Na^+-dependent nucleoside transport in liver: two different isoforms from the same gene family are expressed in liver cells

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Hepatocytes show a Na^+-dependent nucleoside transport activity that is kinetically heterogeneous and consistent with the expression of at least two independent concentrative Na^+-coupled nucleoside transport systems (Mercader et al. Biochem. J. **317**, 835–842, 1996). So far, only a single nucleoside carrier-related cDNA (SPNT) has been isolated from liver cells (Che et al. J. Biol. Chem. **270**, 13596–13599, 1995). This cDNA presumably encodes a plasma membrane protein responsible for Na^+-dependent purine nucleoside transport activity. Thus, the liver must express, at least, a second nucleoside transporter which should be pyrimidine-preferring. Homology cloning using RT-PCR revealed that a second isoform is indeed present in liver. This second isoform turned out to be identical to the 'epithelial-specific isoform' called cNT1, which shows in fact high specificity for pyrimidine nucleosides. Although cNT1 mRNA is present at lower amounts than SPNT mRNA, the amounts of cNT1 protein, when measured using isoform-specific polyclonal antibodies, were even higher than the SPNT protein levels. Moreover, partially purified basolateral plasma membrane vesicles from liver were enriched in the SPNT but not in the cNT1 protein, which suggests that the subcellular localization of these carrier proteins is different. SPNT and cNT1 protein amounts in crude membrane extracts from 6 h-regenerating rat livers are higher than in the preparations from sham-operated controls (3.5- and 2-fold, respectively). These results suggest that liver parenchymal cells express at least two different isoforms of concentrative nucleoside carriers, the cNT1 and SPNT proteins, which show differential regulation and subcellular localization.

INTRODUCTION

Nucleoside transport into mammalian cells is mediated by equilibrative Na^+-independent and concentrative Na^+-dependent transport systems (for a recent review see [1]). Equilibrative uptake involves, at least, two independent agencies (ei and es) which differ in their sensitivity to nitrobenzylthioinosine inhibition [1]. Concentrative transport systems involve at least three biological activities, N1 (cif), N2 (cit) and N3 (cib), which differ in their substrate specificity [1]. Indeed, N1 is purine-preferring, N2 pyrimidine-preferring and N3 shows broad substrate specificity. Although they all translocate adenosine and uridine with similar affinity, N1 is competitively inhibited by formycin B and N2 by thymidine [1]. Na^+-dependent nucleoside transport appears to be a feature of polarized epithelia (kidney and intestine) [1–7], and has also been described in liver parenchymal cells [8–11].

Using liver plasma membrane vesicles it has been shown that Na^+-dependent uridine transport is concentrative and sensitive to membrane potential, which is consistent with electrogenic properties of the carrier translocation activity and with the predicted stoichiometry of 1 Na^+/1 nucleoside [9]. Although this transport activity initially seemed to be accounted for by a single agency, we have recently shown that at least two different transport systems account for nucleoside uptake in isolated hepatocytes [11]. This is based upon the biphasic inhibition of Na^+-dependent uridine transport triggered by other nucleosides such as guanosine and upon the differential sensitivity to inhibitors like formycin B and thymidine. The possibility that two nucleoside carriers are expressed in a single cell type is not unlikely, since this has been reported in absorptive epithelia (jejunum) by kinetic approaches [6] and, more conclusively, after cloning two cDNAs, related to the N1 and N2 types of nucleoside transporters, initially called SPNT and cNT1, which are co-expressed in jejunum [12,13]. Recently, using RT-PCR, it has also been shown that both isoforms may be also expressed in brain [14].

The substrate specificity of the protein product of the hepatic SPNT cDNA, which seems to be responsible for a Na^+-dependent purine-preferring nucleoside transport system, does not really explain the heterogeneous kinetics recently described for the concentrative uridine transport activity found in liver parenchymal cells. From this scenario it is evident that liver cells must express at least one second nucleoside carrier isoform, other than the SPNT gene product.

Using RT-PCR homology cloning we have been able to identify a second nucleoside carrier isoform present in liver, which turned out to be identical to the 'epithelial-specific' isoform known as cNT1. Isoform-specific polyclonal antibodies were generated to prove that the cNT1 protein is expressed in isolated rat liver parenchymal cells. Results are consistent with a differential regulation and subcellular localization of these nucleoside carrier proteins in hepatocytes.

MATERIALS AND METHODS

Materials

[5,6-^{3}H]Uridine (38.5 Ci/mmol) was purchased from DuPont–NEN (Hertfordshire, England). D-[1-^{14}C]Mannitol (57 Ci/mmol), [α-^{32}P]dCTP (10 mCi/ml), [α-^{32}P]dCTP (10 mCi/ml) were from Amersham (Buckinghamshire, England). All cold nucleo-

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sides were from Sigma (St. Louis, MO, U.S.A.). Collagenase A from *Clostridium hystoliticum* was obtained from Boehringer (Mannheim, Germany). 3-Dibutyl phthalate and bis-(3,5,5-trimethylhexyl) phthalate were purchased from Fluka (Buchs, Switzerland). All media used for CHO cell culture were obtained from Whittaker (Verviers, Belgium). Restriction enzymes, RNAse H and buffers were from Boehringer (Mannheim, Germany). The Wizard™ DNA clean-up system, the PolyAtract® mRNA isolation system, the PCR-related Reverse transcription system, Riboprobe® combination system T3/T7, Klenow fragment, T4 DNA ligase and *Taq* polymerase were from Promega (Madison, WI, U.S.A.). All other reagents were of analytical grade.

Hepatocyte isolation, CHO cell culture and uptake measurements

Hepatocytes were isolated from male Wistar rats (200–240 g) by an adaptation of previous standard procedures [15]. CHO-K1 cells were grown, as previously described [16,17], in minimum essential medium supplemented with 4 % (v/v) fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, a mixture of non-essential amino acids and antibiotics.

Nucleoside uptake by isolated rat liver parenchymal cells and CHO cell monolayers was measured as previously described [11,17]. Routinely 1 μM uridine uptake was measured. Inhibitors were added at a final concentration of 100 μM. Uptake rates were expressed as either pmol/min per 10^6 cells or pmol/min per mg protein. Protein was measured according to Bradford [18].

RNA isolation, poly(A)$^+$ RNA purification, cDNA synthesis

Total RNA from liver, kidney, intestine and CHO-K1 cells was isolated using the guanidinium thiocyanate method as previously described [19]. Poly(A)$^+$ RNA was purified from total RNA using the PolyAtract® mRNA isolation system following manufacturer's instructions. Once the poly(A)$^+$ RNA was purified the cDNA was synthesized using the PCR-related Reverse transcription system following the manual but increasing the reaction time to 30 min. Thereafter the cDNA/mRNA hybrid was degraded using RNAse H and the whole reaction mixture was cleaned up using the Wizard™ DNA clean-up system.

PCR cloning, cDNA fragment subcloning and sequencing

cDNA synthesized from liver, kidney and CHO-K1 cells were used. To get the PCR-generated SPNT cDNA, 5 μl of the above cDNA reaction was used as a template. The SPNT oligonucleotides F1 (5′-GCTCAAAGGCCAGAGCAGCTGATC-3′, bp 776–799) and R1 (5′-CAGCTTCACTCCCTCCTTGCTCTT-3′, bp 1465–1442) derived from the published hepatic cDNA sequence [13] were used. The PCR reaction was set up mixing (final concentration): 1 × *Taq* polymerase buffer, 1.5 mM $MgCl_2$, 2.2 mM each dNTPs, 0.4 μM each F1/R1 oligonucleotides, the cDNA and water to 50 μl final volume. Fifty μl of mineral oil was added onto the reaction. The reaction mixture was heated to 94 °C for 5 min and kept to 80 °C. Then, 2.5 units of *Taq* polymerase was added. The PCR conditions were: 1 min, 94 °C; 2 min, 57 °C; 3 min, 72 °C for 40 cycles. Finally, the PCR was heated to 72 °C for 10 min and kept at 4 °C until running the samples in a 1 % agarose gel (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0).

In order to clone the pyrimidine-preferring cNT1-like hepatic cDNA, we used oligonucleotides generated to specific putative transmembrane domains of the jenunum cNT1 cDNA according to the published sequence [12]. The PCR reaction was set as described above. The PCR conditions were: 1 min, 94 °C; 2 min, 42 °C; 3 min, 72 °C for 5 cycles. Then, the annealing temperature was changed to 57 °C and the PCR was run for a further 35 cycles. The cNT1 oligonucleotides used were: F1 (5′-TTTGCAGGCATCTGTGTGTTCCTT-3′, bp 702–725) and R1 (5′-CAACGCACAAGGGGCGGCCATGAC-3′, bp 1295–1272). Once 10 μl of the samples was run as described before, 5 μl of the PCR reaction was used in a new nested PCR using the same conditions. The new cNT1 nested oligonucleotides were: F2 (5′-GTGTTCCTTGTCCTTCTCTTTGCT-3′, bp 717–740) and R2 (5′-GGCCATGACAGAGGCTGCGATTAA-3′, bp 1280–1257). The resulting cDNAs were run in a 1 % agarose preparative gel (40 mM Tris, 20 mM acetic acid. 1 mM EDTA, pH 8.0). The cDNA bands were cut from the gel and purified using the Geneclean kit (Bio 101 Inc., La Jolla, CA). The cDNAs were blunted with Klenow and ligated into an *Eco*RV digested and dephosphorylated BlueScript KS plasmid. The cDNA sequences were confirmed using the Auto Read sequencing kit and the A.L.F. DNA Sequencer (Pharmacia).

Northern blot analysis

Up to 20 μg of total RNA and 2 μg of poly(A)$^+$ RNA were fractionated by electrophoresis through a 1 % agarose gel. The gel was treated as described previously [20] and the RNA transferred overnight and cross-linked to a Hybond-N+ filter (Amersham). The filters were prehybridized and hybridized at high stringency following ref. [21]. The cDNA probes were those obtained by PCR as described above. Where indicated a T3 generated antisense cRNA from cNT1 was used. When cRNA was used as a probe, the prehybridization and hybridization conditions were 50 % formamide and 65 °C. Filters were washed once for 30 min at 65 °C with 3 × SSC and 1 % SDS, once with 1 × SSC and 1 % SDS, and once with 0.2 × SSC and 1 % SDS before autoradiography.

Antipeptide antibodies and Western blot

In order to design oligopeptides suitable for the production of isoform-specific antibodies, the primary structures of the cNT1 and SPNT proteins were analysed on the basis of four criteria: (1) the prediction of transmembrane segments according to the methods of Sander [22] and Aloy [23]; (2) the position of α-helix in the predicted secondary structure using a program that combines the algorithms of Chou-Fasman and Rose [24]; (3) the position of hydrophobicity peaks in the hydrophilicity plot using the Kyte–Doolitle method [25], with a window size of 9 amino acids, and (4) the Surface Probability and Flexibility Index plots, according to the algorithms of Boger [26] and Karplus and Schulz [27], respectively.

Oligopeptides corresponding to residues 45–67 and 30–53 from cNT1 and SPNT proteins respectively were chosen on the basis of their putative antigenicity and their uniqueness in terms of sequence. These oligopeptides were coupled to keyhole limpet haemocyanin, emulsified with an equal volume of Freund's adjuvant and injected into rabbits. Immunoreactive antisera was used for Western blot analysis. Crude membrane extracts from liver, kidney and hepatocytes were obtained by homogenizing these samples in 10 mM Tris (pH 7.4) supplemented with 0.5 % Triton X-100. Liver plasma membrane vesicles, enriched in the basolateral domain were purified using a Percoll density gradient method extensively characterized in our laboratory [28,29]. Twenty μg protein samples were heated for 6 min at 100 °C prior to a 10 % SDS/PAGE. Proteins were transferred onto filters (Immobilon-P, Millipore) and these were blocked in a 5 % dry milk-supplemented 0.2 % Tween-20 phosphate buffer saline (PBS) solution prior to immunoreaction. Filters were then

incubated for 1 h with the diluted antisera (1/500 in a 0.2% Tween-20 PBS solution supplemented with 1% bovine serum albumin). A goat anti-rabbit IgG, coupled to horseradish peroxidase, was used as a second antibody and diluted 1/2000 in dry milk-supplemented 0.2% Tween-20 PBS. To visualize the cNT1 and SPNT proteins ECL (Amersham) was used.

RESULTS

Uptake of 0.5 μM uridine into isolated rat hepatocytes and inhibition by selected nucleosides are shown in Table 1. Results are consistent with the occurrence of at least three independent transport systems: a Na^+-independent one, which is resistant to nitrobenzylthioinosine inhibition (not shown), and two Na^+-dependent transport systems, with similar affinities for uridine and adenosine. Under the experimental conditions detailed above, formycin B and thymidine separately inhibited Na^+-dependent uridine uptake by about 40%, while guanosine inhibition reached 60% of the control values. The combination of either formycin B and thymidine or guanosine and adenosine triggered an inhibition of Na^+-dependent uridine transport greater than 80%. Uridine uptake in CHO-K1 cells was Na^+-independent.

Table 1 Nucleoside transport in isolated liver parenchymal cells and CHO-K1 cells

Results are the mean ± S.E.M. of four independent transport studies. Values are pmol uridine/min per 10^6 cells and pmol uridine/min per mg protein for isolated hepatocytes and CHO-K1 respectively. Uridine transport was 0.5 μM in liver cells and 5 μM in CHO-K1 cells. Selected inhibitors were always used at 100 μM, except guanosine plus adenosine, where adenosine was 50 μM. The Na^+-dependent transport (Na^+-dep) is expressed as a result of the uptake in the presence of sodium (Total) minus the rate in the choline chloride medium (Choline). See Experimental section for details. %, percentage of inhibition.

	Transport rates				
Inhibitor	Total	Choline	%	Na^+-dep	%
Liver cells (pmol uridine/min per 10^6 cells)					
None	1.91 ± 0.34	0.77 ± 0.80	–	1.15 ± 0.31	–
Adenosine	0.50 ± 0.24	0.22 ± 0.06	71	0.12 ± 0.05	90
Formycin B	0.98 ± 0.16	0.42 ± 0.09	45	0.64 ± 0.14	44
Thymidine	0.94 ± 0.21	0.36 ± 0.06	53	0.60 ± 0.15	41
Guanosine	0.98 ± 0.18	0.51 ± 0.09	34	0.47 ± 0.22	60
Formycin B + thymidine	0.59 ± 0.04	0.39 ± 0.01	49	0.20 ± 0.001	83
Guanosine + adenosine	0.57 ± 0.06	0.36 ± 0.04	53	0.21 ± 0.02	82
CHO-K1 cells (pmol uridine/min per mg protein)					
None	5.77 ± 0.50	5.67 ± 0.60	–	0.10 ± 0.10	–

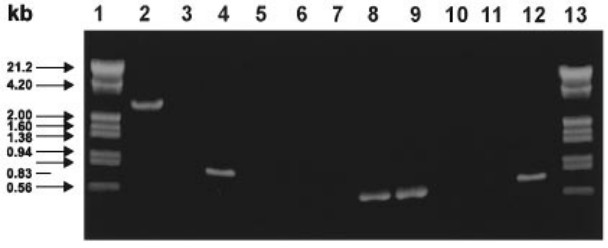

Figure 1 PCR results of the SPNT and cNT1 cDNA from liver, kidney and CHO-K1 cells

The PCR reaction conditions and the oligonucleotides used are described in the Experimental section. A representative 1% agarose in 0.5 × TBE is shown. Lanes 1 and 13, DNA molecular weight markers III (Boehringer–Mannheim); lane 2, *Eco*RV digested BlueScript KS used as a subcloning vector; lanes 3–6, PCR reaction with cNT1 oligonucleotides F1/R1 from H_2O, kidney, liver and CHO-K1 cells, respectively; lanes 7–10, the nested reaction performed with cNT1 oligonucleotides F2/R2 from H_2O, kidney, liver and CHO-K1 cells, respectively; lanes 11, 12, PCR samples from H_2O and liver with F1/R1 SPNT oligonucleotides.

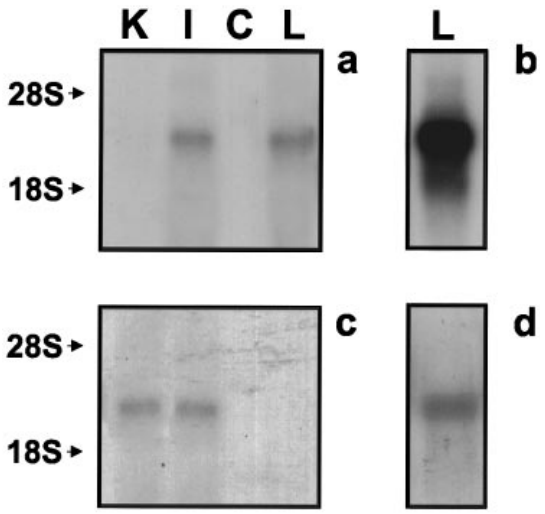

Figure 2 SPNT and cNT1 Northern blot from different tissues and CHO-K1 cells

Up to 20 μg of total RNA and 2 μg of poly(A)$^+$ RNA were analysed as described in the Experimental section. (**a** and **b**), SPNT hybridization; (**c** and **d**), cNT1 results. (**b**) and (**d**) show the specific SPNT and cNT1 hybridization to 2 μg of liver mRNA respectively. K, kidney; I, intestine; C, CHO-K1 cells and L, liver.

The PCR results using liver, kidney and CHO-K1 cDNAs and cNT1-related oligonucleotides are shown in Figure 1. The result using SPNT-related oligonucleotides on liver cDNA is also shown (lane 12). The first cNT1 PCR reaction (oligonucleotides F1/R1) gave a product from kidney cDNA (lane 4) of the expected molecular weight (~ 0.6 kb). No specific bands were observed initially when using liver and CHO-K1 cDNAs (lanes 5 and 6 respectively). However, a subsequent nested PCR with the cNT1 F2/R2 oligonucleotides (lanes 7–10) resulted in a product of about ~ 0.56 kb in both kidney and liver (lanes 8 and 9), but not in CHO-K1 cells (lane 10). An SPNT-specific band was also obtained by PCR in kidney with F1/R1 oligonucleotides in the conditions detailed above (not shown). Subcloning and sequencing of the PCR products revealed that the cNT1-related fragments obtained from kidney and liver were indeed identical, and turned out to be cNT1 itself.

mRNA expression of cNT1 and SPNT from different tissues and CHO-K1 cells is shown in Figure 2. SPNT is present in intestine and liver, but not in kidney or CHO-K1 cells. However, in agreement with the findings from the PCR experiments, an SPNT faint band was detected in kidney but not in CHO-K1 cells when blots were over-exposed (not shown). The cNT1 transcripts were found in kidney and intestine. CHO-K1 and liver RNA did not show any specific hybridization with the cNT1 cDNA probe when analysing it by regular Northern blot. However, when poly(A)$^+$ RNA from liver (2 μg) was hybridized at high stringency with the cRNA cNT1 probe, a transcript of the expected molecular weight appeared. No band was evident when using CHO-K1 poly(A)$^+$ RNA under the same experimental conditions (not shown).

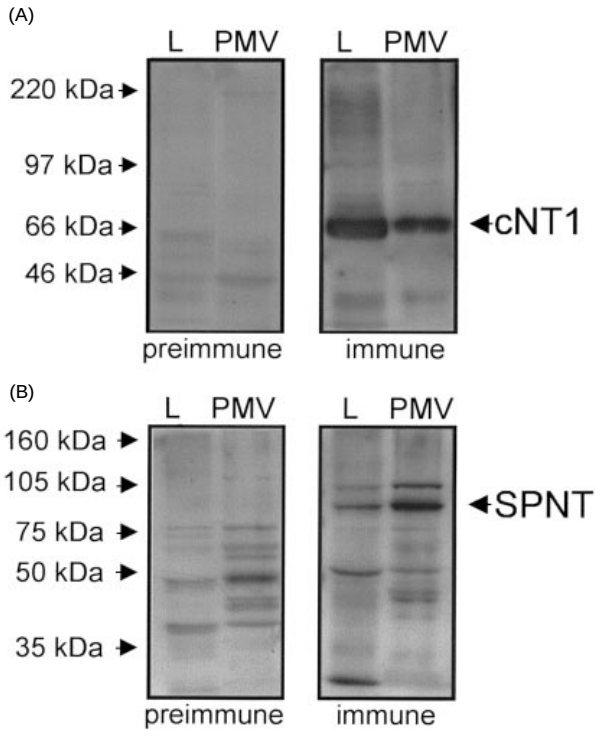

Figure 3 Specificity of anti-cNT1 and anti-SPNT antisera

(**A**) Shows a representative Western blot analysis of 20 μg protein samples from crude homogenates (L) and plasma membrane vesicle preparations (PMV) from rat liver, using the preimmune and immune antisera obtained from the same rabbit immunized with the cNT1-derived oligopeptide. (**B**) shows the same analysis but using the anti-SPNT antisera.

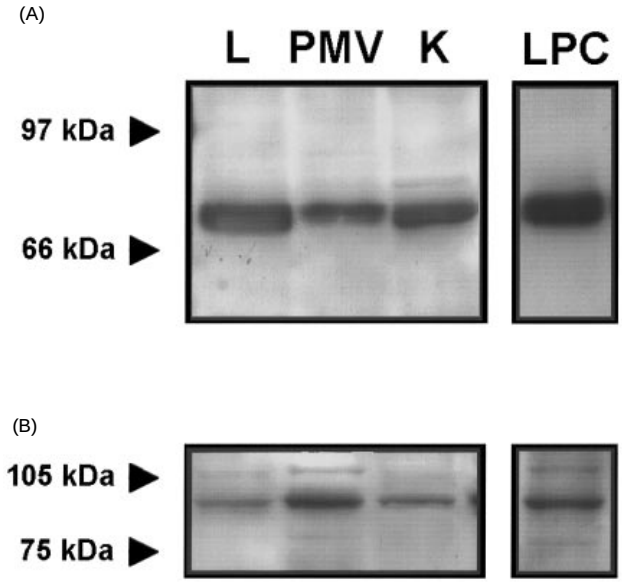

Figure 4 SPNT and cNT1 proteins

Ten μg of protein were used for Western blot analysis, as described in the Materials and methods section. A representative result is shown. (**A**) Shows the cNT1 protein in liver (L), liver plasma membrane vesicles (PMV), kidney (K) and liver parenchymal cells (LPC). (**B**) Shows the SPNT protein in the same samples.

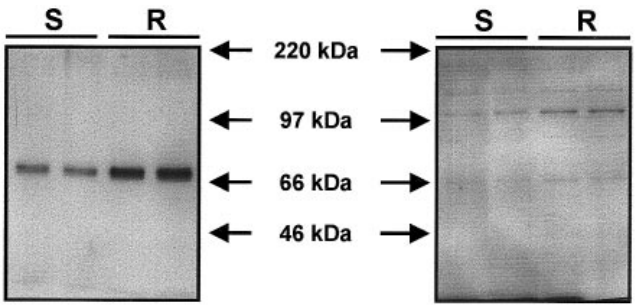

Figure 5 Changes in cNT1 and SPNT protein amounts during liver regeneration after partial hepatectomy

Liver crude membrane extracts (10 μg protein) from either sham-operated (S) or hepatectomized rats (R) 6 h after surgery were used for Western blot analysis as described in the Materials and methods section. A representative result including two independent samples from each experimental group is shown.

Polyclonal antisera reacted with proteins of apparent molecular weights of about 74 and 103 kDa, for cNT1- and SPNT-derived oligopeptides, respectively (Figure 3). Specificity of these major bands is proven by comparing the immune with the preimmune antisera from the same rabbit (Figure 3). Both isoforms were also detected in kidney and liver parenchymal cells (Figure 4). Liver plasma membrane vesicle preparations selectively enriched in the basolateral membrane domain showed an enrichment in SPNT protein amounts. Nevertheless, cNT1 protein levels in these preparations were significantly lower than those detected in whole liver extracts (Figure 4).

Figure 5 shows the cNT1 and SPNT protein amounts in crude extracts from either regenerating rat livers 6 h after partial hepatectomy or from sham-operated controls. Densitometric analysis of Western blots using independent samples showed that the SPNT protein amounts in crude membrane fractions from regenerating rat livers were 3.5-fold higher than in those preparations from sham-operated rats ($340 \pm 50\,\%$, mean $\pm$ S.E.M., $n = 4$). cNT1 protein amounts were also higher in regenerating rat livers than in their controls, but to a lesser extent (about 2-fold) than for the SPNT isoform ($200 \pm 26\,\%$, mean $\pm$ S.E.M., $n = 4$).

DISCUSSION

This study shows that, in accordance with previous kinetic evidence [11], liver parenchymal cells express at least two isoforms of Na^+-dependent nucleoside transporters (N1 and N2 types) that belong to the same gene family and translocate purines and pyrimidines with different affinities. Homology cloning of putative cNT1- and SPNT-related cDNAs in rat liver resulted in the isolation of a second cDNA which, as indicated above, turned out to be cNT1 itself. Generation of isoform-specific antibodies clearly proved that both isoforms are present in hepatocytes. So far, cNT1 has been considered to be an epithelial-specific isoform, because it had been detected exclusively in kidney and jejunum. Nevertheless, very recently, both SPNT and cNT1 mRNAs have been found in brain [14] and, as indicated here, also in liver.

Since kinetic heterogeneity of Na^+-dependent nucleoside transport has been also reported in brush border renal vesicles, kidney cells may also express two different nucleoside carrier isoforms, N1 and N2 types, as reported here for liver. Indeed, as indicated above, an SPNT-related product was isolated from kidney by RT-PCR. This may actually correspond to the 'liver'

purine preferring SPNT gene, since the SPNT protein is also detected in kidney protein extracts. The lack of relationship between the mRNA and protein amounts for both isoforms in liver and kidney suggests that the two isoforms are differentially regulated in both organs. This would also be consistent with translational regulation of both transport systems. Moreover, the relative enrichment of SPNT protein in basolateral plasma membrane vesicles from rat liver compared to the decrease in cNT1 protein amounts supports the view that both carrier isoforms may show a different subcellular localization. This is also consistent with the fact that the SPNT protein may be highly glycosylated but not the cNT1 isoform, as deduced from their apparent molecular weight calculated from their relative electrophoretic mobility on SDS/PAGE. In any case, to ascertain the subcellular localization of both carrier proteins may be relevant for a better understanding of nucleoside metabolism in liver parenchymal cells.

An interesting feature of Na^+-dependent nucleoside transport in liver is that it can be regulated. This transport activity is significantly induced in *in vivo* models of hyperinsulinaemia [30,31] and in the early phase of liver growth after partial hepatectomy [31,32]. Insulin is also able to induce Na^+-dependent uridine uptake in primary cultures of rat hepatocytes [30]. Since two different isoforms are present in liver cells, thus it is interesting to find out which isoform is specifically up-regulated under these physiological conditions. In this report we provide the first evidence of differential regulation of SPNT and cNT1 expression in the early phases of liver growth after partial hepatectomy. Although it does not seem possible to establish a direct relationship between changes in protein amounts and transport activities, it is clear that the magnitude of SPNT increase is higher than the one found for the cNT1 protein. Indeed, it is likely that SPNT up-regulation may be responsible for most of the induced Na^+-dependent uridine uptake reported in plasma membrane vesicles from regenerating rat livers [32]. This is also consistent with the reported increase in SPNT mRNA found in livers from hepatectomized rats 2 h after surgery [31].

In summary, we show that kinetic heterogeneity of Na^+-dependent nucleoside transport in liver is the result of the co-expression of at least two different carrier isoforms, the SPNT-related one, previously cloned by others [13], and that newly identified in this laboratory, which turned out to be identical to the 'epithelial-specific' gene, initially called cNT1. Both SPNT and cNT1 proteins are co-expressed in liver parenchymal cells and appear to be differentially regulated.

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