]. Thus, we hypothesize that the *es* and *ei* Na^+-independent nucleoside transporters are likely to be expressed on the basolateral membrane of the human jejunal epithelium. The methods and findings reported here can be used to isolate human cDNA clones of these transporters.

MATERIALS AND METHODS

Chemicals

[5-^{3}H]Uridine (24 Ci/mmol) and [^{3}H]thymidine (20 Ci/mmol) were obtained from ICN Pharmaceuticals Inc. (Costa Mesa, CA). [8-^{3}H]Guanosine (8.2 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA). NBMPR was obtained from the Sigma Chemical Co. (St. Louis, MO). All other chemicals of the highest analytical grade also were purchased from Sigma.

Procurement of Human Small Intestine and Storage of Mucosae

Human small intestines (from the ligament of Treitz to the cecum) were obtained from breathing adult organ donors (victims of vehicular or cerebrovascular accidents but otherwise in good health) of either sex following removal of any transplantable organs. After separating the proximal one-foot segment that represents the duodenum, the proximal foot of the remaining intestinal segment representing the jejunum was removed and rinsed with ice-cold 0.9% NaCl to remove particulate debris. Then, the entire mucosal layer was removed from a partial jejunal segment (4–6 inches) and immediately frozen in liquid nitrogen and stored at −70° until further use.

Isolation of Poly (A)$^+$ RNA

The frozen mucosae (0.5 g each) were homogenized in 4 M guanidine isothiocyanate solution containing 1 M Tris–HCl (pH 7.5), 0.01 M EDTA (pH 7.5), 0.02 M sarcosyl, 0.02 M β-mercaptoethanol, and antifoam A containing 30% non-ionic emulsifiers and centrifuged at 5000 *g* for 15 min at 4°. The resulting clear supernatant (3 mL) was layered onto a 2-mL cesium chloride cushion (5.7 M) and centrifuged at 120,000 *g* for 20 hr at 20° in an SW 55 rotor to recover the total RNA pellets. The RNA pellets were resuspended in diethylpyrocarbonate (DEPC)-treated (RNAse-free) water and precipitated overnight at −20° in 5 M NaCl and absolute ethanol. The RNA was pelleted by centrifugation (10,000 *g* for 10 min at 20°) and then washed in 70% ethanol and air dried. The RNA pellets were resuspended in DEPC-treated water, and the purity, concentration, and integrity of the preparations were checked by the optical density ratio at 260/280 nm and by gel electrophoresis. Retrieval of poly (A)$^+$ RNA was achieved by two cycles of oligo(dT)-cellulose affinity chromatography using packed oligo(dT)-cellulose columns. Total RNA (500–1000 μg) was applied to the column and washed sequentially with ten 1-mL aliquots of a loading buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl and 0.05% SDS). The poly (A)$^+$ RNA was then eluted from the column with seven 0.4-mL aliquots of an elution buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 0.05% SDS). The poly (A)$^+$ preparations were also checked for concentration and integrity as described above for total RNA.

Preparation of Oocytes and Microinjection of mRNA

Mature oocyte bearing female *X. laevis* were anesthetized with ice, and ovarian lobes were removed, opened, and transferred into sterile MBM medium (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)^2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 2.5 mM sodium pyruvate, 0.05 mg/mL penicillin, and 0.1 mg/mL gentamicin in 10 mM HEPES, pH 7.5). Clumps of oocytes were incubated with gentle agitation in MBM medium containing 8 mg/mL collagenase type I (Sigma), and 1 mg/mL type III trypsin inhibitor (Sigma) at room temperature for approximately 60–90 min to defolliculate the oocytes. Then the oocytes were washed (2 × 5 min) in MBM and incubated for 30 min at room temperature in hypertonic phosphate buffer (100 mM K_2HPO_4, pH 6.5) supplemented with 0.1% bovine serum albumin. Oocytes were allowed to recover

overnight at 20° in 50% Leibovitz L-15 medium containing L-glutamine and 15 mM HEPES (pH 7.4), and mature healthy stage V and VI oocytes were chosen (inspected microscopically) for subsequent injections. Microinjections were carried out using an N_2 driven injection apparatus and a 50-μL capillary pipette with an 11–23 μM diameter opening. Oocytes were injected into the vegetal cytoplasm with either 40 ng (1 ng/nL) intestinal mRNA or 40 nL of water. Injected oocytes were then incubated for 3 days at 20° with daily changes of Leibovitz L-15 medium prior to measuring transport activity. Preliminary studies were done to identify optimal parameters for oocyte incubations and uptake studies including mRNA injection volume, duration, and temperature for oocyte incubation following injections. The results of these preliminary studies indicated that the maximum volume of mRNA, temperature, and duration of incubation necessary to maintain functional oocytes that maximally yield Na^+-nucleoside uptake up to 30 min (using uridine) was 40 nL (1 ng/nL), 20°, and 3 days, respectively (data not shown).

Ooctye Uptake Assays

The initial uptake rates of radioactive (^3H) tracer uridine, thymidine, or guanosine (> 90% radiochemical purity, see below) by oocytes injected with mRNA or water were measured for 30 min in the presence (150 mM NaCl) or absence (150 mM choline chloride) of Na^+ in 0.2 mL of transport assay buffer (2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES, pH 7.4). [In preliminary studies, the uptake of uridine (1 and 4 μM) was found to be linear for up to 1 hr at room temperature in NaCl and choline chloride assay buffer in the presence or absence of NBMPR.] Each experiment was performed in the presence or absence of the Na^+-independent *es* inhibitor, NBMPR (10 μM). The oocytes (8–10 oocytes per well) were incubated with the uptake buffer in a 24-well Falcon tissue culture plate (Becton Dickinson, Lincoln Park, NJ) with gentle agitation on a Gyrotary shaker. All experiments were preincubated for 30 min in choline chloride medium to remove sodium prior to conducting the transport assay. The membrane impermeable marker, [^{14}C]inulin (0.05 μCi), was included in the assay buffer to identify leaky oocytes. Following uptake incubations, the extracellular tracer was removed by three 1-min washes with the ice-cold transport buffer containing the appropriate unlabeled substrate. Individual oocytes were then solubilized in 5% SDS, and the quantity of radioactivity in each oocyte was determined using dual label ($^3H/^{14}C$) liquid scintillation counting (Packard, Downers Grove, IL). For NBMPR inhibition experiments, oocytes were preincubated (30 min) with the inhibitor prior to adding the labeled substrate. In competitive inhibition studies, the oocytes were co-exposed to both the labeled substrate and the unlabeled inhibitor.

Nucleoside Metabolism

Since intracellular metabolism of nucleosides by the oocytes can affect the initial rate of nucleoside uptake, the accumulation of ^3H-nucleosides by the mRNA-injected oocytes during the 30-min incubation used above was followed by HPLC equipped with a reverse phase column (4.6 mm × 25 cm C18, 5 μm, ultrasphere; Beckman, Fullerton, CA). The mobile phase for uridine was methanol:water (3:97) and for thymidine and guanosine was methanol:water (6:94). At a mobile phase flow rate of 1 mL/min, the retention times of uridine, guanosine, and thymidine were 2, 10, and 15 min, respectively. Oocytes (8–10) were incubated with radiolabeled nucleosides (2 μM) as described in the transport assay above. The oocytes were washed free of extracellular radiolabel with transport buffer (100 μL) containing the unlabeled nucleosides (2 μM). Then, the oocytes in 100 μL of the transport buffer were extracted immediately with an equal volume of chloroform and centrifuged (12,000 *g* for 5 min), and 50 μL of the aqueous phase was injected onto the HPLC. Mobile phase fractions corresponding to the UV-detected nucleoside peak were collected at 1-min intervals and counted in a liquid scintillation analyzer. Control experiments were always conducted. In the first control experiment, the nucleoside tracer (2 μM) was incubated in the transport buffer in the absence of oocytes and then processed by HPLC as described above. In the second control experiment, the nucleoside tracer (2 μM) and oocytes were immediately extracted, HPLC-injected and the fractions pertaining to the UV-detectable nucleoside peak were counted. With three different batches of oocytes, the recovery of the unchanged [^3H]uridine when expressed as a percent of radioactivity found in oocytes that were extracted immediately was 90 ± 2% (no degradation of tracer was observed when incubated for 30 min in the absence of oocytes). This indicates negligible metabolism of uridine in the oocytes over the 30-min incubation time. Similar recoveries of unchanged substrates in three different batches of oocytes were observed for thymidine (87 ± 6%) and guanosine (93 ± 3%).

Data Analysis

All uptake values were expressed as means ± SEM of 6–10 oocytes per assay. The net Na^+, *es*, or *ei* uptake was calculated as the difference in uptake by mRNA-injected oocytes in the presence and absence of Na^+ and in the presence and absence of 10 μM NBMPR. Statistical significance of differences between two independent nucleoside uptake experiments was obtained using the nonparametric, Wilcoxon Rank Sum Test. The standard errors of the differences in the mean were generated from the respective standard errors of the individual means. A Michaelis–Menten model was fit to the net mean Na^+, *es*, and *ei* nucleoside uptake values by nonlinear (BMDP, Los Ange-

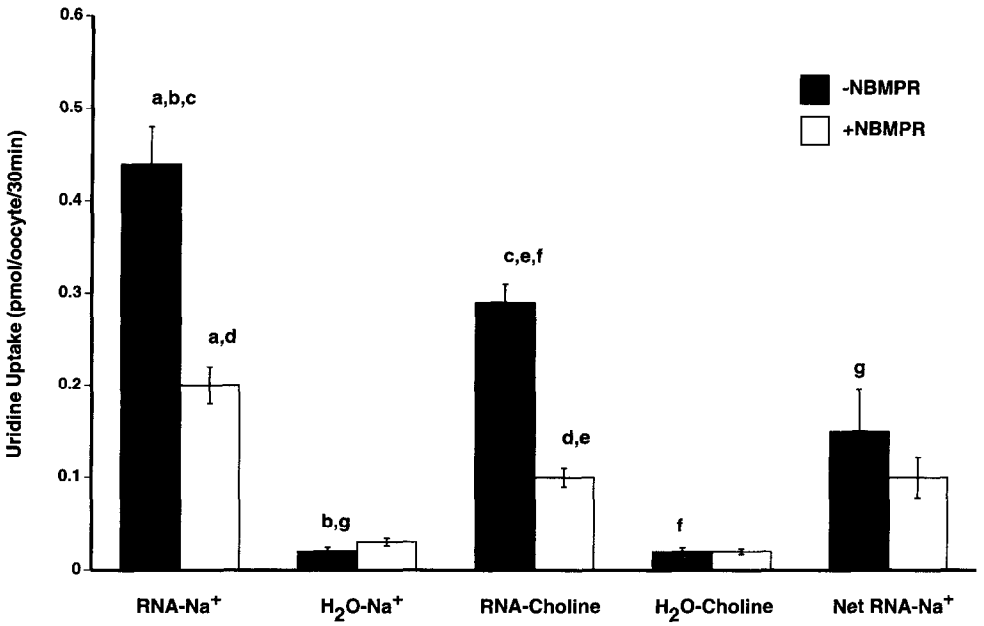

FIG. 1. Na^+-dependent and Na^+-independent uridine uptake by human jejunal mRNA-injected *Xenopus* oocytes. Oocytes were injected with either 40 ng human jejunal mRNA dissolved in water (1 ng/1 nL) or 40 nL water and incubated for 3 days at 20° in 50% Leibovitz-15 medium with daily medium changes. Uptake of 2 μM uridine (30 min uptake) by oocytes was determined at room temperature in uptake buffer containing either 150 mM NaCl or 150 mM choline chloride in the absence (closed column) or presence (open column) of 10 μM NBMPR. The values (pmol/oocyte/30 min) are expressed as means ± SEM of uptake by 8–10 oocytes per experiment. Uptake values that are significantly ($P < 0.05$) different from each other are depicted by labeling the corresponding histograms with the same alphabetical letters. For example, in the absence of NBMPR, the Na^+-dependent uptake of uridine by mRNA-injected oocytes was statistically greater than that by mRNA-injected oocytes in the absence of Na^+ or that in the water-injected oocytes.

les, CA) regression using weight = 1 to estimate the uptake kinetic parameters.

RESULTS

Na^+-Dependent and Na^+-Independent Nucleoside Transport in Oocytes Injected with Human Jejunal mRNA

Since uridine is a substrate for all Na^+-dependent transporters, we used it in our initial studies to first determine and then quantify the functional activity of nucleoside transporters expressed in human jejunal mRNA-injected oocytes (Fig. 1). Surprisingly, we found that in the absence of Na^+, [^{3}H]uridine (2 μM) uptake by mRNA-injected oocytes was 15-fold greater ($P < 0.01$) than that by control (water-injected) oocytes. In addition, 10 μM NBMPR significantly inhibited (by 65%) the Na^+-independent uptake of [^{3}H]uridine, indicating the presence of the NBMPR-sensitive *(es)* transporter in these oocytes. However, even in the presence of 10 μM NBMPR, the uptake of [^{3}H]uridine in the mRNA-injected oocytes was significantly greater (4-fold) ($P < 0.05$) than that in the water-injected oocytes, indicating the expression of the NBMPR-insensitive transporter in the mRNA-injected oocytes.

In the presence of Na^+, [^{3}H]uridine (2 μM) uptake by intestinal mRNA-injected oocytes was found to be 22-fold greater ($P < 0.01$) than that in water-injected oocytes. The contribution of Na^+-dependent transporters to the uptake of [^{3}H]uridine, determined as the difference between [^{3}H]uridine (2 μM) uptake in the presence and absence of Na^+ was found to be significantly greater (7.5-fold; $P < 0.05$) than that in water-injected oocytes. No significant inhibition of net Na^+-dependent uridine uptake was observed in the presence of 10 μM NBMPR (Fig. 1). Collectively, the above data indicate that both the Na^+-dependent and the Na^+-independent nucleoside transporters are translated, targeted into the plasma membrane, and function as integral transport proteins in *Xenopus* oocytes injected with human intestinal mRNA.

Na^+-Independent, NBMPR-Sensitive (es) *and NBMPR-Insensitive* (ei) *Mediated Nucleoside Uptake in Oocytes Injected with Human Jejunal mRNA*

The presence of a significant contribution (65%) of Na^+-independent nucleoside transport to the uptake of uridine led us to further characterize these transporters expressed in mRNA-injected oocytes. To establish that 10 μM NBMPR completely inhibited the *es* nucleoside transporter, we studied the concentration-dependent NBMPR inhibition of Na^+-independent (150 mM choline chloride) guanosine (4 μM; purine) and thymidine (4 μM; pyrimidine) uptake in jejunal mRNA-injected oocytes (Fig. 2). Maximum

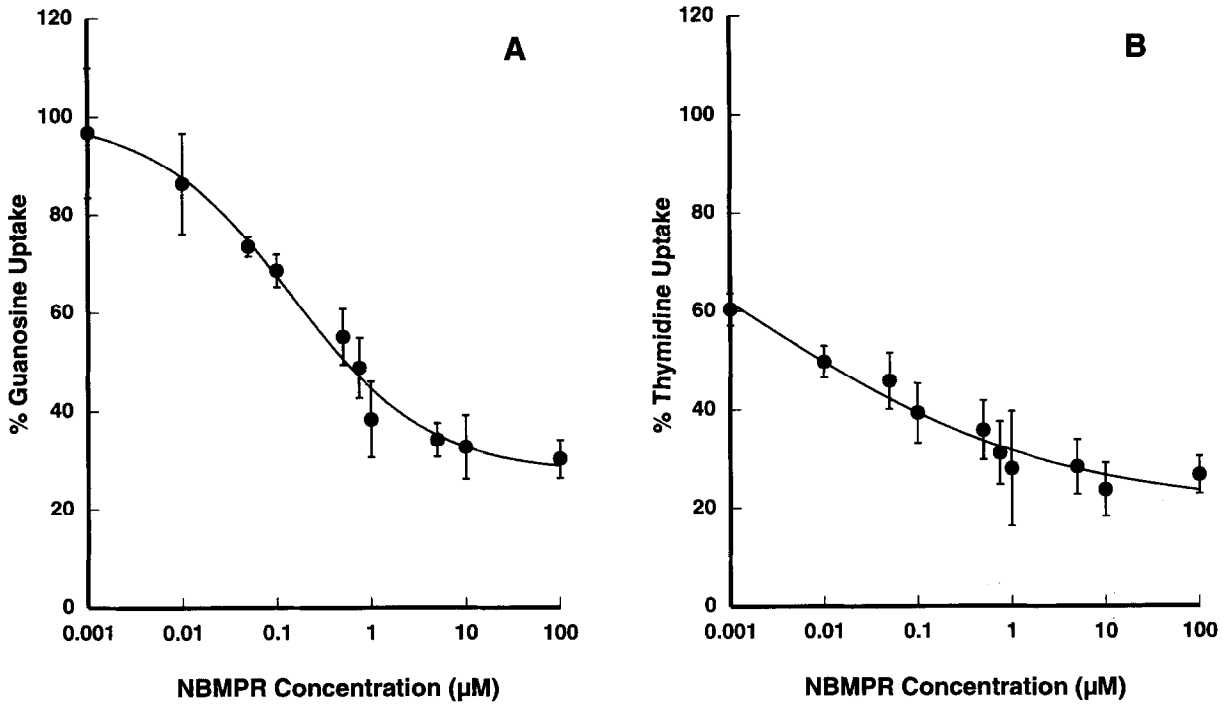

FIG. 2. Concentration-dependent NBMPR inhibition of Na^+-independent nucleoside uptake by human jejunal mRNA-injected *Xenopus* oocytes. Oocytes injected with jejunal mRNA, prepared as described in Fig. 1, were used to measure uptake of guanosine (A) and thymidine (B) (4 μM; 1 μM ^3H tracer with 30-min assay time) in 150 mM choline chloride containing assay buffer in the presence of various concentrations of NBMPR (0–100 μM). The results are expressed as percent of the mean control value (thymidine and guanosine uptake values in the absence of NBMPR were 0.13 ± 0.004 and 0.26 ± 0.03 pmol/oocyte/30 min, respectively). The values are mean ± SEM of uptake by 6–8 oocytes per NBMPR concentration. The mean uptake values for guanosine and thymidine by the water-injected oocytes in the presence of 10 μM NBMPR were 0.04 ± 0.001 and 0.02 ± 0.002 pmol/oocyte/30 min, respectively.

inhibition of uptake of guanosine (IC_{50} 142 nM) or thymidine (IC_{50} 1.5 nM) was observed at 1–100 μM NBMPR. The residual uptake of both substrates at 1 μM NBMPR was not significantly different from that at 10 or 100 μM NBMPR, indicating the expression of both the *es* and *ei* nucleoside transporters in the mRNA-injected oocytes. The presence of the *ei* transporter was supported further by the inhibition of this residual component by 100 μM dipyridamole, a known inhibitor of both the *es* and the *ei* transporter [23] (Fig. 3).

Substrate Specificity of the Na^+-Dependent Nucleoside Transporters Expressed in Oocytes Injected with Jejunal mRNA

Our previous studies with the BBMV from the human intestinal jejunum showed the presence of N1 and N2 Na^+-dependent nucleoside transporters. To determine if both these transporters are also expressed in the human jejunal mRNA-injected oocytes, we determined the inhibition profile of both purine (inosine) and pyrimidine (cytidine) nucleosides (100 μM) on the net Na^+-dependent uptake of the N1 and the N2 model substrates guanosine (Fig. 4A) and thymidine (Fig. 4B), respectively. The net Na^+-dependent guanosine uptake was inhibited significantly by inosine ($P < 0.05$) but not by cytidine. The net Na^+-dependent thymidine uptake was inhibited signif-

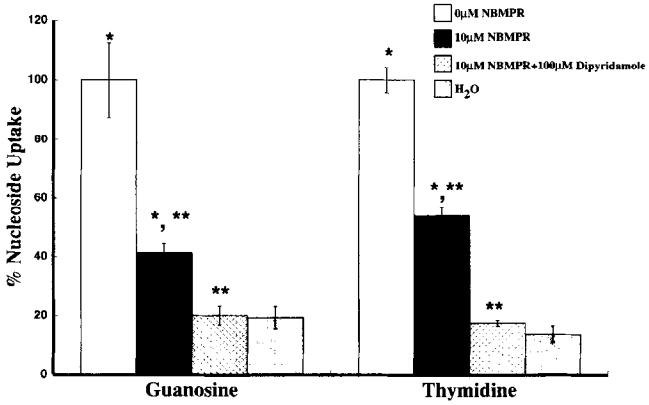

FIG. 3. Effect of dipyridamole on Na^+-independent *ei* uptake of [^3H]guanosine and [^3H]thymidine by human jejunal mRNA-injected *Xenopus* oocytes. The uptake of guanosine and thymidine (4 μM; 1 μM ^3H tracer with 30-min assay time) was measured in oocytes injected with 40 ng human jejunal mRNA in choline chloride assay buffer containing no NBMPR, 10 μM NBMPR, or 10 μM NBMPR plus 100 μM dipyridamole. The results are expressed as percent of the mean control value (thymidine and guanosine uptake values in the absence of NBMPR were 0.15 ± 0.01 and 0.21 ± 0.03 pmol/oocyte/30 min, respectively). The values are means ± SEM of uptake of three different assays with 6–8 oocytes per uptake assay. The mean uptake values for guanosine and thymidine by the water-injected oocytes in the presence of 10 μM NBMPR were 0.04 ± 0.001 and 0.02 ± 0.002 pmol/oocyte/30 min, respectively. Uptake values that were significantly ($P < 0.05$) different from each other are depicted by labeling the corresponding histograms with the same symbol.

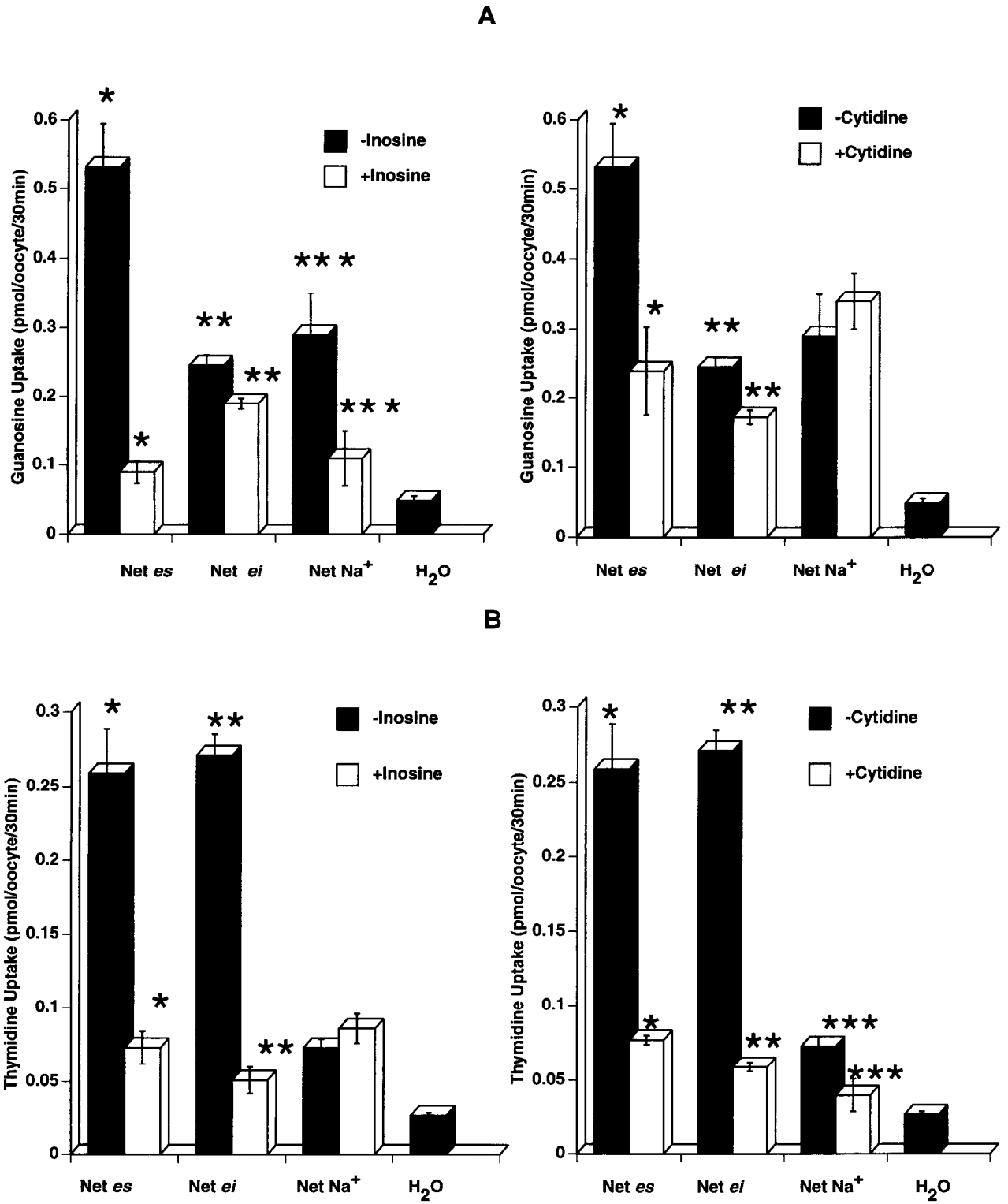

FIG. 4. Effect of purine and pyrimidine on Na^+-dependent and Na^+-independent *es* and *ei* uptake of [^{3}H]guanosine and [^{3}H]thymidine by human jejunal mRNA-injected *Xenopus* oocytes. The uptake of 4 μM guanosine (A) and thymidine (B) (1 μM ^{3}H tracer with 30-min assay time) was measured in oocytes injected with 40 ng human jejunal mRNA in assay buffer containing no NBMPR or 10 μM NBMPR and either NaCl (150 mM) or choline chloride (150 mM) with or without (control) inosine (100 μM) or cytidine (100 μM). The values represent the means ± SEM (pmol/oocyte/30 min) of uptake by 6–8 oocytes and were calculated as described in the text. Uptake values that were significantly ($P < 0.05$) different from each other are depicted by labeling the corresponding histograms with the same symbol. The mean uptake values of guanosine and thymidine by the water-injected oocytes were 0.04 ± 0.002 and 0.045 ± 0.003 pmol/oocyte/30 min, respectively.

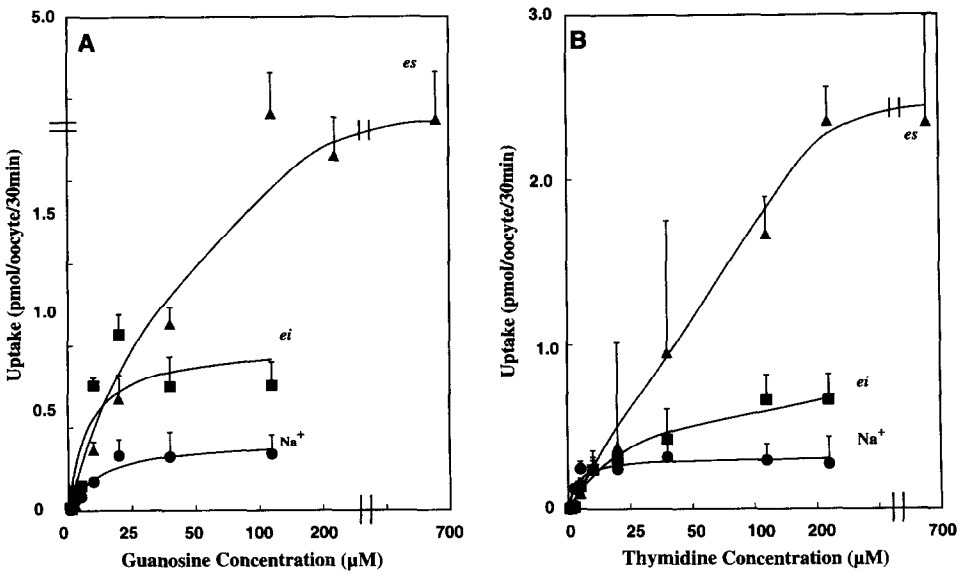

FIG. 5. Concentration-dependent Na^+-dependent and Na^+-independent nucleoside uptake by human intestinal mRNA-injected *Xenopus* oocytes. The rate (pmol/oocyte/30 min) of Na^+-dependent Na^+-independent, *es* and Na^+-independent, *ei* uptake of guanosine (A) or thymidine (B) (1 μM ^3H tracer with 30-min assay) by oocytes injected with 40 ng jejunal mRNA was determined in the presence and absence of 10 μM NBMPR in assay buffer containing NaCl (150 mM) or choline chloride (150 mM). Each value represents the mean ± SEM uptake by 6–10 oocytes. In panel A, the break in the curve represents the discontinuities in both axes.

icantly by cytidine ($P < 0.05$) but not by inosine. These results demonstrate that the Na^+-dependent nucleoside uptake in the human intestinal mRNA-injected oocytes was mediated by both the N1 and the N2 transporters. The Na^+-independent *es* and *ei* uptake of guanosine and thymidine was inhibited significantly ($P < 0.05$) by both inosine and cytidine (Fig. 4). These results suggest that the Na^+-independent *es* and *ei* transporters accept both purines and pyrimidines as substrates.

Concentration-Dependent N1, N2, es *and* ei *Nucleoside Uptake by Oocytes Injected with Human Jejunal mRNA*

The Na^+-dependent (150 mM NaCl) and the Na^+-independent (150 mM choline chloride) guanosine and thymidine uptake by water- or intestinal mRNA-injected oocytes (5–8 oocytes per substrate concentration) was determined as a function of increasing substrate concentration in the presence (10 μM) and absence of NBMPR (to suppress the *es*-mediated guanosine and thymidine uptake). The plots showing the concentration versus the net mean Na^+-dependent, Na^+-independent *es*- and *ei*-mediated uptake of guanosine (Fig. 5A) and thymidine (Fig. 5B) demonstrate that these processes were saturable. The Michaelis–Menten kinetic parameters for these saturable processes are listed in Table 1.

DISCUSSION

The results of our studies indicate that the human jejunal mRNA-injected, *X. laevis* oocytes express four nucleoside transporters, namely the N1 *(cif)* and N2 *(cit)* Na^+-dependent transporters and the *es* and *ei* Na^+-independent nucleoside transporters (Fig. 1). The expression of the N1 and N2 Na^+-dependent nucleoside transporters is consistent with our previous study in which these transporters were found to be present in the brush border membrane of the human jejunum [22]. However, in that study we found no evidence for the presence of the Na^+-independent *es* transporter in the brush border membrane. Collectively, therefore, these observations suggest that the Na^+-independent nucleoside transporters are expressed in the basolateral membrane of the human jejunal epithelium. Based on these data, we hypothesize that the nucleoside transporters are arranged in series in the human jejunal epithelium, with the concentrative transporters in the brush border membrane and equilibrative transporters in the basolateral membrane, to allow efficient vectorial transport of nucleosides from the lumen to the blood (Fig. 6). Such an anatomical arrangement of Na^+-dependent and Na^+-independent transporters has been described for glucose [24], which is actively taken up in the human intestine. If our hypothesis is correct, this will be the first time that such

TABLE 1. Kinetics parameters for N1, N2, *es*, and *ei* nucleoside uptake by human intestinal mRNA-injected *Xenopus* oocytes

Type	Substrate	Experiment	K_m (μM)	V_{max} (pmol/oocyte/30 min)
N1	Guanosine	1	20.7 ± 7.0*	0.42 ± 0.05*
		2	21.2 ± 12.4	0.24 ± 0.05
N2	Thymidine	1	5.8 ± 2.1	0.15 ± 0.01
		2	6.2 ± 2.6	0.19 ± 0.02
ei	Guanosine	1	13.1 ± 9.9	1.00 ± 0.23
		2	48.7 ± 25.7	0.93 ± 0.22
		3	154.5 ± 40.7	2.10 ± 0.20
ei	Thymidine	1	51.2 ± 11.4	0.42 ± 0.03
		2	40.2 ± 16.2	1.71 ± 0.3
		3	43.5 ± 25.6	0.22 ± 0.04
es	Guanosine	3	101.5 ± 39.2	5.90 ± 0.80
es	Thymidine	3	360.5 ± 168.3	3.99 ± 0.93

* Values are estimates ± SD of the estimates.

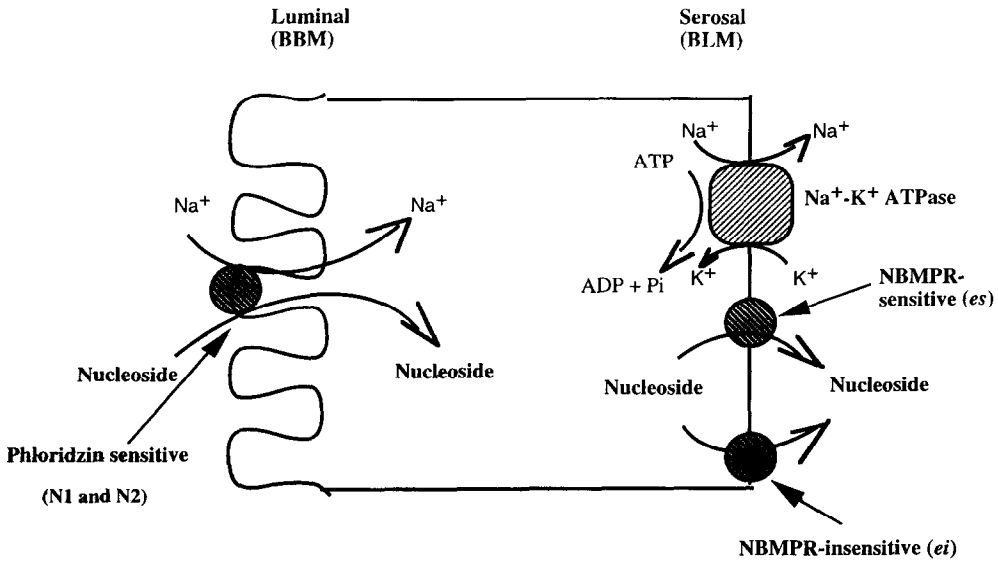

FIG. 6. Proposed scheme for nucleoside transport in the human jejunal epithelium. This proposed arrangement in series of the concentrative transporters in the brush border membrane (BBM) and the equilibrative transporters in the basolateral membrane (BLM) of the human jejunal epithelium would allow for efficient vectorial transport of nucleosides from the lumen to the blood.

a vectorial arrangement of nucleoside transporters would have been found in the intestine. In addition, to our knowledge, this is the first time that these four transporters have been expressed simultaneously in mRNA-injected *Xenopus* oocytes.

The expression of Na^+-dependent nucleoside transporters in the intestine appears to be species specific. While only the N1 and N2 Na^+-dependent nucleoside transporters are found in the human jejunum, evidence of the presence of three (N1, N2 and N3) Na^+-dependent nucleoside transporters has been obtained in expression studies for the rat jejunum with mRNA-injected oocytes [4]. Similarly, oocyte expression studies with rabbit intestinal mRNA have found expression of only the N2 Na^+-dependent nucleoside transporter [25]. In the same study, no expression of Na^+-independent transporters was observed. However, using BBMV, the expression of the N1 and N2 or N3 nucleoside transporters has been reported in the rabbit intestine [17, 19, 20].

The Na^+-dependent nucleoside transporters expressed in mRNA-injected oocytes in this study appear to be of the type that have substrate specificity and kinetic characteristics similar to the transporters previously characterized by us in the brush-border membrane of the human jejunal epithelium [22]. That is, Na^+-dependent guanosine uptake was inhibited significantly by purines and not pyrimidines. Likewise, thymidine uptake was inhibited significantly by pyrimidines and not purines. That is, both the N1 and N2 Na^+-dependent nucleoside transporters found in the brush-border membrane of the human jejunum are also expressed in the mRNA-injected oocytes. However, we found no evidence of the presence of either the N3 or N4 Na^+-dependent nucleoside transporters as reported previously for the rat intestine [4], the rabbit choroid plexus [5–7], and the human kidney [8, 26, 27]. The net Na^+-dependent uptake of guanosine and thymidine by the N1 and the N2 transporters expressed in mRNA-injected oocytes was found to be saturable (Fig. 5) and displayed Michaelis–Menten kinetics (Table 1). These transporters take up guanosine and thymidine with high affinity and low capacity. The similarity in K_m values to those reported earlier by us for the jejunal BBMV (thymidine K_m: 2.74 ± 0.54 μM; guanosine K_m: 12.02 ± 1.34 μM) indicates that the transporters are faithfully transcribed and expressed in the *X. laevis* oocytes. In addition, the K_m values are also similar to those reported for adenosine and thymidine uptake by oocytes expressing the rat canalicular N1 [28], and the rabbit and rat intestinal N2 [25, 29] transporters, respectively.

Uptake of nucleosides by jejunal mRNA-injected oocytes in the absence of Na^+ indicates the expression of Na^+-independent facilitative transporters (Fig. 1). The concentration-dependent NBMPR inhibition (Fig. 2) of thymidine and guanosine fluxes into the oocytes indicates the presence of both the *es* and *ei* equilibrative transporters. Maximal inhibition (~75%) of thymidine and guanosine uptake into oocytes was obtained with 1–100 μM NBMPR, indicating a significant contribution from the *es* transporter. The residual uptake activity was resistant to inhibition by NBMPR concentration as high as 100 μM, but sensitive to dipyridamole (100 μM), an *ei* inhibitor [23], indicating a lesser (~25%) but significant contribution of the *ei* transporter. In contrast to the Na^+-dependent transporters, the *es* and *ei* transporters appear to have a broad substrate selectivity. The uptake of both thymidine and guanosine by these transporters was found to be susceptible to inhibition by both purines and pyrimidines. A similar result has been obtained with BeWo cells where nucleoside uptake by the *es* transporter expressed in *Xenopus* oocytes was found to be inhibitable by both purines and pyrimidines [30]. In contrast to the Na^+-dependent nucleoside uptake, the Na^+-independent transport of nucleosides is of lower affinity and higher capacity. Previous reports on *es* mediated transport of nucleosides have reported a wide range of K_m values [31] ranging from 20 μM

to 2–4 mM. The Michaelis–Menten kinetics of uptake of guanosine and thymidine by the *es* and *ei* transporters expressed in oocytes injected with jejunal mRNA are similar (Table 1) to those reported previously for *es* and *ei* mediated thymidine uptake in BeWo cells [14] and polymyelocytic leukemia NB4 cells [32], respectively. The basis for the large inter-experimental variability in the estimate of the *ei* K_m of guanosine is not clear. However, we believe that this variability is due, in part, to the fact that the *ei* uptake values are obtained after multiple subtractions of the various transporter components and because the relative contribution of the *ei* transporter to the overall uptake of the nucleosides is small.

In this paper, we report for the first time the functional expression and characterization of the human intestinal Na^+-dependent N1 and N2 and the Na^+-independent *es* and *ei* nucleoside transporters in *X. laevis* oocytes injected with mRNA isolated from the human jejunum. Our ability to analyze and quantify this simultaneous expression of four nucleoside transporters should now allow the isolation and characterization of cDNA clones coding for each of these four human nucleoside transporters as has been demonstrated previously for rat liver N1 [28], and intestinal N2 [33] nucleoside transporters. Once isolated, the cDNA clones of these transporters will be invaluable in conducting studies on the fundamental biology of these transporters such as elucidating the mechanistic basis for the difference between the various transporters in their substrate selectivity and Na^+ dependency. Since the intestine is both the site of absorption of orally administered nucleoside drugs and a dose-limiting target of toxicity of nucleoside drugs that inhibit *de novo* nucleoside synthesis, such mechanistic studies have several important implications. For example, such studies could lead to development of therapeutic strategies that target the tumor cells and spare the intestine or target the diseased intestine. In addition, such studies could lead to development of novel nucleoside drug candidates that demonstrate improved absorption characteristics.

NOTE ADDED IN REVISION: Griffiths *et al.* [34] recently cloned the human placental *es* transporter which when functionally expressed in *Xenopus* oocytes demonstrates kinetic (K_m for uridine 240 μM) characteristics and a broad substrate specifity similar to that described above for the human intestinal *es* transporter.

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