Nucleoside transport systems in liver parenchymal cells. Isoform expression pattern in hepatocyte differentiation

Sistemas de transporte de nucleósidos en células parenquimales hepáticas. Patrón de expresión de isoformas durante la diferenciación del hepatocito

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ABSTRACT

Nucleoside uptake into rat hepatocytes is mediated by, at least, three independent transport systems, a Na^+-dependent purine-preferring system (N1-like), a Na^+-dependent pyrimidine-preferring transport system (N2-like) and, finally, a Na^+-independent agency which is NBTI-insensitive. The concentrative component of transport is highly regulated, since it is induced in liver hypertrophia and hyperplasia and up-regulated by pancreatic hormones. Two different cDNAs, SPNT and cNT1, apparently related to N1 and N2 transport activities respectively, have been characterized so far in liver. The SPNT mRNA is more abundant than the cNT1 mRNA, but the biological activities associated with N1 and N2 transport systems are rather equivalent. Transformed liver parenchymal cells (hepatoma cell lines) and fetal hepatocytes show a different pattern of nucleoside transport systems. These highly proliferative cells show high nucleoside transport activity although this is mediated mostly by a Na^+-independent transport component which is mostly sensitive to NBTI inhibition. This equilibrative NBTI-sensitive transport system is not detected in adult hepatocytes. We conclude that the translocation of nucleosides across the plasma membrane of the hepatocyte involves a complex combination of carrier proteins which may differ depending on the differentiated state of the cell rather than by their proliferative status.

Key words: Nucleoside. Transport. SPNT. cNT1. Liver. Hepatoma. Fetus.

RESUMEN

La captación de nucleósidos por el hepatocito está mediada por un mínimo de tres sistemas de transporte, dos dependientes de sodio con especificidades para purinas y pirimidinas (N1 y N2 respectivamente) y un tercer sistema independiente de sodio e insensible a NBTI. La componente de transporte concentrativa está fuertemente regulada, ya que se induce en la hipertrofia e hiperplasia hepática y es estimulada por hormonas pancreáticas. Hasta la fecha se han caracterizado únicamente dos ADNc susceptibles de codificar proteínas transportadoras de nucleósidos de tipo N1 y N2

(SPNT y cNT1 respectivamente). La cantidad de ARNm para SPNT es más elevada que la de cNT1, si bien la actividad biológica de ambos sistemas es similar. Tanto las líneas celulares derivadas de hepatomas como los hepatocitos fetales presentan un patrón de expresión de transportadores de nucleósidos marcadamente distinta a la de un hepatocito adulto. Su elevada tasa de proliferación se acompaña de una elevada capacidad de transporte de nucleósidos, pero ésta se debe, en gran medida o casi exclusivamente, a una componente equilibrativa, independiente de sodio y sensible a NBTI. Esta componente de transporte no se detecta en el hepatocito diferenciado. Concluimos que la translocación de nucleósidos a través de membrana plasmática del hepatocito esta mediada por varias proteínas de membrana, cuyos niveles de expresión dependen más del estado de diferenciación de la célula que de su estado proliferativo.
Palabras clave: Nucleósidos. Transporte. SPNT. cNT1. Hígado. Hepatoma. Feto.

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INTRODUCTION

Hepatocytes retain the ability to proliferate after liver injury. A selective up-regulation of plasma membrane carriers seems to be a characteristic feature of proliferating liver parenchimal cells. In particular, the induction of system A for neutral amino acid transport has been suggested to play a permissive role in liver regeneration (for a recent review see 1). This up-regulation parallels cell cycle and it has been proposed that a major physiological role for system A activation would be the supply of compatible osmolytes for cell volume increase prior to mitosis (2). In this context, it has been shown that system A activity is modulated by hypertonicity in both epithelial and mesenchymal cells (3,4), which supports the hypothesis that amino acids are concentrated far beyond their need for metabolic purposes and may behave as actual compatible osmolytes.

System A activity is high during development (5,6) and its regulation by pancreatic hormones is already established during suckling, although full maximal responses of system A to insulin and glucagon are not still achieved after weaning (7). Induction of system A also occurs during the pre-replicative phase in the regenerating rat liver (8), when a compensatory increase in Na^+,K^+-ATPase activity is associated with an enhancement in the amounts of the $\alpha 1$ and $\beta 1$ subunit mRNAs and proteins (9). Indeed, indirect evidence suggests that the $\alpha 1$ subunit protein of the sodium pump and system A are coordinately regulated at the gene level (10).

A few years ago we decided to check to what extent the up-regulation of hepatic transporters following a mitogenic stimulus was specific to system A. We wondered whether nucleoside transport could be affected by liver growth

promotion. Although liver parenchymal cells show high endogenous synthesis of nucleosides it was not unlikely that their requirements would not be completely fulfilled in a rapid proliferative state, like the one found during liver regeneration after partial hepatectomy or development.

As shown in Figure 1, nucleoside transport systems in mammalian cells have been classified on the basis of their kinetic properties. In thermodinamic terms there are two major types of nucleoside transport systems, equilibrative and concentrative. The former are Na^+-independent and translocate nucleosides downhill a concentration gradient, which, in most cell types, is achieved thanks to the low intracellular nucleoside concentration. These low levels are maintained by the active metabolism of these compounds. In some instances, these equilibrative transport systems may also enable a cell to export nucleosides. Equilibrative transport systems have generally been sub-divided into two kinetic types, a NBTI-sensitive (es) and a NBTI-insensitive (ei). NBTI (nitrobenzylthioinosine) is a nucleoside analogue that can inhibit equilibrative nucleoside transport with high efficiency. Indeed nanomolar concentrations of NBTI can block nucleoside uptake when the concentration of the substrate is in the micromolar range. None of these transport activities has been associated to any cloned cDNA so far. On the contrary, concentrative Na^+-dependent

NUCLEOSIDE TRANSPORT SYSTEMS IN MAMMALIAN CELLS

	EQUILIBRATIVE		CONCENTRATIVE		
	es	***ei***	***cif(N1)***	***cit(N2)***	***cib(N3)***
Na-dependence	−	−	+	+	+
NBTI-inhibition	+	−	−	−	−
Dipyridamol inhibition	+	+	−	−	−
SUBSTRATES					
Formycin B	+	+	+	−	+
Uridine/Adenosine	+	+	+	+	+
Purines	+	+	+	−	+
Pyrimidines	+	+	−	+	+

Fig. 1.—*Kinetic types of nucleoside transport systems in mammalian cells.* As explained in the text, this classification has been established on the basis of kinetic data. At the present time two more Na^+-dependent transport agencies (putative N4 and N5) have been reported, although no extensive characterization of these transport systems has been performed and they are not included yet in this diagram.

transport has been often adscribed to three major kinetic types. N1 (cif) is a purine-preferring transport system which can be inhibited by formycin B. N2 (cit) is a pyrimidine-preferring agency insensitive to formycin B inhibition. N3 seems to correspond to a broad specificity transport system. As indicated below, two cDNAs that are likely to be related to N1 and N2 activities have been cloned so far.

In this manuscript we will summarize and review most of the recent progress we have made in the understanding of the functional, regulatory and molecular properties of nucleoside transporters in liver parenchymal cells.

MATERIALS AND METHODS

Animals and cell lines as experimental tools

To characterize the regulatory and molecular aspects of nucleoside transporters in liver parenchymal cells we have used several *in vivo* models and cultured cell lines. The regulatory properties of these transport systems were assessed in obese Zucker rats (a model of liver hypertrophia), in rats that underwent a partial hepatectomy (a model of liver regeneration —hyperplasia—), in euglycemic hyperinsulinemic rats (a model of experimental hyperinsulinemia previously used in our laboratory to analyze the *in vivo* regulation of system A (11)) and, finally, rat fetuses and neonates, used to monitor the developmental regulation of nucleoside transport activity. For more *in vitro* approaches we used hepatocytes and the established cell lines FAO, HepG2 and Wif-B.

Characterization of nucleoside transport activity

The first characterization of nucleoside transport systems in liver cells was done using plasma membrane vesicles purified as previously described (12) and using a rapid filtration technique to monitor ^{3}H-uridine uptake. Further characterization was performed in either isolated hepatocytes or primary cultures of rat hepatocytes. Nucleoside uptake measurements were performed as previously described for amino acids (7). Transport systems were characterized using inhibitors on the basis of the accepted kinetic types of nucleoside transport systems shown in Table 1.

Molecular characterization of nucleoside transporters in liver cells

A 0.69kb fragment of the purine-preferring nucleoside transporter-related cDNA (N1-like), isolated by Arias and coworkers by expression cloning (13),

Table 1.—Kinetic properties of Na^+-dependent uridine uptake in rat liver. The results have been taken from references 17, 19 and 20 (*,$p<0.001$) and they correspond to measurements performed on either liver plasma membrane preparations or in isolated rat liver parenchymal cells.

	KINETIC PARAMETERS OF URIDINE UPTAKE	
	Km (μM)	*Vmax (pmol/mg protein/3s*
LIVER PLASMA MEMBRANE		
SHAM-OPERATED	6	1.4
REGENERATING	6	3.7*
LEAN ZUCKER	9	2.1
OBESE ZUCKER	11	5.5*
	Km (μM)	Vmax (pmol/10^6cells/3min)
ISOLATED HEPATOCYTES	8-13μm	200-300

was generated by RT-PCR and used for Northern analysis. In parallel, we undertook the homology cloning of a putative second isoform, using RT-PCR and oligonucleotides derived from the cNT1 cDNA published sequence. This cDNA is functionally related to a pyrimidine-preferring nucleoside transport system (N2-like) which is expressed in epithelia (kidney and intestine) but not in liver. Low stringency nested-PCR yielded a 0.56kb fragment that was blunt-ended and subcloned at an Eco-RV site of the Bluescript KS. This fragment was sequenced with an A.L.F. DNA sequencer using the Auto Read sequencing kit and F-dATP (Pharmacia).

RESULTS AND DISCUSSION

Kinetic characterization of nucleoside transport systems in liver plasma membrane vesicles and isolated hepatocytes

A first kinetic approach to the elucidation of which agencies were involved in nucleoside uptake in liver parenchymal cells was developed using plasma membrane vesicles (LPMV) that retained this nucleoside transport activity (14). These fractions have been extensively used in our laboratory and previously characterized (12,14-16). LPMV showed a Na^+-dependent transport activity of apparent broad specificity, which seemed to be electrogenic and sensitive to membrane potential (12). The likely stoichiometry for this transporter was $1Na^+$:1 nucleoside, which is also consistent with the electrogenic nature of the translocation process (12). LPMV were later used in some of the animal models indicated below. Interestingly, no inhibition of Na^+-independent transport activity was found in LPMV. Nevertheless, this seemed unlikely to be true

and we moved to the isolated cell system to further characterize nucleoside uptake (17,18). Results using isolated cells proved that adult rat hepatocytes show, at least, three independent transport system agencies (17). Although Na^+-dependent uridine transport, when plotted as a function of substrate concentration, gives a clear hyperbolic curve, inhibition studies (Figure 2) clearly show that at least two independent transport systems are involved in the concentrative uptake of nucleosides. One is largely insensitive to thymidine inhibition and the other to formycin B inhibition, which is consistent with N1 and N2 transport activities. Since both show similar affinities versus uridine,

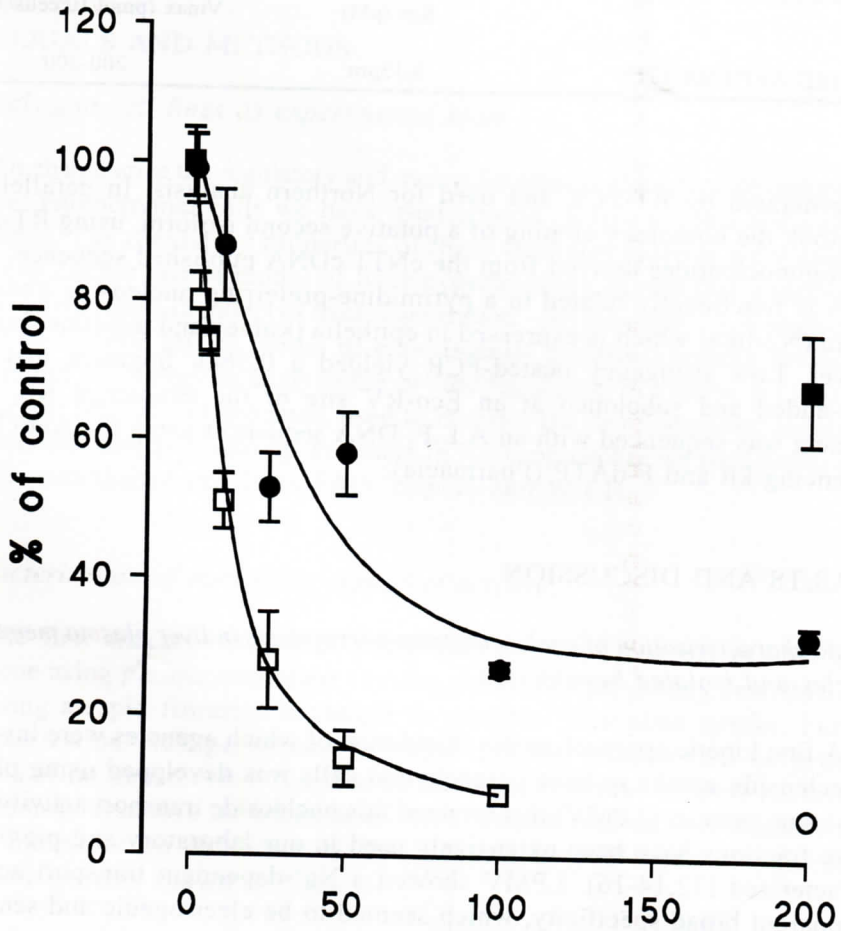

Fig. 2.—*Dose-response inhibition of Na^+-dependent uridine uptake by selected nucleosides.* Na^+-dependent 0.5μM uridine uptake was monitored in isolated hepatocytes either in the absence or in the presence of selected inhibitors. Symbols are: □, adenosine; ●, guanosine; ■, 200μM thymidine; ○, 200μM guanosine plus 25μM adenosine. Basal transport rates were in the range 2-6pmol of uridine per min per 10^6 cells. Results are taken from reference 17.

the Na^+-dependent uptake of this nucleoside is easily fitted with a single transport agency, and indeed, it will probably be referred as this in the section below, devoted to the regulatory aspects of nucleoside transport in liver parenchymal cells. Na^+-independent transport was effectively inhibited by other nucleosides in isolated hepatocytes. This component was shown to be NBTI-insensitive.

Regulatory properties of Na^+-dependent nucleoside transport in liver

What makes a plasma membrane transport system relevant to cell physiology is the possibility of modulating its activity in response to a change in the environment. Na^+-dependent uridine transport was enhanced in a model of liver hypertrophia (Zucker rats that have not underwent obesity symptoms yet) (19) and, as hypothesized, in the pre-replicative phase of liver growth after partial hepatectomy (20). This increase in transport activity was detected in LPMV and resulted from a change in the Vmax without alterations of the affinity constant of the transport system(s) for uridine (Table 1). Obviously, according to the molecular characterization indicated below, it is not clear which is the isoform that is up-regulated in this physiological situation. Nevertheless this adaptation seems to be stable and is consistent with *de novo* synthesis of carrier proteins, not only because of the Vmax effect, but also because this change was detected in LPMV, even after treatment of the membranes with monensin, a known Na^+ ionophore. So far, two hormones seem to modulate the Na^+-dependent hepatic transport of uridine, insulin and glucagon (18). Insulin exerts a long-term effect on this transport activity as determined in primary cultures of rat hepatocytes and in LPMV isolated from rats that underwent an euglycemic hyperinsulinemic clamp (18). This effect is also consistent with *de novo* synthesis of transporters. Nevertheless, glucagon enhances this transport activity in a rapid and transient manner which is mimicked by monensin (Figure 3), thus suggesting that the action of this hormone is mediated by changes in the Na^+ transmembrane gradient and membrane hyperpolarization (18), as previously shown for amino acid transport (21). The evidence that insulin may up-regulate this transport activity in a more permanent manner than glucagon is also supported by the fact that starvation by itself, which is a situation characterized by low insulin levels, is associated with low Na^+-dependent transport activity when measured in LPMV (18).

Molecular characterization of Na^+-dependent nucleoside transporters in liver parenchymal cells

Our laboratory had tried initially to isolate a nucleoside-carrier related

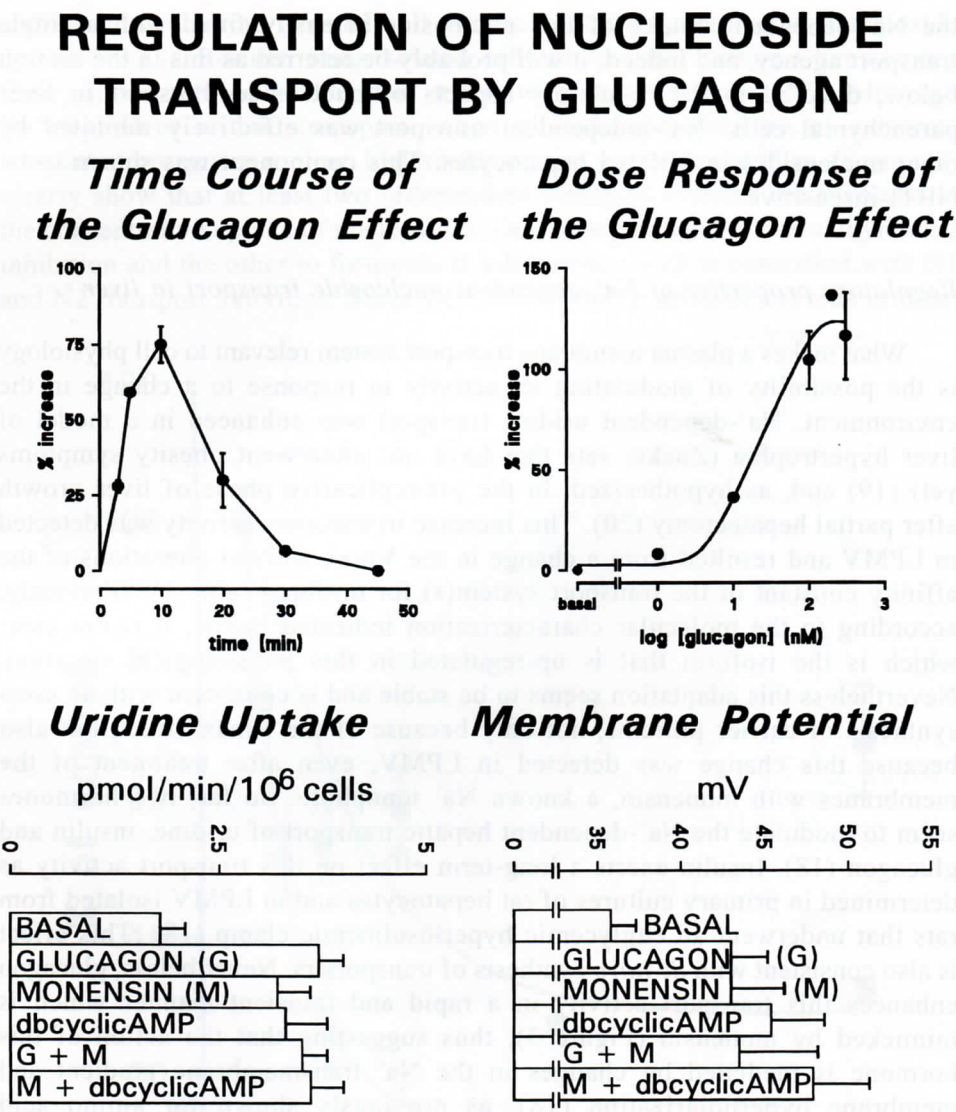

Fig. 3.—*Regulation of nucleoside transport by glucagon.* Upper pannel left: isolated hepatocytes were incubated in the presence of 300nM glucagon and Na^+-dependent 0.5μM uridine uptake was monitored as a function of time after hormone addition (results are shown as %increase above basal values). Upper pannel right: dose-response activation of Na^+-dependent uridine uptake by glucagon. Lower pannels: relationship between the activation of Na^+-dependent 0.5μM uridine uptake by glucagon and other compounds and the change in membrane potential. Adapted from reference 18.

cDNA by means of homology cloning using the SNST1 cDNA as a probe (kindly donated by Ana Pajor, University of Arizona) and a liver λ-zap cDNA library purchased from Stratagene. SNST1 was cloned by sequence homology with the SGLT1 cDNA, known to code for a Na^+-dependent glucose transporter (22). At this stage, we are not sure about the exact physiological role of the protein product of SNST1. Young and coworkers (23) later reported the expression cloning of a nucleoside transporter-related cDNA, cNT1, which was expressed in epithelia (kidney and intestine) but not in liver and did not show any sequence similarity with the SNST1 clone. Meanwhile, Arias and coworkers isolated a purine-preferring transport system related cDNA, SPNT, which was expressed in liver cells (14). Using RT-PCR homology cloning we tried to isolate the cDNA corresponding to the hepatic cNT1-like isoform and, indeed, what we got and sequenced was identical to the cNT1 cDNA reported to be absent (or at least not detected) in liver cells. To make sure that the cNT1 fragment amplified from liver cDNA by nested PCR was not the result of illegitimate transcription we later tried to detect cNT1 expression by Northern blot analysis. As previously reported, a regular Northern analysis using total RNA is not able to detect cNT1 mRNA, but, when using poly-A^+ RNA and the cNT1 fragment as a riboprobe, a band of the right molecular weight (2.5kb) was detected in liver. In our experimental conditions no cross-hybridization occurred between both probes, the one used for cNT1 analysis and the one generated from the SPNT cDNA published sequence. In this context, our results suggested that liver cells express both mRNAs, SPNT and cNT1, which supports the kinetic data proving that two different Na^+-dependent transport systems are expressed in liver parenchymal cells, a purine-preferring and another pyrimide-preferring, both accepting uridine and adenosine at similar affinities. cNT1 and SPNT show high homology (Figure 4) but define by themselves a new gene family. The putative topology of these transporters, deduced from the primary sequence, is consistent with at least 14 transmembrane domains (Figure 4).

Although the increased Na^+-dependent transport activities found in the situations indicated above (genetic obesity, liver regeneration and hyperinsulinemic clamp) correlate fairly well with the increased amounts of SPNT mRNA, the lack of correspondence between the kinetic agencies and the relative amounts of SPNT and cNT1 mRNAs suggests that either there is still another transport system involved in Na^+-dependent nucleoside transport to be cloned or these transporters show post-transcriptional regulation, or both.

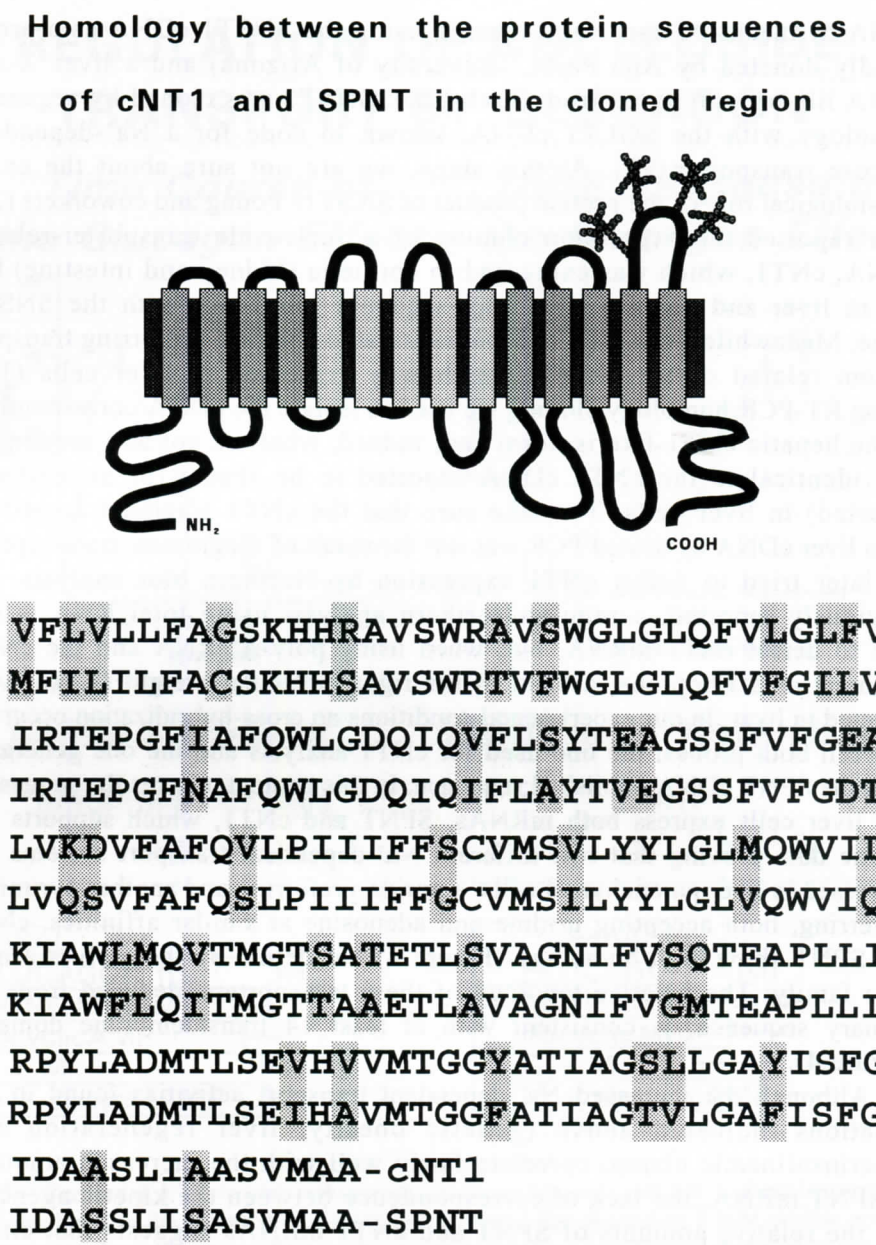

Fig. 4.—*Topology and homology of nucleoside transporters SPNT and cNT1.* The putative topology of the carrier proteins coded by SPNT and cNT1 cDNAs is shown. 14 putative transmembrane domains have been suggested on the basis of the primary sequence. Both N- and C-terminus are in the cytosolic side and a long loop between transmembrane spanning domains 13 and 14 may be glycosylated. The sequence homology of both proteins in the cloned sequence used in our laboratory is also shown. Both SPNT and cNT1 define a new gene family of organic solute transporters.

Characterization of nucleoside transport systems and SPNT expression in fetal hepatocytes and hepatoma cell lines

To determine whether undifferentiated liver parenchymal (fetal rat hepatocytes) or transformed cells showed a different pattern of isoform activities and expression to the one found in adult hepatocytes, we analyzed, first, the Na^+-dependent and independent transport systems present in fetal and hepatoma cells and, second, the amounts of SPNT mRNA in these cell types. The relative contributions of the Na^+-dependent and -independent transport agencies to the overall uridine uptake in hepatocytes and hepatoma cells is shown in Figure 5. Rapidly proliferating hepatoma cell lines show a high basal uridine transport activity which is mostly accounted for by Na^+-independent agencies. Furthermore, hepatoma cells show a NBTI-sensitive equilibrative transport, which is absent in adult hepatocytes. This component of transport is almost the only one detected in the human hepatoma cell line HepG2. The heterokaryont Wif-B, a somatic hybrid obtained from human fibroblasts and FAO cells, shows a similar pattern of transport to that of the parental line FAO. Interestingly the most abundant transport agency present in hepatoma cells, the NBTI-sensitive Na^+-independent component is poorly regulated in HepG2 cells. This is consistent with a constitutive high level of activity and expression of the transporter protein(s) and is completely different to what we have reported in hepatocytes, where most of the uptake relies upon a Na^+-dependent transport activity which is highly modulated, depending on the nutritional and endocrine status of the liver cell. The low or even absent Na^+-dependent transport activities correlate with the levels of SPNT mRNAs, which are not detected by regular Northern blot analysis in HepG2 cells. The expression of cNT1 has not been determined in these cell models.

As expected, proliferating fetal hepatocytes show a higher transport activity for nucleosides than adult hepatocytes, but the transport system pattern is closer to FAO than to differentiated liver parenchymal cells (Figures 6 and 7). Indeed, fetal cells show NBTI-sensitive equilibrative transport, which seems to be a characteristic feature of transformed cell lines. This component is missing in neonatal hepatocytes, although the relative contribution of the Na^+-dependent transport systems is still lower than in the adult. Similarly, the steady-state levels of SPNT mRNA are very low during development and indeed, neither after weaning they reach adult values. Again, since this profile only correlates qualitatively with the transport activity, it could be argued that either cNT1 expression is compensating for the total Na^+-dependent transport or SPNT is under complex post-transcriptonal regulation. Considering that in proliferating adult hepatocytes (model of liver regeneration) the activity which is selectively up-regulated is the Na^+-dependent one and it correlates with increased abundance of the SPNT mRNA, it is highly likely that increased

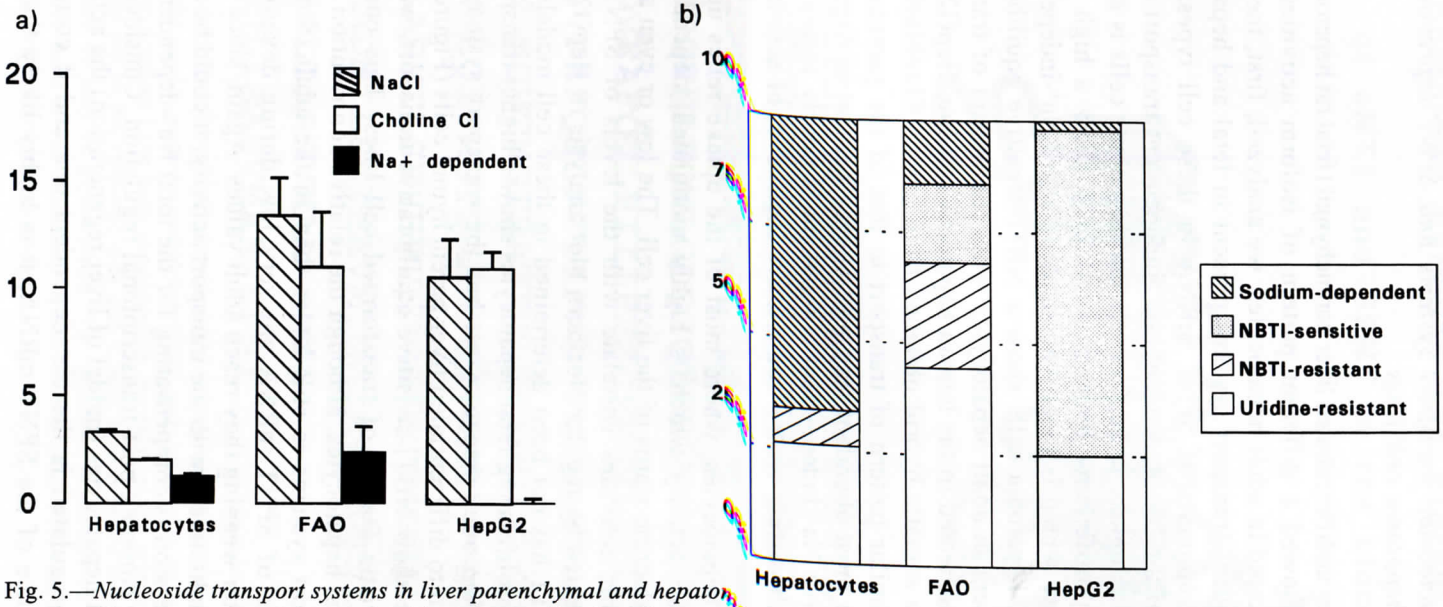

Fig. 5.—*Nucleoside transport systems in liver parenchymal and hepato* *cells.* 24-h cultured rat hepatocytes and the hepatoma cell lines FAO and HepG2 were monitored for nucleoside transport (1μM uridine as s strate). Proliferating hepatoma cells show a much higher nucleoside transport activity than cultured hepatocytes (pannel a), but most of th uptake is accounted for by Na^+-independent equilibrative agencies (pannel b). FAO and HepG2 cells show a NBTI-sensitive equilibrative c nponent of transport which is absent in cultured hepatocytes (pannel b). Adapted from del Santo, Felipe, Casado and Pastor-Anglada (subm ted to Biochem.J.).

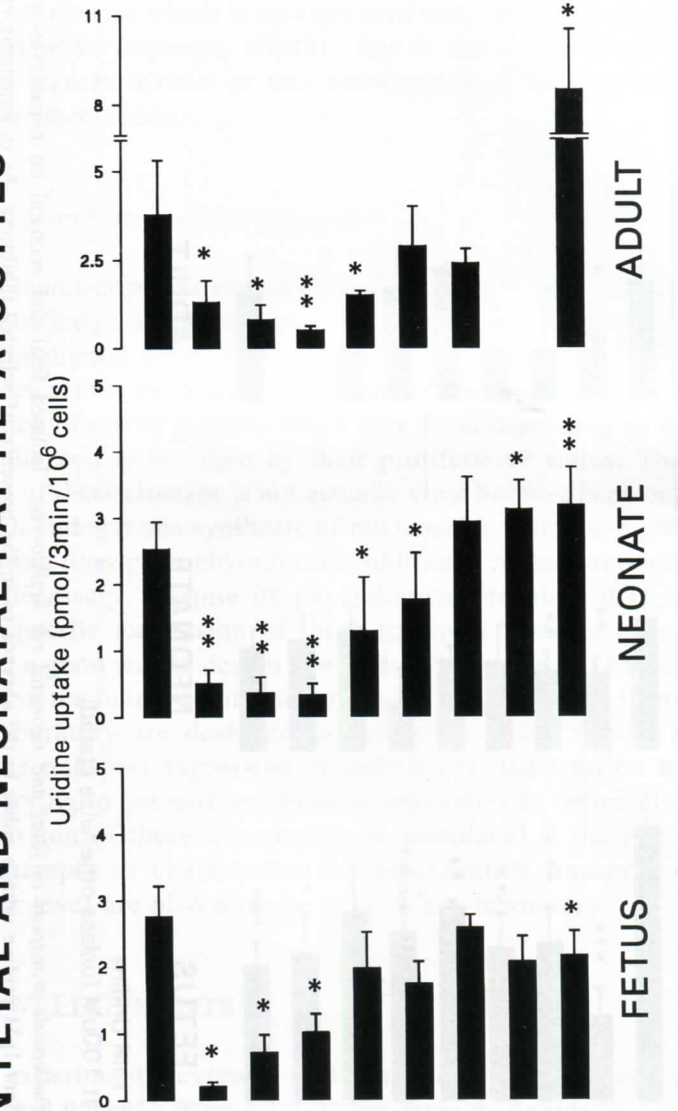

Fig. 6.—*Na^+-dependent nucleoside transport in fetal and neonatal rat hepatocytes.* Isolated fetal and neonatal rat hepatocytes were monitored for Na^+-dependent 1μM uridine uptake either in the absence or in the presence of selected inhibitors. Results are unpublished and correspond to the mean±S.E.M. of at least four independent cell preparations.

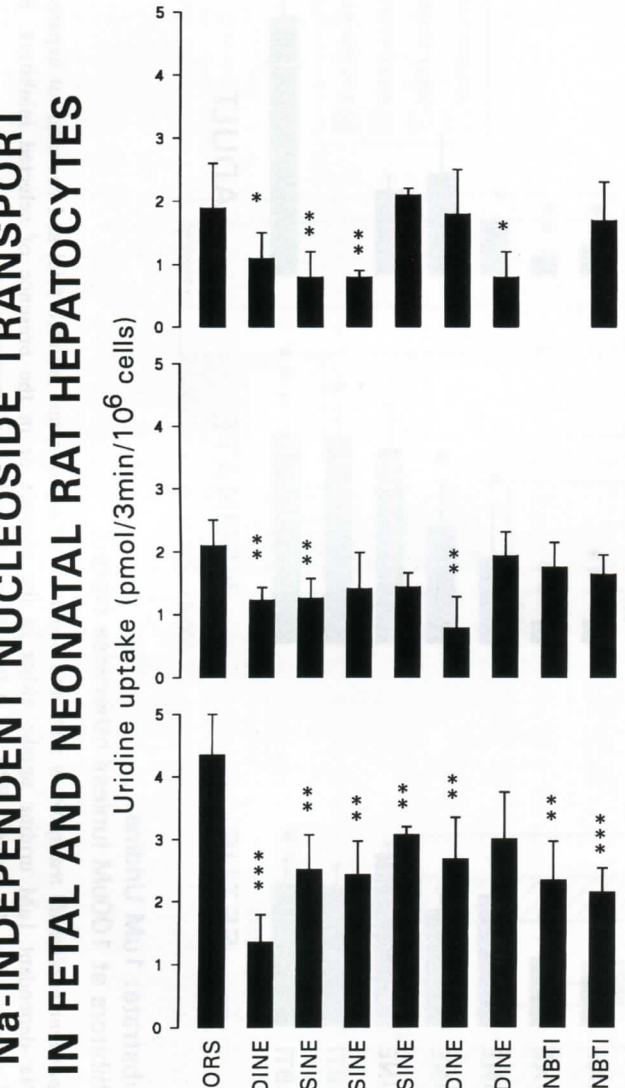

Fig. 7.—*Na⁺-independent nucleoside transport in fetal and neonatal rat hepatocytes.* Isolated fetal and neonatal rat hepatocytes were monitored for Na^+-independent 1μM uridine uptake either in the absence or in the presence of selected inhibitors. As in hepatoma cells, equilibrative transport rates were higher in fetal than in neonatal and adult rat hepatocytes. Nevertheless, a significant portion of this transport activity was sensitive to NBTI inhibition in fetal hepatocyts. This component of transport apparently disappears at birth. Results are unpublished and correspond to the mean±S.E.M. of at least four independent cell preparations.

nucleoside uptake is a general feature of proliferating liver cells, but the type of transport system which is up-regulated may vary according to the nature of the proliferative response, whether this is merely a mitogenic stimulus not involving transformation or this corresponds to liver growth associated to neoplastic phenomena.

Conclusion and prospective projects

This manuscript has reviewed and summarized most of our contributions to the knowledge of the transport systems involved in nucleoside uptake by liver parenchymal cells. At this stage, it is clear that the translocation of nucleosides across the plasma membrane of the hepatocyte involves a complex combination of carrier proteins which may differ depending on the differentiated state of the cell rather than by their proliferative status. The physiological relevance of these changes is not actually clear because hepatocytes themselves show high endogenous synthesis of nucleosides, but it is highly likely that in proliferating liver parenchymal cells additional routes for nucleosides salvage may be necessary. Despite its physiological meaning, it is our bet that the isoform-specific expression of these transport systems in hepatocytes may constitute a good way to design new and early markers of hepatocarcinogenesis and even, in the future, additional targets for chemotherapy. Current experiments in our laboratory are designed to determine which factors are modulating nucleoside transport expression in undifferentiated fetal rat hepatocytes. We are also trying to get isoform-specific antibodies to definetely prove whether the expression of these transporters is modulated at the post-transcriptional level. Attempts to characterize the equilibrative transport systems at the molecular level are also a major goal in our laboratory.

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