Identification of a plasma membrane glutamine transporter from the rat hepatoma cell line H4-IIE-C3

Matthew POLLARD*, David MEREDITH† and John D. MCGIVAN*¹

*Department of Biochemistry, School of Medical Sciences, University Walk, Bristol BS8 1TD U.K., and †Department of Human Anatomy and Genetics, South Parks Road, Oxford OX1 3QX, U.K.

Glutamine is taken up into the rat hepatoma cell line H4-IIE-C3 by a Na⁺-dependent transport system which is specific for glutamine, alanine, serine, cysteine and asparagine and does not tolerate substitution of Na⁺ by Li⁺. Glutamine transport was relatively weakly inhibited by a 50-fold excess of leucine and was not inhibited by phenylalanine or *N*-methyl aminoisobutyrate. These general properties are characteristic of the recently identified ASCT/B⁰ family of transporters. Using a reverse transcriptase PCR-based homology cloning approach, we have characterized a cDNA for a novel member of this transporter family (H4-ASCT2) from H4-IIE-C3 cells. The cDNA encodes a 551-amino acid protein which exhibits similarities of between 75 and 85 % with ASCT/B⁰ transporters previously cloned from other sources. When expressed in *Xenopus* oocytes, this transporter catalyses Na⁺-dependent glutamine uptake with characteristics

very similar to those of glutamine uptake into the H4-IIE-C3 cells. This newly characterized transporter possesses a number of amino acid sequence differences from ASCT2 clones recently isolated from rat astroglial cells and from normal rat liver. In particular, the loop region between transmembrane helices 3 and 4 from H4-ASCT2 shares less than 60 % sequence similarity with ASCT2 from rat liver; furthermore, there are some 25 single amino acid substitutions elsewhere in the H4-ASCT2 sequence compared with that from rat liver. Thus enhanced glutamine uptake in rat hepatoma cells is mediated by the expression of a novel ASCT/B⁰ transporter isoform rather than by increased expression of the ASCT2 mRNA found in normal rat liver.

Key words: Na⁺-dependent, system ASC-like transporter (ASCT), tumour.

INTRODUCTION

In hepatocytes, glutamine is used mainly as a substrate for urea synthesis and gluconeogenesis. Glutamine is hydrolysed by a liver-specific isoform of glutaminase which is activated by ammonia and participates in the regulation of urea synthesis (see [1]). Glutamine is transported into hepatocytes via a specific Na⁺-dependent transporter family termed System N [2]. This transporter is specific for glutamine, asparagine and histidine and tolerates substitution of Na⁺ by Li⁺. A cDNA encoding a protein with System N-like transport activity has been cloned and termed SN1 [3]. More recently a second member of this transporter family has been identified and designated SN2 [4].

In hepatoma cell lines in culture glutamine has a different function and acts as a major energy source. In human hepatoma cells, glutamine is hydrolysed by kidney-type glutaminase. Glutamine transport in human hepatoma cell lines was found to be 15-fold faster than in isolated human hepatocytes [5] and the same is true of rat hepatoma cells with respect to rat hepatocytes [6]. Since hepatoma cell proliferation is dependent on glutamine oxidation it is of importance to characterize the transport system in hepatoma cells that is responsible for the observed elevated rate of glutamine uptake.

In a comparative study of glutamine transport in isolated human hepatocytes and in two human hepatoma cell lines, Bode et al. [7] showed that glutamine transport in hepatocytes was catalysed by a system with the properties of System N. In contrast, in hepatoma cells glutamine transport was mediated mainly by a transport system which was inhibited by alanine, serine and cysteine and did not tolerate Li⁺ for Na⁺ substitution. These findings suggested that hepatoma cells expressed transport

activity similar to that described as System ASC rather than System N. In a later investigation glutamine transport in a rat hepatoma cell line was found to be mediated by two agencies; a minor component similar to System N plus an additional major component similar to System ASC [6].

Recently a family of Na⁺-dependent amino acid transporters of broad specificity has been identified and termed the ASCT ('system ASC-like transporter')/B⁰ family (see e.g. [8] for a review). The ASCT2 clone originally derived from mouse testis [9] encodes a transporter with general specificity similar to that expressed in hepatoma cells. Variants of ASCT2 have been cloned from the human placental tumour cell line JAR [10] from rabbit intestine [11], rat astroglial cells [12] and the bovine epithelial cell line NBL-1 [13]. These clones are all greater than 80 % identical to each other and when expressed heterologously exhibit Na⁺-dependent neutral amino acid transport although with minor differences in amino acid specificity.

It therefore appears that in transformed liver cells expression of System N is largely abolished and increased glutamine uptake is achieved by expression of a member of the ASCT/B⁰ transporter family. The object of the present investigation was to establish the molecular identity of the glutamine transporter expressed in the rat hepatoma cell line H4-IIE-C3.

MATERIALS AND METHODS

Materials

The rat hepatoma cell line H4-IIE-C3 was purchased from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wilts., U.K. Tissue culture reagents were purchased

Abbreviations used: methyl AIB, *N*-methyl aminoisobutyrate; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

¹ To whom correspondence should be addressed (e-mail j.mcgvian@bris.ac.uk).

The sequence of the H4-ASCT2 cDNA has been submitted to the GenBank Nucleotide Sequence Database under the accession no. AY125814.

from Gibco (Paisley, U.K.). Radiolabelled L-[U- 14 C]glutamine was from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Oligonucleotide synthesis and DNA sequencing services were provided by MWG Biotech (Milton Keynes, U.K.).

Cell culture and measurement of glutamine uptake

Cells were cultured in Hams F12 nutrient medium (Gibco) supplemented with 10% (v/v) fetal bovine serum, antibiotics (100 units/ml penicillin G and 0.1 mg/ml streptomycin) and 2 mM glutamine prior to use. Cells were routinely trypsinized and seeded on to 35 mm Petri dishes for experiments. Uptake of amino acids into cells was measured at room temperature as described previously [6]. Briefly, cell monolayers were washed with Na⁺ medium (137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂ and 10 mM Tris/Hepes pH 7.4), Li⁺ medium or choline medium as appropriate (Li⁺ and choline media contained 137 mM LiCl or choline chloride in place of the NaCl). The transport reaction was started by addition of 1 ml of the appropriate medium containing 14 C-labelled glutamine. After 3 min the reaction was terminated by washing with 4 × 1 ml of glutamine-free ice-cold medium. After removing adherent liquid the cells were dissolved in 0.5 ml of 0.5% (w/v) Triton X-100 and aliquots of this solution were used for measurement of radioactivity by scintillation counting and of protein by the method of Bradford [14].

cRNA expression and glutamine transport in oocytes

Isolation of *Xenopus laevis* oocytes and expression of mRNA in this system was performed essentially as described previously [15]. Oocytes were maintained at 18 °C in modified Barth's medium (89 mM NaCl, 1 mM KCl, 0.42 mM MgSO₄, 0.84 mM NaHCO₃, 0.82 mM CaCl₂, 5 mM Hepes pH 7.6, 5 mM sodium pyruvate and 50 µg/ml gentamicin) after type II collagenase separation and manual defolliculation. Healthy stage V and VI oocytes were selected for microinjection with either 50 ng of cRNA (T7 mMessage mMachine *in vitro* transcription kit; Ambion) encoding the transporter or water as a control. Oocytes were then used in transport assays 4 days post injection. Oocytes (a minimum of five per data point) were washed for 1 min at room temperature in the appropriate incubation medium (either 95 mM NaCl or LiCl together with 2 mM KCl, 0.82 mM MgCl₂, 1 mM CaCl₂ and 20 mM Tris/Hepes at pH 7.4). This medium was then changed for one supplemented with 50 µM 14 C-labelled glutamine. Uptake was terminated after 1 h by five sequential washes in ice-cold glutamine-free uptake medium, prior to lysis of single oocytes in scintillation vials with 100 µl of SDS (2%, w/v), addition of scintillation fluid and liquid scintillation counting.

Reverse transcriptase PCR

Total RNA was isolated from monolayers of H4-IIE-C3 cells using TRI reagent (Sigma). cDNA synthesis was performed with either oligo(dT) or gene-specific primers using the Retroscript kit (Ambion). PCR reactions were performed using HiFidelity *Taq* polymerase (Roche). PCR products were ligated into the pGEM-T Easy vector (Promega) and amplified in *Escherichia coli*. Plasmid DNA was isolated using the Genie II miniprep system (Helena Biosciences) and inserts were sequenced using M13 forward and reverse primers in conjunction with gene-specific primers as appropriate.

Figure 1 Strategy for cloning the H4-ASCT2 cDNA by PCR homology approach

Details of the primers shown (F1–F3 and R1–R5) are given in the text.

Isolation and sequencing of a glutamine transporter cDNA (H4-ASCT2) from H4-IIE-C3 cells

Since the general characteristics of glutamine transport in these cells were similar to those of previously cloned transporters of the ASCT/B⁰ family, a PCR homology cloning approach based on these known sequences was used. The strategy is illustrated in Figure 1.

Initially two oligonucleotide primers for the conserved predicted transmembrane regions of members of the ASCT/B⁰ family were used in reverse transcriptase PCR. These were F1, 5'-ATGAACATCCTGGGCATGGTGGTGTTC-3', and R1, 5'-GCGTCACCTTCTACATTGAGGACGGTAC-3'. The resulting 744 bp product was similar to mouse ASCT2 in sequence. The 3' end of the H4-ASCT2 cDNA was subsequently derived using primer F1 in the Clontech 3' rapid amplification of cDNA ends (RACE) protocol. This gave a single 1.5 kb PCR product which included the poly(A)⁺ tail and also shared sequence similarity with mouse ASCT2.

Many attempts to derive the 5' end of the cDNA using 5'-RACE with various reverse primers for the known sequence between F1 and R1 proved fruitless. Extensive attempts to obtain the 5' end by screening a commercially available H4-IIE-C3 bacteriophage cDNA library (Stratagene) with the F1/R1 PCR product also proved futile and it appeared that the library did not contain a full-length H4-ASCT2 clone. Primer R1 was then used in gene-specific reverse transcription. Using this cDNA as a template a further primer pair homologous to conserved regions of the ASCT2 family of clones was found that gave a 422 bp product which overlapped with the 5' end of the sequence already isolated. These primers were F2, 5'-CTGCTCTTTTC-CTGGTCAACCAC-3', and R2, 5'-GGAGTTGAAGAAACGG-ATGAGCAG-3'.

Further attempts at 5'-RACE using reverse primers in the region F2–R2 also proved unsuccessful. Presumably the high GC content of this cDNA towards the 5' end was responsible for the frequent production of spurious 5'-RACE products. At this stage the sequences of ASCT2 cDNA clones from both rat astroglial cells [12] and mouse testis [9] had been published. The N-terminal amino acid sequences of all members of the ASCT/B⁰ family are identical. Comparison of the 5' untranslated regions (UTRs) of the rat and mouse clones indicated a region of identical sequence starting some 60 bp before the start codon. It was assumed that H4-ASCT2 might also contain this sequence at a similar point. A forward primer (F3, 5'-TTGCAAAGTTTCAGCCTCCT-3') was designed for this region with a reverse primer (R3, 5'-GGACGCGAGCAGCGTGGTGA-3') designed for a sequence between F2 and R2. A 491 bp PCR product was obtained. In order to obtain this product it was necessary to heat the RNA with the R1 primer at 85 °C prior to reverse transcription at 52 °C. 60 cycles of PCR amplification were then necessary to obtain the required product.

A preliminary sequence for the full-length cDNA was derived from these overlapping sequences. It proved impossible to

generate the full-length cDNA by PCR using primers in the 5'- and 3'-UTR regions, presumably due to particularly difficult secondary structure between F3 and R3. In order to construct cDNA encoding the full-length protein, gene-specific cDNA was synthesized using a new 3'-UTR reverse primer, R5 (5'-GCCA-TCAGGTAGCATCAAATCACTTGTC-3'), in the reverse-transcription reaction. Two partial clones were then obtained by PCR using this cDNA as template: a 490 bp product using F3 and R3 and a 1468 bp product using the primers F2 and R4 (5'-GGTGACAGAGAATGTCGTCTCCTTGAGTG-3'). These overlapped by some 35 bp. These overlapping clones, which were derived from the same gene-specific reverse transcription, were spliced together by incubation with dNTPs and *Taq* polymerase without added oligonucleotide primers for 30 cycles of PCR (94 °C for 40 s, 58 °C for 40 s and 72 °C for 2 min). In this reaction, each DNA species acted as a primer for PCR amplification of the other. The resulting 1920 bp product was separated from the partial clones by agarose gel electrophoresis and subsequently ligated into the pGEM-T-EASY vector (Promega), amplified in *E. coli* and sequenced on both strands. This insert was then subcloned into pBluescript (Stratagene). Correctly orientated clones downstream of the T7 promoter were linearized with *SalI* and cRNA was produced using the T7 mMessage mMachine kit (Ambion). cRNA was expressed in oocytes as described above.

RESULTS AND DISCUSSION

The initial rate of glutamine uptake by monolayer cultures of H4-IIE-C3 cells was constant over a 3 min time period (results not shown). Table 1 shows that glutamine uptake was largely Na⁺-dependent. Li⁺ could not substitute for Na⁺ since the initial rate of uptake in Li⁺ medium was no greater than that in a choline medium. This indicates that this cell line does not express measurable System N activity. In order to assess the specificity of the transporter, the initial uptake of glutamine (0.1 mM) was measured in the presence of a 50-fold excess of various other amino acids (Table 1). Under these conditions, amino acids entering on the same transporter as glutamine

Table 1 Inhibition profile of glutamine uptake into H4-IIE-C3 cells

The initial rate of uptake of 0.1 mM [¹⁴C]glutamine (3000 d.p.m./nmol) into H4-IIE-C3 cells was measured over 3 min at 20 °C as described in the Materials and methods section. Potential inhibitory amino acids were added at a concentration of 5 mM in Na⁺ medium. Where shown, the Na⁺ medium was replaced with a medium containing an equal concentration of Li⁺ or choline. Results are means ± S.E.M. of uptake from four culture dishes in each case. Significance was assessed by Student's *t* test; ****P* < 0.001 compared with uptake in Na⁺ medium without inhibitors.

Inhibiting amino acid	Rate of glutamine uptake (nmol/min per mg of cell protein)
None (Na ⁺ medium)	1.58 ± 0.08
None (Li ⁺ medium)	0.28 ± 0.01***
None (choline medium)	0.25 ± 0.02***
Asparagine	0.32 ± 0.03***
Alanine	0.28 ± 0.04***
Serine	0.28 ± 0.04***
Cysteine	0.26 ± 0.02***
Methyl AIB	1.32 ± 0.06
Leucine	0.89 ± 0.02***
Valine	0.71 ± 0.07***
Phenylalanine	1.33 ± 0.13
Lysine	1.26 ± 0.14

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1 TTGCAAGATTTCAGCCTCTCTCTAGGCGCACAGGAACCCCTCGTTGCGGCTCAAC
60 ATGGCAGTGGATCCTCCTAAGGCTGACCCCAAGGGGTGGTGGCGTGGATCCACCGGG
   M A V D P P K A D P K G V V A V D P T A
120 AACTGTGGCTCGGGCTGAGGTCCAGAGAGGACAGGAGGAGCAAGCAGGTGGATGCTGC
   N C G S G L R S R E D Q G A K A G G C C
180 AGTTCCCGGGACCAAGTGTGCGGCTTTCGCGCCACCTGCTGCTGCTGCTGCTGCTG
   S S R D Q V C R C L R A N L L V L L T V
240 GCGCGCGGCTGGCTGGCGTGGTGTAGTGTGCGGCTGCTGCGCGGGCGGCTGCTGAG
   A A A V A G V V L G L G V S A A G G A E
300 GCGTGGCGCACGCGCTTACGCGCTTTCGCGCCACCTGCTGCTGCTGCTGCTGCTG
   A L G H A R F T A F A F P G E L L L R L
360 CTGAGATGATCATCTGCGCTGGTGGTGTGAGCTGATCGAGGTGACGACGCTG
   L E M I I L P L V V C S L I G G A A S L
420 GACCTGCGCGCTGCGCGCTTTCGCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
   D P S A L G R L G A W A L L F F L V T T
480 CTGCTGCGCTCGCGCTGCGCTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGG
   L L A S A L G V G L A L A K P G V A V
540 AGCGCCATCAACTGCTGCTGCGGAGCGGACGAGGACAGGACACCCCAAGGAGTG
   S A I N C S V G D G S G H R T P P K E V
600 CTGATTCTCTCTGATCTCATGAGGAATATTTCCCTCCAATCTGGTGTCTGCTGCA
   L D S F L D L M R N I F P S N L V S A A
660 TTCCGCTCTTATTCTACTCATCACTCAGAACTGAGAAACAGCTGACTACTGAC
   F R S Y S T S Y E L R A T S E K Q L T D
720 TGTGCACACAGAGAGACCAACGCCACCTGGTCCAGGTGGGAGGTGAGGTGAGGCGCATG
   C A H R E T N A T L V Q V G G E V E G M
780 AACATCCTGGGCTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGG
   N I L G L V V F A I F T G V A L R K L G
840 CCTGAGGCGCACTGCTCATCGTTTCTTCAACTCCTTCAATGATGCCACCATGGTCTG
   P E G E L L I R F F N S F N D A T M V L
900 GTCTCTGGATTATGTGTACGCCCTGTGGGCTGCTGCTGCTGCTGCTGCTGCTGCTG
   V S W I M W Y A P V G I L F L V A S K I
960 GTGGAGATGGAAGACATCCGCCAGCTGTCATCAGACTGGGCAAGTACGTCCTGTGCTGT
   V E M E D I R Q L F I R L G K Y V L C C
1020 CTCCTGGGCGATGCCATCCAGGGCTCCTGTTTCCGCCCTCACTACTTCTGCTCTCAC
   L L G H A I H G L L T L G C C C T A C T A C T T C G T C T C C
1080 CGCCAAACCCCTATCGATTCTGTGGGGTATCAGCCACTGGCCACGGCTTTTGGG
   R Q N P Y R F L W G I M T P L A T A F G
1140 ACCTCTTAGCTCTGCCAGCTGCGCCTGATGATGAATGTGTAGAGAGAAGAACGGG
   T S S S S A T L P L M M K K V E E K N G
1200 GTGGCCAAACACATCAGCCGGTTCATCCTGCCCTGCGGCCACCGTCAACATGGATGG
   V A K H I S R F I L P I G A T V N M D G
1260 GCGGCGTGTTCAGTGTGTGGCGGCGGTGTCATTGCTCAGCTGAATGGGCTGCTCCTG
   A A L F Q C V A A V F I A Q L N G V S L
1320 GACTCGTGAAGATCATTACCATCTGCTGCTCAGCTACTGCTCAGCGTGGCGGCCA
   D F V K I I T I L V T A T A S S V G A A
1380 GGCATCCTGCGCGGGGCTCCTCAGCTTGCCATCATCTTGAAGCGCTCAGCTCGCT
   G I P A G G V L T L A I I L E A V S L P
1440 GTCAAAGACATCTCCTTGATCTTGGCTGTGGACTGGCTAGTGGACAGGTCTGTACCATC
   V K D I S L I L A V D W L V D R S C T I
1500 ATCAATGTGAAGGTGACGCTTCGGGGCGGAGCTGCTTCAAGTTATGTGGATGGAACC
   I N V E G D A F G A G L L Q S Y V D R T
1560 AAGACGCGAGCTCGGAGCTGACCTGATCAGGTGAAGAACGAACTGCTCTGATCCA
   K T P S S E P E L I Q V K N E T S L N P
1620 CTGCCCCTGGCCAGAGAGGGGAAACCCCTTCTCAAGCAGTACCGAGGACCCCGGG
   L P L A P E E G N P L L K Q Y R G P A G
1680 GACGCTGCGCGCTGTGAAAAGGAGTCTGTGATGTAATAGCAGGAGGGCTTCTGTGCC
   D A A A C E K E S V M *
1740 CTGGTGGCTCTCATTTGAAGTGGATATCATGAGGGCTGGAGAACTCCAGGGGAGGAGT
1800 TTACACAGACCGGAACTAGGGTCCCTGTCTCTTCTGGAACGGATATCCGGGGGGCC
1860 TCATTGCTGGGTACAGTGTATATATGATGTTCTCCACATACCTTCCGCCATCCTGCT
1920 TCOCATCAAGGAGACGACATTCCTGTCAACCCAGAGGACGACGCTACCTGTCTGTCT
1980 ACCACTAGAGATCTTCTTCTTGAAGACAACTGATTGTATGCTACCTGATGGCCAAAG
2040 CTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT
2100 ACACCTGACCTTCCAGTAGTCCCTTTATGACAAACAACTCCCAACAAAGTCCCAA
2160 GGAAGCTAAAGATGAATATTGTCACTCCAGATACATTTTAAAGCAATAAATGGCA
2220 TCAAAAAAAAAAAAAAAAAAAAAA

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Figure 2 Nucleotide sequence and deduced amino acid sequence of H4-ASCT2 cDNA

should completely inhibit Na⁺-dependent glutamine uptake. Alanine, serine, cysteine and asparagine reduced the rate of glutamine uptake to that observed in the absence of Na⁺ ions, while *N*-methyl aminoisobutyrate (methyl AIB; a substrate of

Table 2 Inhibition profile of glutamine uptake into *Xenopus* oocytes injected with H4-ASCT cRNA

The uptake of 50 μ M 14 C-labelled glutamine (38 000 d.p.m./nmol) into cRNA-injected oocytes was measured in Na⁺-containing medium for 1 h in the presence or absence of other amino acids added at 5 mM. Where shown, uptake was measured in a medium where the Na⁺ was replaced by an equal concentration of Li⁺. The results are the means $\pm$ S.E.M. of the values obtained for five oocytes in each case. Significance was assessed using Student's *t* test; **P* < 0.05 and ****P* < 0.001 compared with uptake in Na⁺ medium in the absence of inhibiting amino acids. The corresponding uptake in non-injected oocytes was 15.3 $\pm$ 3.7 pmol/oocyte per h in Na⁺ medium and 5.7 $\pm$ 2.8 pmol/oocyte per h in Li⁺ medium (*n* = 5 in each case).

Inhibiting amino acid	Glutamine uptake (pmol/per h oocyte)
None (Na ⁺ medium)	97 $\pm$ 10
None (Li ⁺ medium)	7.2 $\pm$ 1***
Asparagine	8.7 $\pm$ 1.8***
Alanine	8.2 $\pm$ 2.8***
Serine	8.9 $\pm$ 2.0***
Cysteine	6.0 $\pm$ 2***
Methyl AIB	70 $\pm$ 20
Leucine	14 $\pm$ 3***
Valine	32 $\pm$ 9*
Phenylalanine	79 $\pm$ 18
Lysine	63 $\pm$ 18

System A), lysine and phenylalanine had little effect. Leucine and valine significantly inhibited glutamine uptake but the inhibition was not complete. The lack of inhibition of transport by methyl

AIB, complete inhibition by excess concentrations of serine, cysteine and asparagine and lack of tolerance of substitution of Na⁺ by Li⁺ are all common characteristics of a closely related family of transporters described as the ASCT/B⁰ family. Therefore a PCR-based homology cloning strategy was used to isolate cDNA encoding the glutamine transporter (termed H4-ASCT2 cDNA), as described in the Materials and methods section.

cRNA was synthesized from H4-ASCT2 cDNA as described and injected into *Xenopus* oocytes. After 4 days the uptake of glutamine (50 μ M) was measured over a 1 h period (Table 2). Na⁺-dependent uptake of glutamine into H4-ASCT2 cRNA-injected oocytes was increased by 10-fold over that measured in non-injected oocytes (97 versus 9.6 pmol/oocyte per h, respectively). When Na⁺ was replaced by Li⁺ there was no increase in the rate of uptake over the control oocytes, indicating that, as in the hepatoma cells, Li⁺ could not substitute for Na⁺. As before, the effect of adding an excess of potentially competing amino acids on glutamine uptake was assessed. Alanine, serine, cysteine and asparagine reduced the rate to that observed in the absence of Na⁺ ions. Methyl AIB, phenylalanine and lysine all reduced the rate slightly but this inhibition was not significant. Leucine and valine inhibited glutamine uptake significantly, but to a lesser extent than alanine. These results are largely consistent with the specificity data on cultured cells (Table 1), and indicate that the H4-ASCT2 cDNA encodes the glutamine transporter functionally characterized in H4-IIE-C3 cells.

The cDNA contains an open reading frame encoding a 551-amino acid protein with a calculated molecular mass of 58 055 Da

	1	15 16	30 31	45 46	60 61	75 76	90
H4-ASCT2	MAVDPPKADPKGVVA	VDPTANCGSGLKSRE	DQGAAGGCCSSRDQ	VCRCRLANLLVLLTV	AAAVAGVVLGLGVSA	AGGAELGHARFTAF	
Rat ASCT2	MAVDPPKADPKGVVA	VDPTANCGSGLKSRE	DQGAAGGCCSSRDQ	VCRCRLANLLVLLTV	AAAVAGVVLGLGVSA	AGGAELGHARFTAF	
	91	105 106	120 121	135 136	150 151	165 166	180
H4-ASCT2	AFPGELLRLLEMI	LPLVVCNLIGGAASL	DPSALGRLGAWALLF	FLVTTLLASALGVGL	ALALKPGVAVSAINC	SVGDGSGHRTTPKEV	
Rat ASCT2	AFPGELLRLLEMI	LPLVVCNLIGGAASL	DPSALGRLGAWALLF	FLVTTLLSSALGVAL	ALALKPGAFAAFAINS	SVVDSSVHRAPTKEV	
	181	195 196	210 211	225 226	240 241	255 256	270
H4-ASCT2	LDSFLDLMRNIFPSN	LVSAAFRSYSTSYEL	RTSEKQLTTDCAHRE	TNATLVQVGGEVEGM	NILGLVVFVAVFGVA	LRKLGPEGELLIRFF	
Rat ASCT2	LDSFLELLRNMFPSN	LVSASAAFR---IPC	GACP-----Q	RSNATMDQPHCEMKM	NILGLVVFVAVFGVA	LRKLGPEGELLIRFF	
	271	285 286	300 301	315 316	330 331	345 346	360
H4-ASCT2	NSFNDATMVLVSWIM	WYAPVGVILFLVASKI	VEMEDIRQLFIRLGK	YVLCCLLGHAHIGLL	VLPLIYFVFTRQNPY	RFLWGIMTPLATAFG	
Rat ASCT2	NSFNDATMVLVSWIM	WYAPIGILFLVAGKI	VEMKDIRQLFIRLGK	YIVCCLLGHAHIGLL	VLPLIYFLFTRKNPY	RFLWGIVTPLATAFG	
	361	375 376	390 391	405 406	420 421	435 436	450
H4-ASCT2	TSSSSATLPLMMKCV	EEKNGVAKHISRIFIL	PIGATVNMDGAALFQ	CVAAVFIAQLNGVSL	DFVKIITILVTATAS	SVGAAGIPAGGVLTLL	
Rat ASCT2	TSSSSATLPLMMKCV	EEKNGVAKHISRIFIL	PIGATVNMDGAALFQ	CVAAVFIAQLNGMSL	DFVKIITILVTATAS	SVGAAGIPAGGVLTLL	
	451	465 466	480 481	495 496	510 511	525 526	540
H4-ASCT2	AIILEAVSLPVKDIS	LILAVDWLVDRSCTI	INVEGDAFGAGLLQS	YVDRTKTPSSEPELI	QVKNETSINPLPLAP	EEGNPLLKQYRGPAG	
Rat ASCT2	AIILEAISLPVKDIS	LILAVDWLVDRSCTV	LNVEGDAFGAGLLQS	YVDRTKMPSSEPELI	QVKNDVSLKPLPLAT	EEGNPLLKQCREPSG	
	541						
H4-ASCT2	DAAA-CEKESVM						
Rat ASCT2	DSSATCEKESVM						

Figure 3 Alignment of the derived amino acid sequences of H4-ASCT2 and rat liver ASCT2

The rat liver ASCT2 sequence was taken from [12]. Sequences were aligned using the ClustalW multiple sequence alignment programme. Amino acid residues in H4-ASCT2 that differ from the rat liver sequence are depicted in bold.

(Figure 2). The hydrophobic regions are very similar in sequence to corresponding regions of other members of the ASCT/B⁰ family, indicating a similar secondary structure. One model predicts 10 transmembrane helices but other models suggest 8 helices with a large hydrophobic region in the C-terminus (see [12]). The N-terminal, C-terminal and putative transmembrane regions of the H4-ASCT2 protein are almost identical to the other ASCT2-like clones sequenced and overall the cDNA exhibits 86.4, 83.7, 80.2 and 74.4% similarity to the ASCT2 derived from rat [12], mouse [9], human [10] and bovine [13] sources respectively.

Broer et al. [12] reported the expression and functional characterization of an ASCT2 clone from rat astroglial cells and stated that this was identical to an ASCT2 clone derived from normal rat liver. As shown in Figure 3 the ASCT2 cDNA expressed in rat hepatoma cells possesses regions of different amino acid sequence when compared with that derived from rat liver. In particular, the sequence between amino acids 151 and 240, which represents the putative extracellular loop region between helices 3 and 4, is substantially different. The hepatoma cell sequence contains 13 more amino acid residues than the liver sequence and overall in this entire loop region the two clones show less than 60% similarity. In the remainder of the sequence there are some 25 single amino acid differences between these two clones, nearly all appearing in predicted extramembrane regions.

Extrapolation of data on the ASCT/B⁰ transporters cloned so far indicates that all these clones encode transporters which accept alanine, glutamine, serine, cysteine and asparagine as substrates but do not recognize methyl AIB or the charged amino acids lysine or glutamate. There are subtle differences between the clones with regard to interaction with branched-chain amino acids and phenylalanine. Thus the mouse testis ASCT2 clone catalyses the obligatory exchange of small amino acids but excludes branched-chain amino acids and phenylalanine. Alanine transport catalysed by the bovine renal epithelial NBL-1 B⁰ clone and JAR cell B⁰ clones is inhibited by glutamine, leucine and phenylalanine. Inhibition by phenylalanine is much stronger for the NBL-1 clone than for the JAR cell clone. Threonine transport by the rabbit intestinal B⁰ clone is completely inhibited by excess leucine, but only marginally by phenylalanine. There is no direct evidence that any of these clones encodes a transporter that recognizes and translocates leucine or phenylalanine as substrates. Glutamine transport by the H4-ASCT2 clone described here was relatively weakly inhibited by leucine and was only marginally affected by phenylalanine. This is not substantially different from the properties of ASCT2 from rat astroglial cells or rat liver and it may be that the extracellular loop region between helices 3 and 4 does not play a major role in substrate recognition.

In summary, the rat hepatoma cell line H4-IIE-C3 transports glutamine, a major energy substrate, via the expression of a novel

member of the ASCT/B⁰ transporter family which is not identical to that cloned previously from rat liver and brain. Whether expression of this particular transporter isoform confers a growth advantage on rat hepatoma cells or is a necessary prerequisite for liver cell transformation or proliferation remains to be determined.

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