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Molecular cDNA Cloning and Tissue Distribution of mRNA Encoding a Novel ATP-Binding Cassette (ABC) Half-Transporter

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The majority of proteins belonging to the *A*TP-*b*inding *c*assette (ABC) superfamily catalyzes translocation of substrates across biological membranes. Employing a reverse transcription–PCR approach with degenerate primers, we have identified a full-length cDNA from rat hepatocytes encoding a novel ABC transporter termed umat (*u*biquitously expressed *m*ammalian *A*BC half-*t*ransporter). The deduced sequence of 836 amino acids comprises an N-terminal membrane anchor domain and a single conserved C-terminal nucleotide binding fold, specifying umat as an ABC half-transporter. While the first 250 amino acid positions are highly divergent from other ABC transporters, clusters of conserved residues are evident along the rest of the protein. The greatest sequence similarity was observed with the fission yeast heavy metal tolerance protein hmt1 (44.5% identity in a 626-amino-acid overlap). Umat mRNA, expressed in all tissues analyzed, was most abundant in testis. Substantial umat mRNA expression in cultured primary rat hepatocytes suggests that hepatocyte cultures should represent an adequate model for investigation of umat function and regulation.

The superfamily of ATP-binding cassette (ABC) proteins consists of numerous members identified throughout prokaryotic and eukaryotic kingdoms which exhibit domains highly conserved during evolution [1]. In particular, ABC proteins are characterized by one or two nucleotide binding folds which comprise the conserved Walker A and B motifs [2] and the SGG(Q) ABC-family signature [3]. The majority of ABC proteins catalyzes ATP-dependent transport of endogenous or exogenous substrates across biological membranes. Over 80 ABC protein genes are encoded by the *Escherichia coli* genome alone [4] and the existance of 29 ABC proteins has been predicted for *Saccharomyces cerevisiae* [3]. Several ABC family members have been identified in humans, most of which have been associated with clinically relevant phenotypes [5]. Intensively characterized mammalian transporters include several proteins of the full-transporter type, consisting of two transmembrane domains alternating with two nucleotide binding folds. Examples are the human MDR (multidrug resistance) and MRP (multidrug resistance associated) proteins, overexpression of which contributes to a multidrug resistant phenotype in tumor cells [6], or the CFTR (cystic fibrosis transmembrane conductance regulator) protein, which has been associated with inherited cystic fibrosis [7]. Mammalian ABC proteins of the half-transporter type exhibit one transmembrane anchor domain and a single nucleotide binding fold, and are functional as components of dimeric structures. Characterized hemitransporters include peroxisomal transporters [8] or the ER membrane-situated TAP proteins which are involved in antigen presentation [9]. Some ABC-family members have been shown to play a protective role by altering the localization of potentially toxic substrates; this is exemplified by the fission yeast hmt1 transporter (mediating compartmentalization of heavy metal ion complexes into the vacuole) [10] or MDR1 and MRP proteins which catalyze extrusion of xenobiotics (MDR1) or conjugates (MRP) through the cytoplasmic membrane [6]. Mammalian liver, as a major site of biotransformation and detoxification of xenobiotics, has been shown to express several members of the ABC transporter superfamily [11]. To identify novel mammalian ABC transporters in hepatocytes, an RT(reverse transcription)-PCR approach was used, employing degenerate primers corresponding to regions highly conserved in ABC proteins for initial cDNA fragment amplification. After completion of 3′- and 5′-ends, a full-length cDNA sequence coding for a novel ABC

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hemitransporter displaying greatest similarity to the fission yeast heavy metal tolerance protein hmt1 was obtained. Ubiquitous expression of corresponding mRNA in all analyzed rat tissues led to the name umat (*u*biquitously expressed *m*ammalian *A*BC half-*t*ransporter).

MATERIALS AND METHODS

Isolation of cDNA fragments from hepatocytes by degenerate PCR. Primary rat hepatocytes from adult male Wistar rats (220 g b.w.) were isolated by collagenase perfusion [12]. Total RNA was purified from freshly isolated hepatocytes by guanidinium thiocyanate phenol extraction according to [13]. First strand cDNA was synthesized using Superscript II RNase H^- reverse transcriptase (Gibco/BRL, Eggenstein, Germany) with 1 μg of total RNA primed by oligo (dT). Degenerate oligonucleotide primers, complementary to regions conserved among ABC protein members [14], were used to amplify ABC protein cDNA fragments from rat hepatocyte cDNA. The degenerate forward primer was S1: 5′-GGC GGA TCC TCN GGN KSN GGN AAR AGY AC-3′ the reverse primer S2 : 5′-CGG GAA TTC TCN ARN GCR CTN GTN GSY TCR TC-3′ (N for A/G/C/T; K for G/T; S for G/C; R for A/G and Y for C/T) [14]. PCR conditions were: 1 min at 94 °C, 2 min at 45 °C and 2.5 min at 72 °C for 35 cycles using 50 pmol primer. For discrimination of PCR products, the cDNA fragments were cloned by the TA cloning method (Invitrogen, Carlsbad, CA, USA) and sequenced. Sequence-PCR and automatic sequencing were performed according to the manufacturers' instructions with the Dye Terminator sequencing kit in an automated fluorescence model 377 sequencer (Applied Biosystems, Foster City, CA, USA).

Completion of 5′- and 3′- cDNA ends by RACE. The 5′-cDNA terminus of the cDNA fragment clone 29 which represented a novel sequence was completed by the 5′-RACE (rapid amplification of cDNA ends) method [15, 16] using the system obtained from Gibco/BRL. In brief, first strand cDNA synthesis was performed with total hepatocyte RNA with the 29p oligonucleotide in reverse orientation as the gene-specific primer (primer position indicated in Figure 1). Following addition of an oligo(dC)-anchor sequence to the 5′-end, the first strand cDNA was subjected to PCR using an anchor primer (Gibco/BRL) and the 29b primer in reverse orientation (position indicated in Figure 1). The PCR conditions for long template PCR were 20 sec at 94°C, 1 min at 60 °C and 5 min at 68 °C for 30 cycles, using the Expand™ polymerase mixture purchased from Boehringer, Mannheim, Germany. After reamplification, the resulting 5′-cDNA fragment was cloned and sequenced. The 3′-cDNA terminus was obtained by the 3′-RACE method [15]. In brief, after generation of first strand cDNA with an oligo(dT)-adaptor primer (Gibco/BRL), the 3′-end was amplified using the universal amplification reverse primer (Gibco/BRL) and the 29b forward primer, followed by a second and third round of amplification with the primer 29p in forward orientation. The 3′-cDNA amplification product was cloned and sequenced. The full-length cDNA encoding the novel ABC transporter was assembled from overlapping 5′-, 3′- and intermediate cloned cDNA fragments. The full sequence was confirmed by independent RT-PCR amplification and sequencing of new overlapping fragments at least twice: overlapping PCR fragments were generated with the primer pairs (29e for/29p rev) and (29c for/29f rev), respectively, whose positions are indicated in Figure 1.

Primary rat hepatocyte cell culture. Primary rat hepatocytes isolated by collagenase perfusion were cultured in serum-free MX-83 medium [17] in the presence of 1 μM insulin and 20 μM hydrocortisone as described previously [18] for up to 4 days.

Northern blot analysis. Total RNA was isolated from rat tissues and hepatocyte cultures according to [13] and was separated by electrophoresis through formaldehyde/agarose gels (20 μg/lane). Staining of gels with ethidium bromide was performed to ensure equal loading of lanes. Subsequently, RNA was transferred to Hybond N nylon membranes (Amersham, Braunschweig, Germany) by capil-

FIG. 1. Nucleotide sequence of the rat ABC transporter cDNA encoding the mammalian ABC half-transporter umat. The start codon (*), stop codon (**), and a putative polyadenylation signal are indicated in bold. Regions corresponding to primers used for amplification of cDNA fragments by PCR (29b, 29c, 29e, 29f, 29p, S1 and S2) are in bold and underlined, "for" denoting forward and "rev" indicating reverse primer orientation. The nucleotide sequence shown here has been deposited in the EMBL Database under Accession No. AJ003004.

FIG. 2. Amino acid alignment of the deduced rat umat protein product in comparison to the yeast atm1 protein [14], the fisson yeast heavy metal transporter hmt1 [10], the known partial sequence of the murine ABC7 transporter [25] (EMBL U43892), and the rat mdr1b sequence [26]. Alignment was performed with the ClustalW program [27]. Conserved residues are indicated in bold; motifs of the ATP-binding cassette are designated by A (Walker A), B (Walker B), and C (ABC family signature). Four of the eight predicted membrane-spanning sections whose positions appear conserved in respect to other ABC transporters are underlined.

lary blotting and hybridized to individual umat-specific antisense oligonucleotide probes (designated 29b, 29p and 29c), the positions of which are indicated in Figure 1. The probes had been end-labelled by T4-polynucleotide kinase (Boehringer, Mannheim, Germany) [19].

RESULTS AND DISCUSSION

An RT-PCR strategy was employed with degenerate primers to amplify cDNA fragments of sequences encoding ABC proteins in hepatocytes. The degenerate primers corresponded to conserved regions of the ABC protein nucleotide binding fold, namely to the Walker A motif (primer S1) and to part of the Walker B motif (primer S2). Multiple PCR products were obtained, ranging from approximately 370 to 450 bp in length. Discrimination of differing cDNA fragments was achieved by cloning and sequencing. Of 23 cDNA clones analyzed, 22 sequences were identified as partial ABC protein encoding regions, of which 11 clones represented rat mdr2 or tap transporters. By comparison to the EMBL database, the clone designated 29 was shown to represent a novel sequence. The full-length cDNA was obtained by rapid amplification of cDNA ends and assembly of overlapping 5′-, 3′- and intermediate cDNA fragments.

The full-length cDNA consists of 2842 bp (up to the poly-A tail). The longest open reading frame begins with the sequence GCCATGG which matches the consensus eukaryotic translation initiation motif [20] at nucleotide position 155 (ATG) and ends with a TAA termination codon at nucleotide position 2663 (Figure 1). A putative polyadenylation signal (AATAAA) [21] is located in the 3′-untranslated region at nucleotide position 2823 (Figure 1). Provided that the first in-frame ATG encodes the amino terminal methionine, a deduced polypeptide of 836 amino acids with a predicted molecular weight of 93.3 kDa would result. The predicted peptide was analyzed in respect to potential membrane spanning segments applying a modification of the method according to [22] using the PSORT program [23]. Eight putative membrane spanning regions were defined, which is in line with a number of 5-10 transmembrane helices predicted for other eukaryotic ABC half-transporters [3]. The N-terminal membrane-anchoring domain and the following conserved single ATP- binding fold (Figure 2) specify the novel protein as an ABC half-transporter. Comparison to protein database sequences using the FASTA 3 program [24] revealed closest similarity to other half-transporters previously identified: to the hmt1 protein in *Schizosaccharomyces pombe* [10] (44.5 % identity in 626 amino acid overlap), to a mouse transporter with unknown function (ABC7 partial sequence [25], 41.2 % identity in 640 amino acid overlap) and to the atm1 protein of *Saccharomyces cerevisiae* [14] (38.2 % identity in 637 amino acid overlap), Figure 2. The novel ABC protein demonstrated a more distant relationship to human and rodent MDR/mdr transporter isoforms, as exemplified by comparison to the rat mdr1b protein in Figure 2. The N-terminal part of the umat protein comprising the first 250 amino acids represents a non-conserved, individual feature of the umat transporter, as very little similarity to other ABC transporters was found (only approximately 11 % identity in residues compared to the hmt1 protein in a 210 amino acid overlap, Figure 2). However, multiple clusters of conserved residues were evident along the rest of the umat protein. Homology to other ABC transporters was highest in the ATP binding fold domain, the novel hemitransporter exhibiting the typical features conserved among ABC transporters, the Walker A and B regions and the intermediate ABC family signature (Figure 2). In the protein region flanked by Walker A and B motifs which had been defined by the initial degenerate PCR strategy, 70 % identity over 134 residues was observed between the umat protein and the hmt1 half-transporter.

In analogy to other ABC half-transporters, it is assumed that the umat protein is functional as part of a dimeric transporter structure. Amino acid sequence analysis by the PSORT program [23] did not suggest a definite sequence targeting the primary umat translation product to a specific membrane structure. However, as all mammalian hemitransporters identified so far have been shown to reside in an intracellular membrane, including the related hmt1 transporter (situated in the fisson yeast vacuolar membrane) or the atm1 protein (localized in the yeast inner mitochondrial membrane) [14], it appears likely that umat may be associated with an intracellular membrane structure rather than with the cytoplasmic membrane.

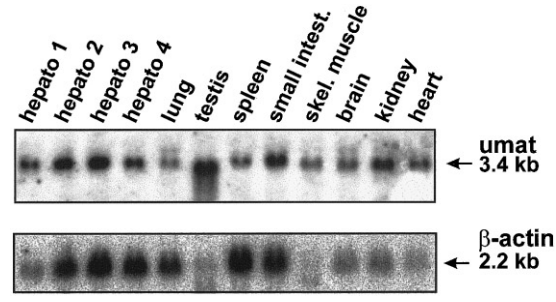

FIG. 3. Expression pattern of umat mRNA in primary rat hepatocyte cultures and in rat tissues. (Top) Representative Northern blot hybridization of total RNA (20 μg/lane) isolated from hepatocytes or from tissues of an adult male rat (intest., intestine; skel. muscle, skeletal muscle). Hepato 1, 2, 3, and 4 denote hepatocytes cultivated for 1, 2, 3, or 4 days, respectively. Hybridization was performed to the 29b oligonucleotide. (Bottom) A rehybridization of the blot with a rat β-actin oligonucleotide probe (phosphorimaging analysis).

The tissue distribution of umat mRNA expression was investigated by Northern blot analysis using total RNA derived from a panel of male rat tissues. Hybridization of blots to the umat oligonucleotide probe 29b (position indicated in Figure 1) led to detection of mRNA with an approximate molecular weight of 3.4 kb (Figure 3). MRNA of the same molecular weight was also detected by independent hybridizations to other umat oligonucleotide probes (29c, 29p) which were complementary to different cDNA regions (data not shown). Umat mRNA was expressed in all tissues analyzed (lung, testis, spleen, small intestine, skeletal muscle, brain, kidney and heart (Figure 3) and also in large intestine and total liver (data not shown). Ubiquitous expression led to the name umat (ubiquitously expressed mammalian ABC half-transporter). Noteworthy, exceptionally high expression was found in testis (Figure 3). The initial umat cDNA fragment had been obtained by RT-PCR using total RNA isolated from rat hepatocytes. Moreover, umat mRNA was stably expressed in primary hepatocytes cultured for up to 4 days (Figure 3). Thus, primary hepatocytes constitute an isolated cell population exhibiting high umat expression and may represent an adequate model for elucidation of umat regulation and for specification of function.

The umat-related hmt1 transporter has been shown to confer heavy metal tolerance, presumably by trans-

port of phytochelatin-heavy metal ion complexes through the vacuolar membrane into the vacuole [10]. Recently, the mitochondrial atm1 protein has been demonstrated to participate in yeast mitochondrial iron homeostasis [4]. Due to sequence similarity of umat to the hmt1 and atm1 proteins, it may be hypothesized that the novel ABC protein might be involved in metal ion homeostasis. Possible participation of umat in biometal homeostasis, e. g. of zinc or iron, would be in line with ubiquitous expression of the transporter mRNA.

In short, we have identified a full-length cDNA sequence from rat hepatocytes encoding a new ABC half-transporter which was termed umat. Ubiquitous expression of umat mRNA in all tissues investigated supports the notion that the corresponding transporter protein fulfills an important role in tissue homeostasis.

REFERENCES

1. Higgins, C. F. (1992) *Annu. Rev. Cell Biol.* **8,** 67–113.
2. Walker, J. E., Seraste, M., Runswick, M. J., and Gay, N. J. (1982) *Embo J.* **1,** 945–951.
3. Decottignies, A., and Goffeau, A. (1997) *Nature Genetics* **15,** 137–145.
4. Kispal, G., Csere, P., Guiard, B., and Lill, R. (1997) *FEBS Lett.* **418,** 346–350.
5. Allikmets, R., Gerrard, B., Hutchinson, A., and Dean, M. (1996) *Human Mol. Genet.* **5,** 1649–1655.
6. V. Ling (1997) *Cancer Chemother. Pharmacol.* **40,** S3–S8.
7. Riordan, R. J., Alon, N., Grzelczak, Z., Dubel, S., and Sun, S. Z. (1991) *Adv. Exp. Med. Biol.* **290,** 19–29.
8. Valle, D., and Gartner, J. (1993) *Nature* **361,** 682–683.
9. Spies, T., Bresnahan, M., Bahram, S., Arnold, D., Blanck, G., Mellins, E., Pious, D., and DeMars, R. (1990) *Nature* **348,** 744–747.
10. Ortiz, D. F., Kreppel, L., Speiser, D. M., Scheel, G., McDonald, G., and Ow, D. W. (1992) *Embo J.* **11,** 3491–3499.
11. Childs, S., Yeh, R. L., Georges, E., and Ling, V. (1995) *Cancer Res.* **55,** 2029–2034.
12. Seglen, P. O. (1976) *Methods Cell Biol.* **13,** 29–83.
13. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
14. Leighton, J., and Schatz, G. (1995) *Embo J.* **14,** 188–195.
15. Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8998–9002.
16. Loh, E. Y., Elliott, J. F., Cwirla, S., Lanier, L. L., and Davis, M. M. (1989) *Science* **243,** 217–220.
17. Hoffmann, B., Piasecki, A., and Paul, D. (1989) *J. Cell. Physiol.* **139,** 654–662.
18. Hirsch-Ernst, K. I., Ziemann, C., Schmitz-Salue, C., Foth, H., and Kahl, G. F. (1995) *Biochem. Biophys. Res. Commun.* **215,** 179–185.
19. Omiecinski, C. J., Walz, F. G., and Vlasuk, G. P. (1985) *J. Biol. Chem.* **260,** 3247–3250.
20. Kozak, M. (1986) *Cell* **44,** 283–292.
21. Keller, W. (1995) *Cell* **81,** 829–832.
22. Klein, P., Kanehisa, M., and DeLisi, C. (1985) *Biochim. Biophys. Acta* **815,** 468–476.
23. Nakai, K., and Kanehisa, M. (1992) *Genomics* **14,** 897–911.
24. Pearson, W. R., and Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2444–2448.
25. Savary, S., Allikmets, R., Denizot, F., Luciani, M.-F., Mattei, M.-G., Dean, M., and Chimini, G. (1997) *Genomics* **41,** 275–278.
26. Silverman, J. A., Raunio, H., Gant, T. W., and Thorgeirsson, S. S. (1991) *Gene* **106,** 229–236.
27. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680.