Cloning, characterization and tissue distribution of the rat ATP-binding cassette (ABC) transporter ABC2/ABCA2

Li-Xia ZHAO*, Cheng-Ji ZHOU⁺, Arowu TANAKA⁺, Masanori NAKATA^{*}, Takahiro HIRABAYASHI[§], Teruo AMACHI⁺, Seiji SHIODA⁺, Kazumitsu UEDA⁺ and Nobuya INAGAKI^{*1}

*Department of Physiology, Akita University School of Medicine, 1-1-1, Hondo, Akita 010-8543, Japan, †Department of Anatomy, Showa University School of Medicine, Tokyo 142-8555, Japan, ‡Laboratory of Biochemistry, Division of Applied Life Sciences, Kyoto University Graduate School of Agriculture, Kyoto 606-8502, Japan, and §Center for Biotechnology, Showa University, Tokyo 142-8555, Japan

The ABC1 (ABCA) subfamily of the ATP-binding cassette (ABC) transporter superfamily has a structural feature that distinguishes it from other ABC transporters. Here we report the cloning, molecular characterization and tissue distribution of ABC2/ABCA2, which belongs to the ABC1 subfamily. Rat ABC2 is a protein of 2434 amino acids that has 44.5 %, 40.0 % and 40.8 % identity with mouse ABC1/ABCA1, human ABC3/ABCA3 and human ABCR/ABCA4 respectively. Immunoblot analysis showed that proteins of 260 and 250 kDa were detected in COS-1 cells transfected with ABC2 having a haemagglutinin tag, while no band was detected in mock-transfected cells. After incubation with N-glycosidase F, the mobilities of the two proteins increased and a single band was detected, suggesting

that ABC2 is a glycoprotein. Photoaffinity labelling with 8azido- $[\alpha$ -³²P]ATP confirmed that ATP binds to the ABC2 protein in the presence of Mg²⁺. RNA blot analysis showed that ABC2 mRNA is most abundant in rat brain. Examination of brain by *in situ* hybridization determined that ABC2 is expressed at high levels in the white matter, indicating that it is expressed in the oligodendrocytes. ABC2, therefore, is a glycosylated ABC transporter protein, and may play an especially important role in the brain. In addition, the N-terminal 60-amino-acid sequence of the human ABC1, which was missing from previous reports, has been determined.

Key words: apoptosis, brain, flippase, oligodendrocyte.

INTRODUCTION

The ATP-binding cassette (ABC) transporter superfamily is one of the largest gene families known; it encodes highly conserved proteins involved in the energy-dependent transport of a variety of substrates across membranes, including ions, sugars, amino acids, peptides, polysaccharides and proteins [1]. Over 500 members have been identified to date in prokaryotes and eukaryotes [1,2]. In mammalian cells, the typical ABC protein consists of four domains: two membrane-spanning domains (MSDs), usually containing six transmembrane segments through which solute crosses the membrane and determines substrate specificity, and two nucleotide-binding domains (NBDs) located at the cytosolic face of the membrane, which couple ATP hydrolysis to solute movement [3]. Another type of ABC protein, a half molecule, consists of one NBD and one MSD, and exerts its function as a dimer with the same or structurally similar polypeptides [3].

ABC proteins have diverse functions: the multidrug resistance proteins MDR1 and MDR2 function as an active transporter of chemotherapeutic drugs and as a flippase respectively [4,5]; the cystic fibrosis transmembrane conductance regulator CFTR functions as a chloride channel [6]; and the sulphonylurea receptor SUR regulates potassium channel function [7,8]. ABC proteins have received considerable attention because many mammalian ABC proteins have been associated with clinically relevant phenotypes, such as cystic fibrosis [6], adrenoleukodystrophy [9], persistent hyperinsulinaemic hypoglycaemia of infancy [10], congenital jaundice [11] and intrahepatic cholestasis [12].

Among several subfamilies in the ABC transporter superfamily, the ABC1 (ABCA) subfamily is unique in that the members have a structure distinct from that of the other subfamilies: they have a large domain between the first NBD and the second MSD, interrupted by an extra hydrophobic segment [13]. To date, four members have been identified as belonging to the ABC1 subfamily: ABC1/ABCA1 [13], ABC2/ABCA2 [13], ABC3 (ABC-C)/ABCA3 [14], and ABCR/ABCA4 [15-17]. Recently the genes encoding ABC1 and ABCR have been determined to be the causal genes for Tangier disease [18-20] and Stargardt's macular dystrophy [15] respectively, and are proposed to be flippases [21] for intracellular cholesterol and protonated N-retinylidene-phosphatidylethanolamine [22] respectively, suggesting that the members of this subfamily function as flippases. Intriguingly, the nematode Caenorhabditis elegans gene ced-7, which is involved in the engulfment of cell corpses during programmed cell death, encodes a protein similar to the ABC1 subfamily proteins [23], suggesting that this subfamily is also associated with the engulfment of mammalian apoptotic cells. ABC2, a member of the ABC1 subfamily, is known to be abundant in the brain [13]. In a previous paper, ABC2 gene expression was amplified in an ovarian carcinoma cell line

Abbreviations used: ABC, ATP-binding cassette; MSD, membrane-spanning domain; NBD, nucleotide-binding domain; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase-PCR; HA, haemagglutinin.

¹ To whom correspondence should be addressed (e-mail inagaki@med.akita-u.ac.jp).

The nucleotide sequences for rat ABC2 and human ABC1 have been deposited in the DDBJ/EMBL®/GenBank/GSDB Nucleotide Sequence Databases under accession numbers AB037937 and AB037924 respectively.

resistant to the chemotherapeutic agent oestramustine [24], suggesting that ABC2 may function as a transporter of the oestradiol-mustard conjugate, but the complete structure and function of ABC2 remain unknown.

In the present study we cloned and sequenced the full-length rat cDNA for ABC2, and studied its molecular properties and distribution in various tissues. Since ABC2 mRNA is expressed abundantly in the brain, we examined the distribution of ABC2 mRNA in the brain by *in situ* hybridization. During the alignment of the amino acid sequences of the ABC1 subfamily, we assumed that the N-terminal region of human ABC1 is missing, and thus we isolated and determined the nucleotide sequence of the 5'-end of the cDNA encoding the N-terminus of the human ABC1 protein.

MATERIALS AND METHODS

Cloning of the ABC2 cDNA

A fragment of mouse ABC2 cDNA (corresponding to nucleotides +2957 to +5219 relative to the putative translation start of the rat ABC2 cDNA) [13] was amplified by reverse transcriptase-PCR (RT-PCR) using total RNA extracted from mouse brain, as described previously [25]. A total of 7.2×10^5 plaques of a rat cDNA library (Stratagene) were screened using a ³²P-nicktranslated partial mouse cDNA fragment as a probe under the standard hybridization conditions: hybridizations were carried out in $5 \times SSC$ ($1 \times SSC$ is 0.15 M NaCl/0.015 M sodium citrate), 50% formamide, 2 × Denhardt's solution (1 × Denhardt's is 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.002 % BSA), 20 mM sodium phosphate buffer, pH 6.5, 0.1 % SDS, 100 μ g/ml sonicated and denatured salmon testis DNA, 10 % (w/v) dextran sulphate and 1×106 c.p.m./ml ³²P-labelled probe at 42 °C for 16 h [24]. The nylon membranes were washed in $0.1 \times SSC/0.1 \%$ SDS at 50 °C for 1 h before autoradiography. To obtain the upstream portion of the rat ABC2 cDNA, the same library was rescreened using the DNA fragment of the isolated rat ABC2 clone as a probe. DNA sequencing was performed on an ABI377 automated DNA sequencer (Perkin Elmer Applied Biosystems) by using a Bigdye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer Applied Biosystems) after subcloning of appropriate DNA fragments into pGEM3Z (Promega). Both strands were sequenced.

To obtain the 5'-end of the rat ABC2 cDNA, rapid amplification of cDNA ends (RACE) was carried out by the using the 5'-RACE system (version 2.0; Life Technologies, Inc.) according to the manufacturer's instructions. Briefly, the first-strand DNA was synthesized from rat brain total RNA using the antisense primer 5'-TCCAGACGTTGGTGCAGTGC-3' (nucleotides 383–364 relative to the putative translation start site of the rat ABC2 cDNA). The original mRNA template was removed by treatment with RNase H, and then a homopolymeric tail was added to the 3'-end of the cDNA using terminal deoxynucleotidyl transferase and deoxyCTP. The tailed cDNA end was amplified by PCR using the second antisense primer 5'-CAGGAAGCC-AAACTCATCAC-3' (nucleotides 255-236 relative to the putative translation start site of the rat ABC2 cDNA), and the amplified DNA was cloned into pGEM-T vector (Promega) followed by sequencing.

To obtain the 5'-end of the human ABC1 cDNA, nested PCR was carried out using Marathon-Ready cDNA according to the manufacturer's instructions (Clontech). In the nested PCR, the antisense primer 5'-GAACCCAAGGAAGTGTTCCTGC-3' (nucleotides 151–130 of the registered human ABC1 cDNA) and the antisense primer 5'-GTAGCTCAGCCGAACAGAGATC-3' (nucleotides 78–57 of the registered human ABC1 cDNA)

were used for the first and second PCR respectively. The PCR product of approx. 400 bp was inserted into pCR2.1 (Invitrogen), and the sequence was determined.

RNA blot analysis

To study the tissue distribution of the ABC2 mRNA, total cellular RNA was prepared from various rat tissues by the guanidinium isothiocyanate/CsCl procedure [25]. For RNA transfer blots, $20 \ \mu g$ of total RNA from the various rat tissues was denatured with formaldehyde, subjected to electrophoresis on a 1% (w/v) agarose gel, and transferred to a nylon membrane [25]. Hybridization was carried out as described above with a ³²P-labelled DNA fragment (nucleotides 1369–1888 relative to the putative translation start site of the rat ABC2 cDNA). The membrane was washed as described above and exposed to X-ray film with an intensifying screen at -80 °C for 4 days.

Cell culture and transfection

The culture and transfection of COS-1 or COS-7 cells was carried out as described previously [7,8]. Briefly, cells were plated on 35 mm culture dishes at a density of 2×10^5 cells per dish 24 h before transfection, and cultured in Dulbecco's modified Eagle's medium (450 mg/dl glucose) supplemented with 10% (v/v) fetal calf serum. A portion of $2\mu g$ of rat ABC2 expression vector (pCMVrABC2) was transfected into cells with Lipofectamine and Opti-MEM I (Life Technologies, Inc.), according to the manufacturer's instructions. For immunoblot and ATP-binding analyses, pCMVrABC2-HA was constructed, in which DNA encoding the human influenza haemagglutinin (HA) tag (YPYDVPDYA) was introduced into the 3'-end of the coding region of ABC2 in pCMVrABC2 using PCR.

Preparation of crude membranes

Crude membranes were prepared as described previously [26]. Briefly, for immunoblot analysis, COS-1 cells were taken 3 days after transfection with pCMVrABC2-HA or pCMV vector alone and washed three times with PBS, suspended in buffer A [consisting of 50 mM Tris (pH 7.5), 1 mM EDTA and a protease inhibitor cocktail (10 μ l/ml) (Sigma)], homogenized and then centrifuged at 10000 g at 4 °C for 1 h. The pellets were resuspended in 500 μ l of buffer A and stored at -80 °C until immunoblot analysis. For ATP-binding analysis, crude membranes were prepared from COS-7 cells under the same conditions, except that nitrogen cavitation was used as described previously [27].

Immunoblot analysis

Crude membranes were prepared from COS-1 cells transfected with pCMVrABC2-HA or pCMV alone, as described above. The crude membrane proteins $(20 \ \mu g)$ were boiled in SDS reducing sample buffer, electrophoresed on an SDS/7%polyacrylamide gel, and transferred to a nitrocellulose membrane (Hybond[®]; Amersham) by electroblotting overnight at 4 °C at 200 mA [26]. For N-glycosidase F treatment, a 20 μg portion of crude membrane proteins was incubated with 4 units of Nglycosidase F (Boehringer Manheim) for 3 h at 37 °C, and then subjected to SDS/PAGE and electroblotting as described above. The membrane was blocked for 1 h at room temperature in 5 % defatted milk in 1 × PBS-T [1 × PBS (136.9 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄ and 1.8 mM KH₂PO₄), pH 7.4, and 0.1% Tween 20]. After washing with 1 × PBS-T, the membrane was incubated with 1:1000-diluted high-affinity anti-HA rat antibody (Boehringer Manheim) for 2 h at room temperature. After washing with $1 \times PBS$ -T, the membrane was incubated with 1:800-diluted horseradish peroxidase-conjugated anti-rat IgG (Biosys) for 1 h. After washing with $1 \times PBS$ containing 0.3% Tween 20 and then with $1 \times PBS$ -T, proteins were detected using an enhanced chemiluminescence system (ECL[®]; Amersham), according to the manufacturer's instructions. Protein concentrations were determined using the BCA assay (Pierce).

Interaction of ABC2 with 8-azido- $[\alpha - {}^{32}P]ATP$

Crude membrane proteins (50 μ g) from COS-7 cells transiently expressing ABC2–HA fusion protein were incubated with 50 μ M 8-azido-[α -³²P]ATP (ICN Biomedicals), 2 mM ouabain, 0.1 mM EGTA and 40 mM Tris/HCl (pH 7.5) in a total volume of 6 μ l for 10 min at 0 °C in the presence or absence of 3 mM MgSO₄ (or 1.5 mM EDTA) or 2 mM ATP. Proteins were UV-irradiated for 5 min (at 254 nm; 5.5 mW/cm²) on ice. ABC2–HA fusion protein was immunoprecipitated with anti-HA antibody. Samples were electrophoresed on an SDS/7 %-polyacrylamide gel and autoradiographed.

In situ hybridization

Sprague-Dawley rats (2 months old) were anaesthetized with sodium pentobarbital (50 mg/kg body weight; intraperitoneal) and killed by decapitation. The brain was quickly removed and frozen in liquid nitrogen, and stored at -70 °C until used. Frozen 7-µm-thick sagittal sections were cut with a cryostat (Microm HM500) and thaw-mounted on 3-aminopropyltriethoxysilane-coated slides. The plasmid containing a 311-bp ABC2 cDNA fragment (nucleotides 4593-4903 relative to the putative translation start site of the rat ABC2 cDNA) was transcribed with SP6 RNA polymerase and labelled with [³⁵S]CTP (1250 Ci/mmol; NEN) to generate the antisense cRNA probe. The same plasmid was transcribed with T7 RNA polymerase (Boehringer Mannheim) to generate the sense probe. The hybridization histochemistry was performed as described previously [28]. In brief, after being fixed with 4 % paraformaldehyde in PBS for 30 min, the sections were treated with proteinase K $(20 \,\mu g/ml$ for 10 min) and acetylated with 0.25 % acetic anhydride in triethanolamine. Hybridization was carried out at 55 °C overnight in a solution containing 50 % formamide, 300 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA, 1 × Denhardt's solution, 10 mM dithiothreitol, 0.25 % SDS, 200 µg of Escherichia coli tRNA (Sigma) and 1×10^5 c.p.m./µl riboprobe. The hybridized sections were treated with 20 µg/ml RNase A, and washed at 55 °C with $2 \times SSC$ and then $0.2 \times SSC$ for 40 min each. After dehydration through a graded series of ethanol baths (containing 300 mM ammonium acetate) and air drying, the sections were exposed to Beta-max Hyperfilm (Amersham) for 1 week at room temperature.

RESULTS

Cloning of rat ABC2 cDNA

Based on the partly known sequence of the mouse ABC2 cDNA, a 2260-bp DNA fragment corresponding to the first NBD was amplified from mouse brain mRNA by RT-PCR and then radiolabelled. Using the radiolabelled DNA fragment as a probe, a rat brain cDNA library was screened, and 14 λ clones were isolated from over 100 positive clones. Two overlapping clones, λ rABC2-1-1 and λ rABC2-1-23, were subcloned and sequenced. The nucleotide sequence identity between the mouse ABC2 cDNA fragment we used as a probe and the corresponding region of the rat ABC2 cDNA is 93.5%. To obtain more of the sequence upstream, a 520-bp DNA fragment located at the 5'end of rat ABC2-1-1 (corresponding to nucleotides 1369–1888 relative to the putative translation start site of the rat ABC2 cDNA) was radiolabelled and then used as a probe for further screening of the rat brain cDNA library; 26 λ clones were isolated, and one clone, λ rABC2-3-16, was subcloned and sequenced.

The composite sequence of 8040 bp contains a single open reading frame beginning with the first ATG in the cDNA sequence, which predicts the amino acid sequence of a 2434residue protein (molecular mass 270913.5 Da) (Figure 1). No termination codon was found either in the 44 bp of DNA upstream from the first ATG or in the additional 23 bp of DNA further extended by 5'-RACE. However, the first ATG is thought to be a translation start site, because the nucleotide sequence around it is consistent with the Kozak box [29]. The predicted amino acid sequence of ABC2 shows 44.5%, 40.0% and 40.8%identity with mouse ABC1, human ABC3 and human ABCR respectively (Figure 1). Hydropathy analysis predicts that 14 hydrophobic segments (H1-H14 in Figure 1), seven in the first MSD (H1-H7) and seven in the second MSD (H8-H14), are conserved among ABC1, ABC2, ABC3 and ABCR. The amino acid sequences of these proteins are conserved not only in the NBD but also in the MSD, except for the regions between H1 and H2 and between H8 and H9. In addition, an approx. 40amino-acid sequence at the N-terminus of these proteins is well conserved, further suggesting that the first ATG of ABC2 is a translation start site. Although the membrane topology of ABC2 is still unknown, there are four possible cAMP-dependent phosphorylation sites (Ser-1118, Ser-1132, Ser-1454 and Ser-2196) and seven possible protein kinase C-dependent phosphorylation sites (Ser-1115, Ser-1242, Ser-1340, Ser-1382, Ser-1428, Ser-2064 and Thr-2146), at least in the two NBDs on the cytoplasmic side. There also is a possible tyrosine kinase-dependent phosphorylation site (Tyr-1389) in the first NBD.

Isolation of the first methionine codon of human ABC1

It has been reported that the full-length cDNA for human ABC1 contains a 6603-bp open reading frame, which encodes a polypeptide of 2201 amino acids [30]. Although the N-terminal regions are conserved among the members of ABC1 subfamily, as shown in Figure 1, this region is missing from the reported sequence of the human ABC1 [30]. We thought that the reported sequence might not be the full-length cDNA for human ABC1, because the reported human ABC1 cDNA was cloned by an RT-PCR method using primers derived from mouse ABC1 cDNA [13]. We have found that there is no termination codon upstream of the reported ATG initiator codon, and that the putative open reading frame continues to the 5'-end of the reported human ABC1 cDNA sequence (EMBL/NCBI/GenBank accession no. AJ012376). To determine the sequence of the full-length human ABC1 cDNA, we attempted to isolate the N-terminal region of the human ABC1 cDNA by a 5'-RACE reaction using human placental cDNA. We isolated a 400-bp cDNA fragment which contains another ATG codon 180 bp upstream from the reported putative translation start site. There is a termination codon 9 bp upstream from the newly identified ATG. Mouse ABC1 cDNA contains ATG at the same position on the cDNA sequence (EMBL database accession no. X75926), and the deduced 60amino-acid sequence of the 180-bp region upstream from the reported putative translation start codon is identical with that of the N-terminus of mouse ABC1 protein, except for the fourth

		H1
rABC2	1	MGFLHQLQLLLWKNVTLKRRSPWVLAFEIFIPLVLFFILLGLRQKKPTISVKEAFYTAAPLTSAGILPVMQSLCPDGQRDEFGFLQYANSTVTQLLERLNRVVEESNLFDPERPSL
mABC1	1	MACXPQLRLLLWKNLTFRRQTCQLLLEVAWPLFIFLILISVRLSYDPYEQHECHFPNKAMPSAGTLPWVQGIICNANNPCFRYPTPGEAPGVVGNFNKSIVSRLFSDAQRLLLYSQR
hABC3		MAVLRQLALLIMKNYTLQKRKVLVTVLELFLPLLFPGILIWLRLK
hABCR	1	MGFVRQIQILLIWKNWTLRKRQKIRFVVELVWPLSLFLVLIWLRNANPLYSHHECHFPNKAMPSAGMLPWLQGIFCNVNNPCFQSPTPGESPGIVSNYNNSILARVYRDFQELLMNAPE
rABC2	117	${\tt GSELEALHQRLEALSSGPGTweselsarpavssfsldsvardkrelwrflwqmlslpnstaqallaarwdpsevyrllfgplpdldgklgflrkqepwshlgsnplfqmeelllapallequeselsarpavssfsldsvardkrelwrflwqmlslpnstaqallaarwdpsevyrllfgplpdldgklgflrkqepwshlgsnplfqmeelllapallequeselsarpavssfsldsvardkrelwrflwqmlslpnstaqallaarwdpsevyrllfgplpdldgklgflrkqepwshlgsnplfqmeelllapallequeselsarpavssfsldsvardkrelwrflwqmlslpnstaqallaarwdpsevyrllfgplpdldgklgflrkqepwshlgsnplfqmeelllapallequeselsarpavssfsldsvardkrelwrflwqmlslpnstaqallaarwdpsevyrllfgplpdlgklgflrkqepwshlgsnplfqmeelllapallequeselsarpavssfsldsvardkrelwrflwqmlslpnstaqallaarwdpsevyrllfgplpdlgklgflrkqepwshlgsnplfqmeelllapallequeselsarpavssfsldsvardkrelwrflwqmlsdnstaqallaarwdpsevyrllfgplpdlgklgflrkqepwshlgsnplfqmeelllapallequeselsarpavssfsldsvardkrelwrflwqmlsdnstaqallapallequeselsarpavssfsldsvardkrelwsflwqmlsdnstaqallapallequeselsarpavssfsldsvardkrelwrflwqmlsdnstaqallapallequeselsarpavssfsldsvardkrelwsfsldsvardkrelwrflwqmlsdnstaqallapallequeselsarpavssfsldsvardkrelwrflwqmlsdnstaqallapallequeselsarpavssfsldsvardkrelwsfsldsvardkrelwrflwqmlsdnstaqallapallequeselsarpavssfsldsvardkrelwrflwqmlsdnstaqallapallequeselsarpavssfsldsvardkrelwrflwqmlsdnstaqallapallapallequeselsarpavssfsldsvardkrelwrflwqmlsdnstaqallapallaqallapallaqallapallaqallapallaqallapallaqallapallaqalla$
mABC1	119	DTSIKDMHKVLRMLRQIKHPNSNLKLQDFLVDNETFSGFLQHMLSLPRSTVDSLLQXNVGLQKVFLQGYQLF
hABC3		
hABCR	119	SQHLGRIWTELHILSQFMDTLRTHPERIAGRGIRIRDILKDEETLTLFLIKNIGLSDSVVYLLINSQVRPEQFAHGVPDLALKDIACSEALLERFIIFSQRRGA
rABC2	237	LTCAPGSGELGRILTMPEGHQVDLQGYRDAVCSGQATARAQHFSDLATELRNQLDIAK-IAQQLGFNVPNGSDPQPQAPSPQSLQALLGDLLDVQKVLQDVDVLSALALLLPQGAC2
mABC1	191	$\texttt{LASLCNGSKLEEI} \texttt{IQLGDAEVSALCGLPRKKLDAAERVLRYNMD} \texttt{LKPVVTKLNSTSHLPTQHLAEATTVLLDSLGGLAQELFSTKSWSDMRQEVMFLTNVNSSSLEEI} \texttt{LASLCNGSKLEEI} \texttt{LQLGDAEVSALCGLPRKKLDAAERVLRYNMD} \texttt{LKPVVTKLNSTSHLPTQHLAEATTVLLDSLGGLAQELFSTKSWSDMRQEVMFLTNVNSSSLEEI} \texttt{LASLCNGSKLEEI} \texttt{LQLGDAEVSALCGLPRKKLDAAERVLRYNMD} \texttt{LKPVVTKLNSTSHLPTQHLAEATTVLLDSLGGLAQELFSTKSWSDMRQEVMFLTNVNSSSLEEI] \texttt{LASLCNGSKLEEI} \texttt{LQLGDAEVSALCGLPRKKLDAAERVLRYNMD} \texttt{LASLCNGSKLEEI} \texttt{LASLCNGSKLEEI \texttt{LASLCNGSKLEEI} \texttt{LASLCNGSKLEEI} \texttt{LASLCNGSKLEEI} \texttt{LASLCNGSKLEEI} \texttt{LASLCNGSKLEEI \texttt{LASLCNGSKLEEI} LASLCNGSKLEEI \texttt{LASLCNGSKLEEI \texttt{LA$
hABC3	46	1020005
habcr	223	KTVRYALCSLSQGTLQWIEDTLYANVDFFKLFRVLPTLLDSRSQGINLRSWGGILSDMS-PRIQ-EFIHRPSMQDLLWVTRPLMQNGGPETFTKLMGILSDLLCG
rABC2	353	grapap- Qagspsgpanstgvgantgpnttveegtqspvtpaspdtlqgqcsafvqlwaglqpilcgn-nrtiepealrrgnmsslgftskeqrniggllvhimtsnpkilyapagseadher and the statemeter of the sta
mABC1		SSSTQIYQAVSRIVCGHPEGGGLKIKSLMYEDNNYKALFGGN-NTEEDVDTFYDNSTTPYCNDLMKNLESSPLSRIIWKALKPLLVGKILYTPDTPATRC
hABC3	53	NATIYPQQSIQELPLFPTFPPPGDTWELAYIPSHSDAAKTVTETVRRALVINMRVRGFPSEKDFEDYIRYDNCSSSVLAAVVFEHPFNHSKEPLPLAVKYHLRFSYTRRNY
habcr	326	YPEGGGSRVLSFNWYEDNNYKAFLGIDSTRKDPIYSYDRRTTSFCNALIQSLESNPLTKIAWRAAKPLLMGKILYTPDSPAARR
rABC2	471	VILKANETFAFVGNVTHYAQVWLNISAEIRSFLEQGRLQQHLHWLQQYVADLRLHPEAMNLSLDELPPALRLDYFSLPNGTALLQQLDTIDNAACGWIQFMSKVSVL
mABC1	395	VMAEVNKTFQELAVFHDLEGMWEELSPQIWIFMENSQEMDLVRTLLDSRGNDQFWE-QKLDGLDWTAQDIMAFLAKNPEDVQSPNGSVYTWREAFNETNQAIQTISRFMECVNLN
hABC3		
hABCR		ILKNANSTFEELEHVRKLVKAWEEVGPQIWYFFDNSTQMMMIRDTLGNPTVKDFLNRQ-LGEEGITAEAILNFLYKGPRESQADDMANFDWRDIFNITDRTLRLVNQYLECLVLDKFES-DOMANFDWRDIFNITDRTRTLRLVNQYLECLVLDKFES-DOMANFDWRDIFNITDRTRTRTRTRTRTRTRTRTRTRTRTRTRTRTRTRTRTR
rABC2		
rabc2 mABC1	5/8	IFKGFPDEESIVNYTLNQAYQDNVTVFASVIFQTRKDGSLPPHVHYKIRQNSSFTEKTNEIRRAYWRPGPNTGGRFYFLYGFVWIQDMIERAIINTFVGHDVVEPGNYVQM
hABC3		KLEPIPTEVRLINKSMELLDERKFWAGIVFTGITPDSVELPHHVKYKIRMDIDNVERTNKIKDGYWDPGPRADPFEDMRYVWGGFAYLQDVVEQAIIRVLTGSEKKTGVVVQQ
hABCR		LFPLFPNPGPRELTSPDGGEP-GYIREGFLAVQHAVDRAIMEYHADAATRQLFQRLTVTIKR YNDETQLTQRALSLLEENMFWAGVVFPDMYPWTSSLPPHVKYKIRMDIDVVEKTNKIKDRYWDSGPRADPVEDFRYIW3GFAYLQDMVEQGITRSQVQAEAPVGIYLQQ
	220	
rABC2	689	H2 H4 FPYPCYTRDDFLFVIEHMMPLCMVISWVYSVAMTIQHIVAEKEHRLKEVMKTMGLMNAVHWVAWFITGFVQLSISVTALTAILKYGQVLMHSHVLIIWLFLAVYAVATIMFCFLV
mABC1	622	MPYPCYVDDIFLRVMSRSMPLFMTLAWIYSVAVIIKSIVYEKEARLKETMRIMGLDNGILWFSWFVSSLIPLLVSAGLLVVILKLGNLLPYSDPSVVFVFLSVFAMVTILQCFLI
hABC3	245	FPYPPFIADPFLVAIQYQLPLLLLLSFTYTALTIARAVVQEKERRLKEYMRMMGLSSWLHWSAWFLLFFLFLLIAASFMTLLFCVKVKPNVAVLSRSDPSLVLAFLLCFAISTISFSFMV
hABCR	637	MPYPCFVDDSFMIILNRCFPIFMVLAWIYSVSMIVKSIVLEKELRLKETLKNQGVSNAVIWCTWFLDSFSIMSMSIFLLTIFIMHGRILHYSDPFILFLFLLAFSTATIMLCFLLAFSTATIMLTFLAFSTATIMLTFLAFSTATIMLTFLAFSTATIMLTFLAFSTATIMLTFLAFSTATIMTTATIMTTATIMTTATIMTTATIMTTATIMTTATIMTTATIMTTATIMTTATIMTTATIMTTATIMTTATIMTTATITATI
rABC2	804	
mABC1	737	SVLYSKAKLASACGGIIYFLSYVPYMYVAIREEVAHDKITAFEKCIASLMSTTAFGLGSKYFALYEVAGVGIQMHTFSQSPVEGDDFNLLLAVTMLMVDTVVYGVLTWVIEAVFPGMYGL STLFSRANLAAACGGIIYFTLYLPYVLCVAWQDYVGFSIKIFASLLSPVAFGFGCEYFALFEEQGIGVQWDNLFESPVEEDGFNLTTAVSMMLFDTFLYGVMTWYIEAVFPGQYGI
hABC3	365	STIFFSKANMAAAFGGFLYFFTYIPYFFVAPRYNNMTLSQKLCSCLLSNVAMAMGAQLIGKFEAKGMGIQWRDLLSPVNVDDDFCFGQVIGMLLLDSVLYGLVTWYMEAVFPGQFGV
habcr	752	STFFSKASLAAACSGVIYFTLYLPHILCFAWQDRMTAELKKAVSLLSPVAFGFGTEYLVRFEEQGLGLQWSNIGNSPTEGDEFSFLLSMQMMLLDAACYGLLAWYLDQVF9GDYGT
rABC2	924	Walker A PRPWYFPLQKSYWLGSGRTETWEWSWPWAHAPRLSVMEEDQACAMESRHFEETRGMEEDPTHLPLVVCVDKLTKVYKNDKKLALNKLSLNLYENQVVSFLCHNGAGKTTTMSILTGLF
mABC1	853	PRPWYPCTKSYWFGEEIDEKSHPGSSQKGVSEICMEEEPTHLRLGVSIQNLVKVYRDGMKVAVDGLALNFYEQQITSFLGHNGAGKTTTMSILTGLF
hABC3	481	PQPWYFFIMPSYWCGKPRAVAGKEEEDSDPEKALRNEYFEAEPEDLVAGIKIKHLSKVFRVGNKDRAAVRDLNLNLYEGQITVLLGHNGAGKTTTLSMLTGLF
hABCR	868	PLPWYFLLQESYWLSGEGCSTREERALEKTEP-LTEETEDPEHPEGIHDSFFEREHPGWVPGVCVKNLVKIFEPCGRPAVDRLNITFYENQITAFLGHNGAGKTTTLSILTGLL
rABC2	1042	Walker B PPTSCSATIYGHDIRTEMDEIRKNLGMCPQHNVLFDQLTVEEHLWFYSRLKSMAQEEIRKEMDKMIEDLELS-NKRHSLVQTLSCGMKRKLSVAIAFVGGSRAIILDEPTAGVDPYARRA
mABC1	951	PPTSGTAYILGKDIRSEMSSIRQNLGVCPQHIVLFDMLTVEEHIWFYARLKGLSEKHVKAEMEQMALDVGLPPSKLKSKTSQLSGGMQRKLSVALAFVGGSKWILDEPTAGVDPYSRRG
hABC3	584	PPTSGRAYISGYEISQDMVQIRKSLGLCPQHDILFDNLTVAEHLYFYAQLKGLSRQKCPEEVKQMLHIIGLE-DKWNSRSRFLSGGMRKLSIGIALIAGSKVLILDEPTSGMDAISRRA
hABCR	981	
	201	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKWILDEPTSGVDPYSRRS
rABC2		PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRRS
rABC2 mABC1	1161	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRRS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLKCCGSPLFLKGAYGDGYRLTLVKRPAEPGTSQEPGMASSP5GRPQLSNCSEMOVSOFIRKH
	1161 1071	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRRS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLKCCGSPLFLKGAYGDGYRLTLVKRPAEPGTSQEPGMASSP5GRPQLSNCSEMQVSQFIRKH IWELLLKYRQGRTIILSTHHMDEADILGDRIAIISHGKLCCVGSSLFLKNQLGTGYYLTLVKKDVESSLSSCRNSSSTVSCLKKEDSVSOSSSDAGLGSDHESDTLTIDVSAISNIJERH
mABC1	1161 1071 703	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRRS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLKCCGSPLFLKGAYGDGYRLTLVKRPAEPGTSQEPGMASSPSGRPQLSNCSEMOVSOFIRKH
mABC1 hABC3	1161 1071 703 1100	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLKCCGSPLFLKGAYGDGYRLTLVKRPAEPGTSQEPGMASSPSGRPQLSNCSEMQVSQFIRKH IWELLLKYRQGRTIILSTHHMDEADILGDRIAIISHGKLCCVGSSLFLKNQLGTGYYLTLVKKDVESSLSSCRNSSSTVSCLKKEDSVSQSSSDAGLGSDHESDTLTIDVSAISNLIRKH IWDLLQRQKSDRTIVLTHHMDEADLLGDRIAIISHGKLCCCGSSLFLKNQLGTGYHLTLVKEPHCNP
mABC1 hABC3 hABCR	1161 1071 703 1100 1261	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSOGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRKS IWDLILKYKPGRTIILSTHHMDEADLIGDRIAIISHGKLKCCGSPLFLKGAYGDGYRLTLVKRPAEPGTSQEPGMASSPSGRPQLSNCSEMQVSQFIRKH IWELLLKYRQGRTIILSTHHMDEADLIGDRIAIISHGKLCCCGSSLFLKQAYGDGYRLTLVKKDVESSLSSCRNSSSTVSCLKKEDSVSQSSSDAGLGSDHESDTLTIDVSAISNLIRKH IWDLLQRQKSDRTIVLTHFMDEADLIGDRIAIISHGKLCCCGSSLFLKQAYGAGYHMTLVKEPHCNP
mABC1 hABC3 hABCR rABC2	1161 1071 703 1100 1261 1191 781	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLMGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRRS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLCCCGSPLFLKGAYGDGYRLTLVKRPAEPGTSQEPGMASSPSGRPQLSNCSE
mABC1 hABC3 hABCR rABC2 mABC1	1161 1071 703 1100 1261 1191 781	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLMGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRKS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLKCCGSPLFLKGAYGDGYRLTLVKRPAEPGTSQEPGMASSPSGRPQLSNCSE
mABC1 hABC3 hABCR rABC2 mABC1 hABC3	1161 1071 703 1100 1261 1191 781 1216	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRRS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLCCGSPLFLKGAYGDGYRLTLVKRPAEPGTSQEPGMASSPSGRPQLSNCSE
mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR	1161 1071 703 1100 1261 1191 781 1216 1381	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRRS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLKCCGSPLFLKQQLGTGYYLTLVKKPAEPGTSQEPGMASSPSGRPQLSNCSE
mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR rABC2	1161 1071 703 1100 1261 1191 781 1216 1381 1275	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRRS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLKCCGSPLFLKGAYGDGYRLTLVKRPAEPGTSQEPGMASSPSGRPQLSMCSE
mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR rABC2 mABC1	1161 1071 703 1100 1261 1191 781 1216 1381 1275 870	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLMGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRKS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLKCCGSPLFLKNQLGTGYYLTLVKKPAEPGTSQEPGMASSPSGRPQLSNCSE
mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR	1161 1071 703 1100 1261 1191 781 1216 1381 1275 870 1313	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQINILLFHHLTVAEHMLFYAQLMGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLCCGSPLFLKGAYGDGYRLTLVKRPAEPGTSQEPGMASSPSGRPQLSNCSE
mABC1 hABC3 hABC7 mABC2 mABC1 hABC3 hABC7 rABC2 mABC1 hABC3	1161 1071 703 1100 1261 1191 781 1216 1381 1275 870 1313 1500	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQINILLFHHLTVAEHMLFYAQLMGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRRS IWDLILKYKPGRTILLSTHHMDEADILGDRIAIISHGKLCCGSPLFLKGAYGDGYRLTLVKRPAEPGTSQEPGMASSPSGRPQLSNCSE
mABC1 hABC3 hABCR mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR rABC2 rABC2	1161 1071 703 1100 1261 1191 781 1216 1381 1275 870 1313 1500 1391	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQINILLFHHLTVAEHMLFYAQLMGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRRS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLKCCGSPLFLKQQGTYRLTLVKRPAEPGTSQEPGMASSPSGRPQLSNCSE
mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR rABC2 mABC1	1161 1071 703 1100 1261 1191 781 1216 1381 1275 870 1313 1500 1391 954	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRRS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLCCGSPLFIKGAYGDGYRLTLVKRPAEPGTSQEPGMASSPSGRPQLSNCSE
mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR hABC3 hABC3	1161 1071 703 1100 1261 1191 781 1216 1381 1275 870 1313 1500 1391 954 1417	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRS IWDLILKYKPGRTILLSTHHMDEADLIGDRIAIISHGKLCCGSPLFLKGAYGDGYRLTVKRPAEPGTSQEPGMASSPSGRPQLSNCSE
mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABC3 hABC3 hABC3 hABC3 hABC3 hABC3 hABCR rABC2 rABC2	1161 1071 703 1100 1261 1191 781 1216 1381 1275 870 1313 1500 1391 954 1417 1620	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRS IWDLILKYKPGRTILLSTHHMDEADLLGDRIAIISHGKLKCCGSPLFLKGAYGDGYRLTLVKRPAEPGTSQEPGMASSPSGRPQLSNCSE
mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR rABC2 mABC1 hABC3 hABCR hABC3 hABC3	1161 1071 703 1100 1261 1191 781 1216 1381 1275 870 1313 1500 1391 954 1417 1620 1444	PPTSGTVLVGGRDIETSLDAVRQSLGMCPQHNILFHHLTVAEHMLFYAQLKGKSQEEAQLEMEAMLEDTGLH-HKRNEEAQDLSGGMQRKLSVAIAFVGDAKVVILDEPTSGVDPYSRRS IWDLILKYKPGRTILLSTHHMDEADLIGDRIAIISHGKLCCGSPLFLKGAYGDGYRLTVKRPAEPGTSQEPGMASSPSGRPQLSNCSE

π0

				н9
rABC2	1721 KIAVRRV	/AQVLY NN KGY H SMPTYLNSLNN/	AILRANLPKSKGNPAAYGITVTNHP1	NKTSASLS-LDYLLQGTDVVIAIFIIVAMSFVPASFVVFLV
mABC1	1559 KKLLKLTKDTSADRFLSSLGRFMAGLDTKNN	IVKVWF NN KGW H AISSF LN VIN N	AILRANLQKGE-NPSQYGITAFNHP	NLTKQQLSEVALMTTSVDVLVSICVIFAMSFVPASFVVFLI
hABC3	1010 FRASVEGGGFNERCLVAASFRDVGERTV	VNALF NN QAY H SPATA L AVVDNI	LFKLLCGPHASIVVSNFPQP	SALQAAKDQFNEGRKGFDIALNLLFAMAFLASTFSILAV
hABCR	1584 GRIMNVSGGPITREASKEIPDFLKHLETEDN	IIKVWF NN KGW H ALVSFLNVAHN	AILRASLPKDR-SPEEYGITVISQP	NLTKEQLSEITVLTTSVDAVVAICVIFSMSFVPASFVLYLI
		110	H11	H12
rABC2	1816 AEKSTKAKHLQFVSGCNPVIYWLANYVWDMI	NYLVPATCCIIILFVFDLPAYT	PTNFPAVLSLFLLYGWSITPIMYPA	SFWFEVPSSAYVFLIVINLFIGITATVATFLLQLFEHDKDL
mABC1	1678 QERVSKAKHLQFISGVKPVIYWLSNFVWDMC	NYVVPATLVIIIFICFQQKSYV	STNLPVLALLLLYGWSITPLMYP	SFVFKIPSTAYVVLTSVNLFIGINGSVATFVLELFTNNK-L
hABC3	1121 SERAVQAKHVQFVSGVHVASFWLSALLWDLI	SFLIPSLLLLVVFKAFDVRAFT	DGHMADTLL LLLYGW AIIPLMYL	NFFFLGAATAYTRL/TIFNILSGIATFLM/TIMRIPA/KL
habcr	1703 QERVNKSKHLQFISGVSPTTYWVTNFLWDIM	INYSVSAGLVVGIFIG F QKKAYTS	PENLPALVALLLLYGWAVIPMMYP	SFLFDVPSTAYVALSCANLFIGINSSAITFILELFDNNRTL
	H13		H14	
rABC2	1936 KVVNSYLKSCFLIFPNYNLGHGLMEIAYNE-	YINEYYAKIGQFDH	MKSPFEWDIVTRGLVAMTVEGF	GFFLTIMCQYNFLRQPQRLPVSTKPV-ED
mABC1	1797 NDINDILKSVFLIFPHFCLGRGLIDMVKNQ-	AMADALERFGENRI	V-SPLSWDLVGRNLFAMAVEGV	FFLITVLIQYRFFIRPRPVKAKLPPLNDE
hABC3	1239 EELSKTLDHVFLVLPNHCLGMAVSSFYENYE	TRRYCTSSE-VAAHYCKKYNIQ)	QENFYAWSAPGVGRFVASMAASGCA	YLILLFLIETNLLQRLRGILCALRRRRTLTELYTRMPVLPE
habcr	1823 LRFNAVLRKLLIVFPHFCLGRGLIDLALSQ-	AVTDVYARFGEEHS	-ANPFHWDLIGKNLFAMVVEGV	YFLLTLLVQRHFFLSQWIAEPTKEPIVDE
			Walker A	
rABC2				TTGGEAFVNGHSVLKDLLQVQQSLGYCPQFDALFDEL/TARE
mABC1				VTRGDAFLNKNSILSNIHEVHQNMGYCPQFDAITELLTGRE
hABC3				LTSGDAFVGGHRISSDVGKVRQRIGYCPQFDALLDHMTGRE
hABCR	1918 DDDVAEERQRIITGGNKTDILRLHELTK	IYLGTSSPAVDRLCVGVRPG	ECFGLLGVNGAGKTTTFKMLTGDT1	VTSGDATVAGKSILTNISEVHQNMGYCPQFDAIDELLTGRE
			Walker B	
rABC2				NLILDLIKTGRSVVL TSHSMEECEAVCTRLAIMV NGRLRCL
mABC1	2006 HVEFFALLRGVPEKEVGKFGEWAIRKLGLVK	YGEKYASN YSGGNKRKLST AM AI	IGGPPVVFLDEPTTGMDPKARRFLM	NCALSIVKEGRSVVL TSHSMEECEALCTRMAIMVNG RFRCL
hABC3	1476 MLVMYARLRGIPERHIGACVENTLRGLLLEP	HANKLVRT YSGGNKRKLST GI AI	IGEPAVIFLDEPSTGMDPVARRLLM	DTVARARESGKAIIITSHSMEECEALCTRLAIMVQGQFKCL
hABCR	2032 HLYLYARLRGVPAEEIEKVANWSIKSLGLTV	YADCLAGT YSGGNKRKLST AI AI	IGCPPLVLLDEPTTGMDPQARRMLM	NVIVSIIRKGRAVVLTSHSMEECEALCTRLAIMVKGAFRCM
rABC2	2268 GSIQHLKNRFGDGYMITVRTKSSQNV	KDVVR F FNRN FP EAMLKER H HTK	VQYQLKSEHI SLA QV F SKMEHVVGV	LGIED YSV SQTTLDN VF VN FA KKQSDNVEQQEAEPSTLP S P
mABC1	2126 GSVQHLKNRFGDGYTIVVRIAGSNPDL	KPVQEFFGLAFPGSVLKEKHRNM	LQYQLPSSLS SLA RI F SILSQSKKR	LHIEDYSVSQTTLDQVFVNFAKDQSDD
hABC3	1596 GSPQHLKSKFGSGYSLRAKVQSEGQQEAL	EEFKAFVDLTFPGSVLEDEHQGM	VHYHLPGRDLSWAKVFGILEKAKEK	YGVDDYSVSQISLEQVFLSFAHLQPPTAEEGR*1704
hABCR	2152 GTIQHLKSKFGDGYIVTMKIKSPKDDLLPDL	NPVEQ FF QGN FP GSVQRER H YNM	LQFQVSSS SLA R IF QLLLSHKDS	LLIEEYSVTQTTLDQVFVNFAKQQTESHDLPLHPRAAGASR
rABC2	2383 LGLLSLLRPRPAPTELRALVADEPEDLDTED			
mABC1	2228DHLKDLSLHKNQTV-VDV	AV L T SF LQD E KVKE S YV*226	1	
hapon	2270 0300+ 2272			

hABCR 2270 QAQD*....2273

Figure 1 Comparison of the amino acid sequences of rat ABC2, mouse ABC1, human ABC3 and human ABCR

Amino acids are indicated in single-letter code. Identical amino acid residues among these proteins are shown in **bold** type. Predicted hydrophobic segments (H1–H14) and consensus Walker A and Walker B motifs conserved among these proteins are indicated. It is not known which of these hydrophobic segments actually span the membrane (see the text). Potential N-linked glycosylation sites in the region upstream from H1, between H1 and H2, and between H8 and H9 in ABC2 are indicated by closed circles. The deduced 60-amino-acid sequence of the N-terminus of human ABC1 is identical with that of the mouse protein, except for the fourth amino acid residue, which is tryptophan in human ABC1 but is uncertain (shown as X) in the mouse protein.

amino acid residue, which is tryptophan in human ABC1 but is unknown in the mouse sequence. In addition, the nucleotide sequence downstream from the newly identified ATG of human ABC1 is highly similar to that of mouse ABC1, while upstream it is quite different (results not shown), indicating that the newly identified ATG is the first translation start site of human ABC1.

Characterization of ABC2 protein

To characterize the ABC2 protein, the HA epitope (YPYD-VPDYA) was introduced into the C-terminus of the protein, and immunoblot analysis was performed using anti-HA antibody. The total membrane fraction of COS-1 cells transfected with ABC2 expression vector having an HA tag (pcMVrABC2-HA) revealed two bands of molecular mass 260 kDa and 250 kDa (Figure 2, lane 3), while those transfected with pCMV vector alone showed no band (Figure 2, lanes 1 and 2). Next, to determine if these proteins are glycosylated, the membrane protein was incubated with N-glycosidase F. After treatment with N-glycosidase F for 3 h at 37 °C, the mobilities of the two proteins increased, and a single band, of molecular mass slightly less than 250 kDa, was observed (Figure 2, lane 4), indicating that ABC2 is a glycoprotein.

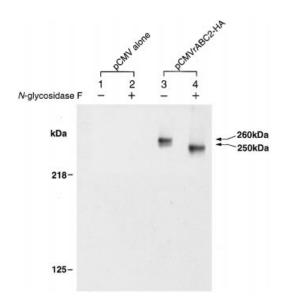
To determine if the expressed ABC2 is functional, we examined its ATP-binding ability. Membrane proteins from COS-7 cells transiently expressing ABC2–HA fusion protein were incubated with 50 μ M 8-azido-[α -³²P]ATP for 10 min at 0 °C in the presence or absence of excess ATP (2 mM). The proteins were then irradiated with UV light, and ABC2–HA fusion protein was precipitated with anti-HA antibody. As shown in Figure 3 (lane 3), ABC2–HA fusion protein was photoaffinity-labelled as proteins of 260 and 250 kDa in the presence of Mg²⁺. The photoaffinity labelling was strongly inhibited in the absence of Mg²⁺ (Figure 3, lane 4) and in the presence of excess ATP (Figure 3, lanes 5 and 6). No photoaffinity-labelled protein was observed in membrane proteins from untransfected COS-7 cells (Figure 3, lanes 1 and 2). These results suggest that 8-azido-[α -³²P]ATP binds to the ABC2 expressed in COS-7 membranes in the presence of Mg²⁺.

Tissue distribution of ABC2 mRNA

An RNA blotting study revealed a single 10.5-kb transcript of ABC2 that was expressed at high levels in the brain, at moderate levels in the heart, kidney and lung, and at low levels in the skeletal muscle, stomach, spleen, colon and pancreas (Figure 4). No band was detected in the liver or small intestine. These results are consistent with a previous report on the distribution pattern of ABC2 mRNA in mouse tissues [13], except that an ABC2 transcript was detected in the liver, but not in the spleen, in the mouse.

Expression of ABC2 mRNA in the brain

Because ABC2 mRNA is most abundant in the brain, we further examined the expression levels in rat brain by *in situ* hybridization



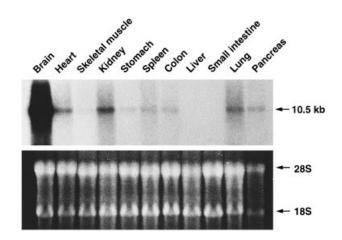


Figure 4 RNA blot analysis of ABC2 mRNA in various rat tissues

Figure 2 Immunoblot analysis of ABC2 protein expressed in COS-1 cells

Membrane proteins prepared from COS-1 cells transfected with pCMV vector alone (lanes 1 and 2) or pCMVrABC2-HA (lanes 3 and 4) were electrophoresed on a 7%-polyacrylamide gel in the presence of SDS. Before electrophoresis, proteins were incubated in the absence (lanes 1 and 3) or the presence (lanes 2 and 4) of N-glycosidase F for 3 h at 37 °C. For immunoblot analysis, 1:1000-diluted anti-HA rat antibody and 1:800-diluted horseradish peroxidase-conjugated anti-rat IgG (Biosys) were used as the primary and secondary antibodies respectively. Proteins were detected using an enhanced chemiluminescence system. The molecular masses of marker proteins are indicated on the left, and the detected 260-kDa and 250-kDa bands are indicated by arrows.

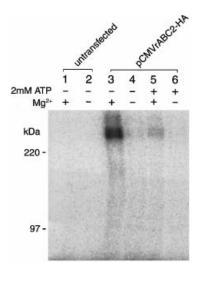


Figure 3 Photoaffinity labelling of ABC2 with 8-azido- $[\alpha$ -³²P]ATP

Membrane proteins (50 µg) from untransfected COS-7 cells (lanes 1 and 2) or COS-7 cells expressing ABC2–HA fusion protein (lanes 3–6) were incubated with 50 µM 8-azido-[α - 32 P]ATP in the presence of Mg²⁺ (lanes 1, 3 and 5) or EDTA (lanes 2, 4 and 6) for 10 min at 0 °C, followed by UV irradiation. For samples in lanes 5 and 6, reactions were carried out in the presence of 2 mM ATP. ABC2–HA fusion protein was immunoprecipitated by anti-HA antibody.

histochemistry (Figure 5). The signals for ABC2 mRNA were detected intensely in the white matter of the corpus callosum, anterior commisure, stria medullaris, optic tract, longitudinal fasciculus pons and medulla, as well as the granular layer of the

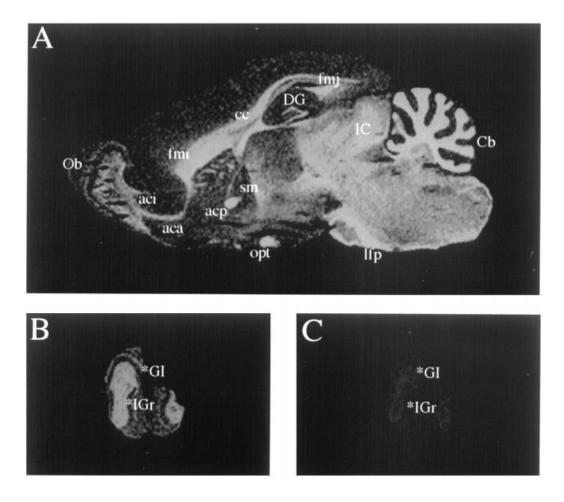
Portions of 20 μ g of total RNA from various rat tissues were denatured and electrophoresed in a 1% (w/v) agarose gel, blotted on a nylon membrane, and hybridized with ³²P-labelled rat ABC2 cDNA. For autoradiography, the nylon membrane was exposed to X-ray film with an intensifying screen at -80 °C for 4 days. The ethidium bromide-stained gel before transfer is shown below the autoradiograph. The sizes of the hybridizing transcript and of 28 S and 18 S rRNA markers are indicated.

cerebellum, and moderately in the glomerular layer and internal granular layer of the olfactory bulb, the dentate gyrus of the hippocampus, the inferior colliculus and the brain stem. Many silver grains of the ABC2 mRNA signal were found over the oligodendrocytes.

DISCUSSION

In the present study we have isolated the full-length cDNA for rat ABC2, and have characterized the molecular properties of the protein. Rat ABC2 is a glycosylated protein of 2434 amino acids that has 40–45 % amino acid identity with other members of the ABC1 subfamily, including mouse ABC1 [13], human ABC3 [14] and human ABCR [15]. In addition, we have shown that ABC2 is most abundant in the brain, and that, in the brain, ABC2 mRNA is generally expressed in the white matter and is absent from the grey matter and the glia limitans. Almost all of these features are quite similar to the gene expression pattern of myelin basic protein, but dissimilar to that of glial fibrillary acidic protein [31,32]. This suggests that ABC2 gene expression in white matter can be ascribed to the oligodendrocytes, and that ABC2 may be involved in the regulation of myelin formation and/or synthesis, although it is possible that ABC2 is also expressed in astrocytes, microglias or neurons.

Furthermore, we isolated the cDNA encoding the N-terminal 60 amino acids of human ABC1 that were missing from the previously reported sequence [13,30]. Alignment of the amino acid sequences of human and mouse ABC1 [13,29], rat ABC2, human ABC3 [14], and human [15], bovine [16] and mouse [17] ABCR indicates that the approx. 40-amino-acid sequence at the N-terminus, including the first hydrophobic segment (H1), is strongly conserved among all the members of ABC1 subfamily – this feature, as well as the existence of H8, uniquely distinguishes the ABC1 subfamily from other subfamilies of the ABC transporter superfamily [13,15]. Hydropathy analysis predicts that at least 14 hydrophobic segments (H1–H14 in Figure 1), seven in the first MSD (H1–H7) and seven in the second MSD (H8–H14), are conserved among ABC1, ABC2, ABC3 and ABCR. However, since the membrane topology of the ABC1



871

Figure 5 Expression of ABC2 mRNA in rat brain

Localization of ABC2 mRNA in rat brain by *in situ* hybridization with antisense (**A** and **B**) and sense (**C**) ³⁵S-labelled cRNA probes. (**A**) X-ray film autoradiogram of the signals for ABC2 mRNA in a sagittal brain section. Signals were densely localized in the forceps minor and major corpus callosum (fmi, fmj), the corpus callosum (cc), the dentate gyrus (DG), the anterior (aca), intrabulbar (aci) and posterior (acp) parts of the anterior commissure, the stria medullaris (sm), the optic tract (opt), the longitudinal fasciculus pons (lfp), the inferior colliculus (IC), and the granular layer and the medulla of the cerebellum (Cb). (**B**) Signals of ABC2 mRNA in the cross-section of the olfactory bulb, which were dense in the glomerular layer (GI) and the internal granular layer (IGr). (**C**) A control section was hybridized with a sense cRNA probe for ABC2. No specific labelling is visible in the olfactory bulb.

subfamily has not been determined, it is not known which of these hydrophobic segments actually spans the membrane. The number of transmembrane segments in the second MSD should be even, because the second MSD is located between the two NBDs. Illing et al. [16] have proposed a model for ABCR in which there are two large extracellular loops, between H1 and H2 and between H8 and H9. By contrast, according to the model proposed for ABC3 by Klugbauer and Hofmann [14], both H1 and H8 are not in the membrane and, therefore, there is a large N-terminal domain and a large loop following the first NBD on the cytoplasmic side. As proposed for ABC1 and ABCR by Luciani et al. [13] and by Azarian and Travis [17] respectively, H8 may not span the whole length of the membrane, but only dip into or cross the membrane twice on the cytoplasmic side. In the present study, we show that ABC2 is a glycoprotein. In ABC2 there is one N-linked glycosylation site at the N-terminus (Asn-14), and clusters of putative N-linked glycosylation sites in the large loops between H1 and H2 (16 sites) and between H8 and H9 (six sites), as in other members of the ABC1 subfamily. Determination of the N-linked glycosylation sites should provide important insight into the membrane topology of the ABC1 subfamily.

Mutation in the ced-7 gene is known to block the engulfment of cell corpses during programmed cell death in C. elegans. Recently, Wu and Horvitz [23] identified the ced-7 gene, and showed that the structure of the CED-7 protein is similar to that of members of the ABC1 subfamily. Since mosaic analysis has revealed that CED-7 functions in both dying cells and engulfing cells during the engulfing process, these authors propose that CED-7 functions to translocate molecules that mediate homotypic adhesion between the cell surfaces of the dying cell and the engulfing cell [23]. Luciani and Chimini [33] have suggested that ABC1 might be a mammalian homologue of ced-7, by showing that the expression of ABC1 during embryonic development correlates spatially and temporally with the areas of programmed cell death, and that the abilities of macrophages to ingest apoptotic cell bodies are severely impaired after blockade of ABC1 by injected antibody against this protein [33]. Recently, however, ABC1 has been shown to be the causal gene of Tangier disease [18–20], the familial deficiency of high-density lipoprotein. It was suggested that ABC1 functions as a flippase for cholesterol from the inner leaflet of the lipid bilayer to the outer one, and that the flipped cholesterol is then removed by the major highdensity lipoprotein, apolipoprotein A-I [34]. In addition,

mutations of the ABCR gene are responsible for Stargardt's disease [15], a recessive form of macular degeneration, and a study using ABCR knockout mice has revealed that ABCR might function as a flippase for protonated *N*-retinylidene-phosphatidylethanolamine [22]. We have shown that ABC2 is strongly expressed in the white matter of the brain. Therefore ABC2, which is homologous to ABC1 and ABCR, may also function as a flippase for lipids or phospholipids, and/or may be involved in the engulfment of cell corpses during programmed cell death in mammalian cells, especially in the brain. Further studies are needed to identify the cells expressing ABC2, as well as to characterize of the function of this transporter protein.

We are grateful to K. Yamada and T. Furukawa for useful discussions, and to S. Seino (Chiba University, Japan) for providing us with the rat brain cDNA library. This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture, Japan, a grant provided by the Ichiro Kanehara Foundation, and a grant from the Japan Foundation of Cardiovascular Research.

REFERENCES

- Higgins, C. F. (1992) ABC transporters: from microorganism to man. Annu. Rev. Cell Biol. 8, 67–113
- 2 Allikmets, R., Gerrard, B., Hutchinson, A. and Dean, M. (1996) Characterization of the human ABC superfamily: isolation and mapping of 21 new genes using the Expressed Sequence Tags database. Hum. Mol. Genet. 5, 1649–1655
- 3 Dean, M. and Allikmets, R. (1995) Evolution of ATP-binding cassette transporter genes. Curr. Opin. Genet. Dev. 5, 779–785
- 4 Gottesmann, M. M. and Pastan, I. (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu. Rev. Biochem. 62, 385–427
- 5 Smit, J. J. M., Schinkel, A. H., Oude Elferink, R. P. J., Groen, A. K., Wagenaar, E., van Deemter, L., Mol, C. A. A. M., Ottenhoff, R., van der Lugt, N. M. T., van Roon, M. A. et al. (1993) Homozygous disruption of the murine *mdr2* P-Glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell **75**, 451–462
- 6 Sheppard, D. N. and Welsh, M. J. (1999) Structure and function of the CFTR chloride channel. Physiol. Rev. 79, 23–45
- 7 Inagaki, N., Gonoi, T., Clement, IV, J. P., Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S. and Bryan, J. (1995) Reconstitution of I_{KATP}: an inward rectifier subunit plus the sulfonylurea receptor. Science **270**, 1166–1170
- 8 Inagaki, N., Gonoi, T., Clement, IV, J. P., Wang, C.-Z., Aguilar-Bryan, L., Bryan, J. and Seino, S. (1996) A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K⁺ channels. Neuron **16**, 1011–1017
- 9 Mosser, J., Douar, A.-M., Sarde, C.-O., Kioschis, P., Feil, R., Moser, H., Poustka, A.-M., Mandel, J.-L. and Aubourg, P. (1993) Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. Nature (London) **361**, 726–730
- 10 Thomas, P. M., Cote, G. J., Wohllk, N., Haddad, B., Mathew, P. M., Rabl, W., Aguilar-Bryan, L., Gagel, R. F. and Bryan, J. (1995) Mutations in the sulfonylurea receptor gene in familial persistent hyperinsulinemic hypoglycemia of infancy. Science 268, 426–429
- 11 Paulusma, C. C., Bosma, P. J., Zaman, G. J. R., Bakker, C. T. M., Otter, M., Scheffer, G. L., Scheper, R. J., Borst, P. and Oude Elferink, R. P. J. (1996) Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. Science 271, 1126–1128
- 12 Strautnieks, S. S., Bull, L. N., Knisely, A. S., Kocoshis, S. A., Dahl, N., Arnell, H., Sokal, E., Dahan, K., Childs, S., Ling, V. et al. (1998) A gene encoding a liverspecific ABC transporter is mutated in progressive familial intrahepatic cholestasis. Nat. Genet. **20**, 233–238
- 13 Luciani, M. F., Denizot, F., Savary, S., Mattei, M. G. and Chimini, G. (1994) Cloning of two novel ABC transporters mapping on human chromosome 9. Genomics 21, 150–159

Received 14 March 2000/6 June 2000; accepted 12 July 2000

- 14 Klugbauer, N. and Hofmann, F. (1996) Primary structure of a novel ABC transporter with a chromosomal localization on the band encoding the multidrug resistanceassociated protein. FEBS Lett. 391, 61–65
- 15 Allikmets, R., Singh, N., Sun, H., Shroyer, N. F., Hutchinson, A., Chidambaram, A., Gerrard, B., Baird, L., Stauffer, D., Peiffer, A. et al. (1997) A photoreceptor cell-specific ATP-binding transporter gene (*ABCR*) is mutated in recessive Stargardt macular dystrophy. Nat. Genet. **15**, 236–246
- 16 Illing, M., Molday, L. L. and Molday, R. S. (1997) The 220-kDa Rim protein of retinal rod outer segments is a member of the ABC transporter superfamily. J. Biol. Chem. 272, 10303–10310
- 17 Azarian, S. M. and Travis, G. H. (1997) The photoreceptor rim protein is an ABC transporter encoded by the gene for recessive Stargardt's disease (ABCR). FEBS Lett. 409, 247–252
- 18 Brooks-Wilson, A., Marcil, M., Clee, S. M., Zhang, L.-H., Roomp, K., van Dam, M., Yu, L., Brewer, C., Collins, J. A., Molhuizen, H. O. F. et al. (1999) Mutations in *ABC1* in Tangier disease and familial high-density lipoprotein deficiency. Nat. Genet. **22**, 336–345
- 19 Bodzioch, M., Orsó, E., Klucken, J., Langmann, T., Böttcher, A., Diederich, W., Drobnik, W., Barlage, S., Büchler, C., Porsch-Özcürümez, M. et al. (1999) The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. Nat. Genet. 22, 347–351
- 20 Rust, S., Rosier, M., Funke, H., Real, J., Amoura, Z., Piette, J.-C., Deleuze, J.-F., Brewer, H. B., Duverger, N., Denèfle, P. and Assmann, G. (1999) Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. Nat. Genet. **22**, 352–355
- Higgins, C. F. (1994) Flip-flop: the transmembrane translocation of lipids. Cell 79, 393–395
- 22 Weng, J., Mata, N. L., Azarian, S. M., Tzekov, R. T., Birch, D. G. and Travis, G. H. (1999) Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in *abcr* knockout mice. Cell **98**, 13–23
- 23 Wu, Y.-C. and Horvitz, H. R. (1998) The *C. elegans* cell corpse engulfment gene *ced-*7 encodes a protein similar to ABC transporters. Cell **93**, 951–960
- 24 Laing, N. M., Belinsky, M. G., Kruh, G. D., Bell, D. W., Boyd, J. T., Barone, L., Testa, J. R. and Tew, K. D. (1998) Amplification of the ATP-binding cassette 2 transporter gene is functionally linked with enhanced efflux of estramustine in ovarian carcinoma cells. Cancer Res. **58**, 1332–1337
- 25 Inagaki, N., Yoshida, H., Mizuta, M., Mizuno, N., Fujii, Y., Gonoi, T., Miyazaki, J.-I. and Seino, S. (1994) Cloning and functional characterization of a third pituitary adenylate cyclase-activating polypeptide receptor subtype expressed in insulinsecreting cells. Proc. Natl. Acad. Sci. U.S.A. **91**, 2679–2683
- 26 Suzuki, M., Fujikura, K., Inagaki, N., Seino, S. and Takata, K. (1997) Localization of the ATP-sensitive K⁺ channel subunit Kir6.2 in mouse pancreas. Diabetes 46, 1440–1444
- 27 Ueda, K., Inagaki, N. and Seino, S. (1997) MgADP antagonism to Mg²⁺-independent ATP binding of the sulfonylurea receptor SUR1. J. Biol. Chem. **272**, 22983–22986
- 28 Shioda, S., Shuto, Y., Somogyvari-Vigh, A., Legradi, G., Onda, H., Coy, D. H., Nakajo, S. and Arimura, A. (1997) Localization and gene expression of the receptor for pituitary adenylate cyclase-activating polypeptide in the rat brain. Neurosci. Res. 28, 345–354
- 29 Kozak, M. (1991) An analysis of vertebrate mRNA sequences: intimations of translational control. J. Cell Biol. 115, 887–903
- 30 Langmann, T., Klucken, J., Reil, M., Liebisch, G., Luciani, M.-F., Chimini, G., Kaminski, W. E. and Schmitz, G. (1999) Molecular cloning of the human ATP-binding cassette transporter 1 (hABC1): evidence for sterol-dependent regulation in macrophages. Biochem. Biophys. Res. Commun. 257, 29–33
- 31 Lewis, S. A. and Cowan, N. J. (1985) Temporal expression of mouse glial fibrillary acidic protein mRNA studied by a rapid in situ hybridization procedure. J. Neurochem. 45, 913–919
- 32 Higgins, G. A., Schmale, H., Bloom, F. E., Wilson, M. C. and Milner, R. J. (1989) Cellular localization of 1B236/myelin-associated glycoprotein mRNA during rat brain development. Proc. Natl. Acad. Sci. U.S.A. 86, 2074–2078
- 33 Luciani, M.-F. and Chimini, G. (1996) The ATP binding cassette transporter ABC1 is required for the engulfment of corpses generated by apoptotic cell death. EMBO J. 15, 226–235
- 34 Lawn, R. M., Wade, D. P., Garvin, M. R., Wang, X., Schwartz, K., Gordon Porter, J., Seilhamer, J. J., Vaughan, A. M. and Oram, J. F. (1999) The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. J. Clin. Invest. **104**, 25–31