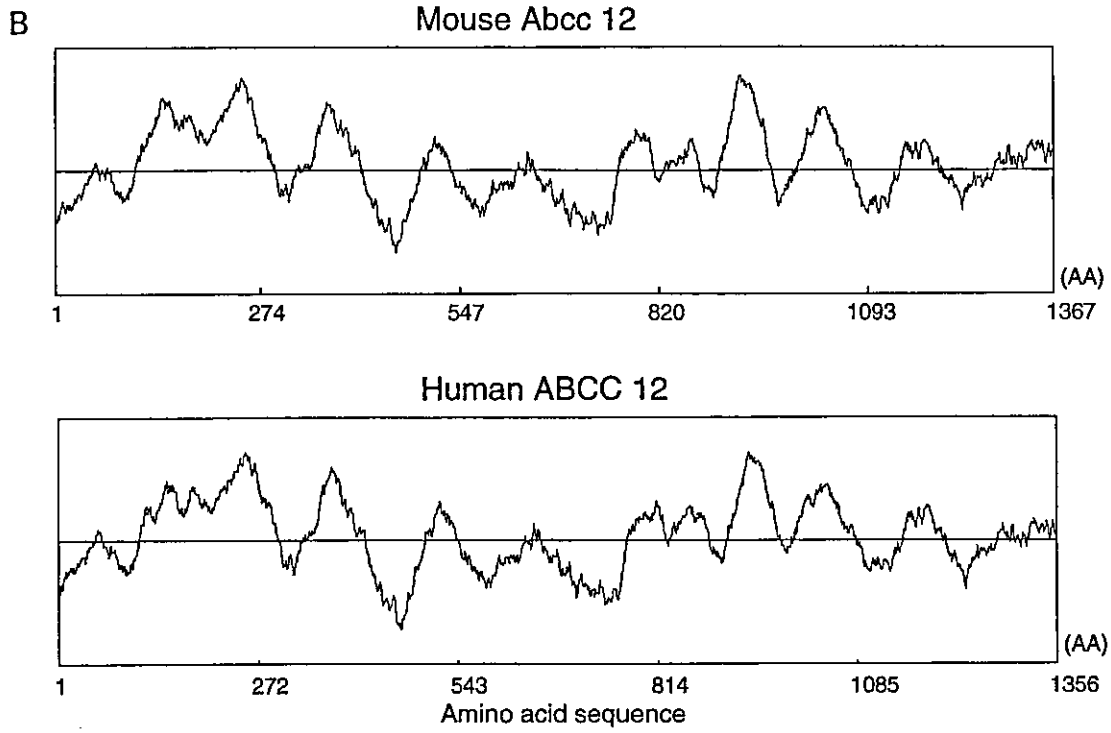

Fig. 2 (*continued*)

then rinsed with 0.1% DEPC-treated water. The following biotin-labeled oligonucleotide DNA probes were synthesized, i.e. the sense probe: 5′-AGCCTGACTCTGCATTTGCGATGTTACTAGCTGCAG-3′ and the anti-sense probe: 5′-CTGCAGCTAGTAACATCGCAAATGCAGAGTCAGGCT-3′. The probes were diluted in the DAKO in situ hybridization solution (DAKO S3304) at a final concentration of 1 ng/ml. The hybridization with the sense or the anti-sense probe was carried out on the thin section at 37 °C overnight. Thereafter, the slides were incubated in 0.1× SSC (300 mM sodium chloride and 1.5 mM sodium citrate, pH 7.0) at 37 °C for 20 min twice and washed in TBS at room temperature for 3 min.

In situ hybridization signals were visualized by a tyramide amplification signal detection system using the DAKO GenPoint system (DAKO K0620), according to manufacturer's instructions. Finally, the sections were counterstained with Mayer's hematoxylin (Sigma, USA).

2.6. Data analysis

DNA sequences were analyzed with the GENETYX-MAC software ver.11 and compared with other ABCC transporter genes registered in the NCBI database. The hydropathy profile of the protein deduced from the cDNA sequence was calculated with the Kyte and Doolittle hydropathy algorithm (Kyte and Doolittle, 1982), and the SOSUI program (http://sosui.proteome.bio.tuat.ac.jp/sosuimenu0.html) was used to predict transmembrane domains. Phylogenic relationships were calculated by using the distance-based neighbor-joining method (Saitou and Nei, 1987).

3. Results

3.1. Cloning and characterization of mouse Abcc 12 cDNA

Fig. 1 depicts the strategy of cloning mouse Abcc12 cDNA. The sequence of human ABCC12 cDNA was applied to the currently available mouse EST database on an NCBI BLAST search to discover ESTs encoding partial sequences of mouse Abcc 12. Thereby, the following EST clones were extracted: BB616859, BB615294, AI427812, AI614586, BE864084, AW060464, BB013432, BB014467, BB717705, and BB209897. In addition, in a search of the FANTOM 2 database of RIKEN, we found one cDNA clone (ID number = 4932443H13) that exhibited a high sequence homology with human ABCC12 cDNA. Based on those ESTs as well the partial cDNA clone (ID = 4932443H13), we designed four sets of PCR primers to clone the mouse Abcc12 cDNA (see Section 2 for experimental details). By PCR, we obtained a total of four cDNA fragments (Fig. 1) and assembled them to construct the full cDNA encoding mouse Abcc12.

Table 1
Amino acid sequence identity of the mouse Abcc 12 with human ABC proteins in the ABCC sub-family

ABC protein	Identity (%)
ABCC1	33.4
ABCC2	32.1
ABCC3	31.1
ABCC4	40.2
ABCC5	43.7
ABCC6	28.5
CFTR (ABCC7)	27.9
ABCC8	30.6
ABCC9	27.9
ABCC10	34.6
ABCC11	47.8
ABCC12	84.5

The amino acid sequences of human ABC proteins were acquired from the NCBI database (refer to the accession numbers given in the legend of Fig. 3).

3.2. Characterization of mouse Abcc12 cDNA in comparison with members of the human ABCC sub-family

The cloned mouse Abcc12 cDNA (GenBank accession number: AF502146) was 4511 bp long, containing a 4101 bp open reading frame (ORF). The Abcc12 cDNA has a Kozak consensus initiation sequence (Kozak, 1991) for translation around the first ATG region, namely, 5′-ATCAAGATGG-3′. The amino acid sequence deduced from the cDNA sequence with the GENETYX-MAC program revealed that the cDNA encodes a single peptide consisting of 1366 amino acid residues (Fig. 2A). Motif analysis predicted the existence of two sets of ATP-binding cassettes (Walker et al., 1982): namely, Walker A (amino acids 514–521 and 1161–1168), Walker B (amino acids 624–628 and 1284–1288), and signature C motifs (amino acids 604–618 and 1264–1278) (Fig. 2A). Fig. 2B shows the hydropathy plots of mouse Abcc12 and human ABCC12, demonstrating a remarkable similarity between these transporters.

Table 1 shows that the amino acid sequence of mouse Abcc12 has the highest identity with human ABCC12 among the hitherto known members of the human ABCC sub-family. The sequence identity of mouse Abcc12 with human ABCC12 was 84.5%, whereas its identity with human ABCC11 was 47.8%. The identity of mouse Abcc12 with other human members was relatively low, in the range of 27.9 to 43.7% (Table 1).

Fig. 3 shows the phylogenic relationship among the members of the human and mouse ABCC subfamily. Mouse Abcc12, as well as human ABCC12, apparently belongs to a cluster named 'Class D' that comprises ABCC4, ABCC5, ABCC11, Abcc4, and Abcc5 (see Section 4 for the classification).

3.3. Chromosomal location of the mouse Abcc 12 gene

Fig. 4 shows the location of the *Abcc12* gene on the mouse chromosome 8. The mouse Abcc12 gene spans a 65 kb length and is located between two microsatellite markers, D8Mit347 and D8Mit348, at the D3 region of the mouse chromosome 8 (Fig. 4), as referred to in the mouse genome databases of NCBI and EMBL/UCSC (Mouse Genome Sequencing Consortium, 2002). This genome region of the mouse chromosome 8 is reportedly related to human chromosome 16q12.1, where the human *ABCC11* and *ABCC12* genes are tandemly located (Yabuuchi et al., 2001; Tammur et al., 2001). Comparison of the cloned

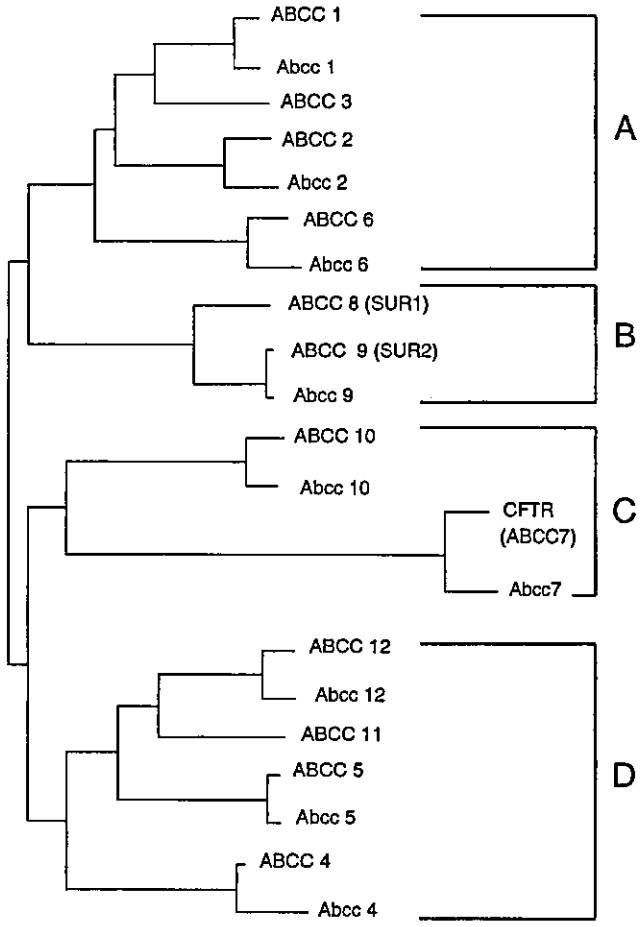

Fig. 3. The phylogenetic relationship among hitherto characterized members of the human and mouse ABCC sub-family. The phylogenetic distance was calculated according to the distance-based neighbor-joining method (Saitou and Nei, 1987). For the sequences of those ABC transporters, accession numbers are as follows: human ABCC1 (NM004996), ABCC2 (NM000392), ABCC3 (Y17151), ABCC4 (NM005845), ABCC5 (NM005688), ABCC6 (NM001171), ABCC7 (NM000492), ABCC8 (NM000352), ABCC9 (NM005691), ABCC10 (AK000002), ABCC11 (AF367202), ABCC12 (NM033226) mouse Abcc1 (NM008576), Abcc2 (NM013806), Abcc4 (D630049P08), Abcc5 (NM013790), Abcc6 (NM018795), Abcc7 (NM021050), Abcc8 (XM133448), Abcc9 (NM011511), Abcc10 (AF406642), Abcc12 (AF502146).

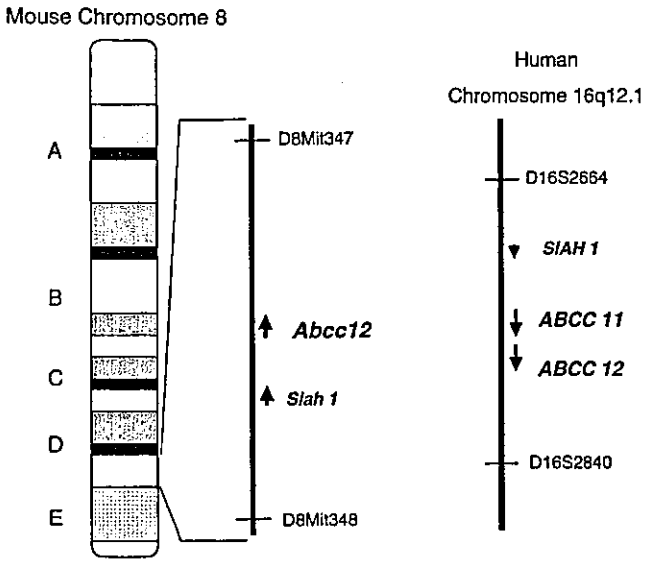

Fig. 4. Location of the *Abcc12* gene on mouse chromosome 8. *Abcc12* and *Siah 1* genes are located in the mouse chromosomal region between two microsatellite markers, D8Mit347 and D8Mit348. Human *ABCC11*, *ABCC12*, and *SIAH 1* genes are located in the region between D16S2664 and D16S2840 on the human chromosome 16q12.1.

Abcc12 cDNA with the mouse genome data revealed that the *Abcc12* gene consists of at least 29 exons, where the translation start codon (ATG) was found in exon 1. In addition, two sets of ATP-binding cassettes were detected. The first Walker A motif is located in the exon 10, whereas the second Walker A motif spreads over exons 24 and 25. Two Walker B motifs are encoded in exons 13 and 28, and two signature C motifs are located in exons 13 and 27.

Table 2 summarizes the exon and intron boundaries with partial sequences at each splicing site of the *Abcc12* gene; however, the partial sequences of the introns proximal to both 5′- and 3′-ends of exon 3 are presently not available (Table 2). These results suggest that the splicings of the mouse Abcc12 gene follow the conventional GT–AG rule, except for the exon 19.

3.4. Tissue-dependent expression of the mouse Abcc 12 gene

Fig. 5A shows the expression levels of the mouse Abcc12 gene in different organs as detected by RT–PCR with two different sets of PCR primers #1 and #2 (see Fig. 1 and Section 2 for details). The products of PCR reactions with primers #1 and #2 were 486 and 288 bp, respectively. Among the organs tested, the highest expression (mRNA) of

Table 2
Partial sequences of intron/exon and exon/intron boundaries in the mouse *Abcc 12* gene

Exon	Size (bp)	Intron/Exon		Exon/Intron	
1	152	tgtcccgaag	CCAAGAGTCG	CCCGTGCAAG	gtgagccagg
2	156	tttgctctag	GTTGGCACCC	ACGCCAAGAG	gtaccaggct
3[a]	147	nnnnnnnn	ATTCCAGATC	CTTGGGGCCG	nnnnnnnn
4	238	tgttttacag	ACAGTTCTCA	TGCAGGCGAG	gtaagcaggg
5	174	ttctttctag	GTACTCAATA	CCCGATCCAG	gtaagttggg
6	148	tcgatttcag	ATGTTTATGG	ACCATTCACG	gtaagatgag
7	149	tcttttgcag	ACATAAGAAA	TGCCCCTGTG	gtaagagtta
8	108	cctccttcag	GCATTTAGTG	GAGAATGAAG	gtataagtaa
9	279	ttaatctcag	AAAATCCTCA	GGTGAGAAAG	gtgagtgtat
10	72	tctctgacag	GGGAAGGTCT	CCTAGGACAG	gtgagtgtgt
11	125	atcgctctag	ATGCAGTTAC	ACCACCAAAG	gtattattaa
12	73	atgtctacag	GTACCAACAC	CCTGACTGAG	gtaagcagag
13	204	ctgtccacag	ATTGGAGAGC	CCAGTTGCAG	gtgactggga
14	135	gtctctgcag	TTCCTGGAGT	GCAATTCAAG	gtaaactgca
15	76	ttatctccag	GATCCAGAGC	GAAGACGCTG	gtacagtcag
16	69	ctcatcctag	TCTTGGCTTC	GACACAAACG	gtatttacca
17	90	gtctctgcag	CTCCCGCTCA	GCTTCTGGAG	gtttagtata
18	104	tctccggcag	GGTACCTGGT	GGGTTCCCAG	gtgagtttcc
19	194	tgtgttgcag	GTCGTCTGTG	GAGTATTTAATA	caaggtagaa
20	229	ttctaccctc	AGATCGTCAG	TTCTTTTACG	gtaggattat
21	138	tcccccacag	CATCTTCCAT	GCATCAGCAA	gtgagtggat
22	187	ttgactttag	GTTTAAGACA	CATCATCCAG	gtaacggctg
23	90	ttcccaacag	CTCAGTGGAT	GTACATTTTG	gtaaggaatg
24	190	tgcttttcag	ACCTGTGTTC	ACGGGCTCCG	gtgaggacag
25	160	ttgtccccag	GAAAATCATC	GTACAGTAAG	gtagctgttt
26	79	ttcgttgcag	GTACAACTTG	GAGAGACACA	gtacgtcttg
27	114	tgttttatag	ATAATGAAAC	TAATTCAAAA	gtaaggaaac
28	165	tccttaacag	ATCATTCTCC	AAATGGGAAG	gtgcaggaaa
29	464	tgattttcag	GTGATTGAGT	CCTGGATTTT	gttaccagac

[a] The partial sequences of the introns proximal to the 5′- and 3′-ends of exon 3 are not available, therefore they are represented by 'nnnn'.

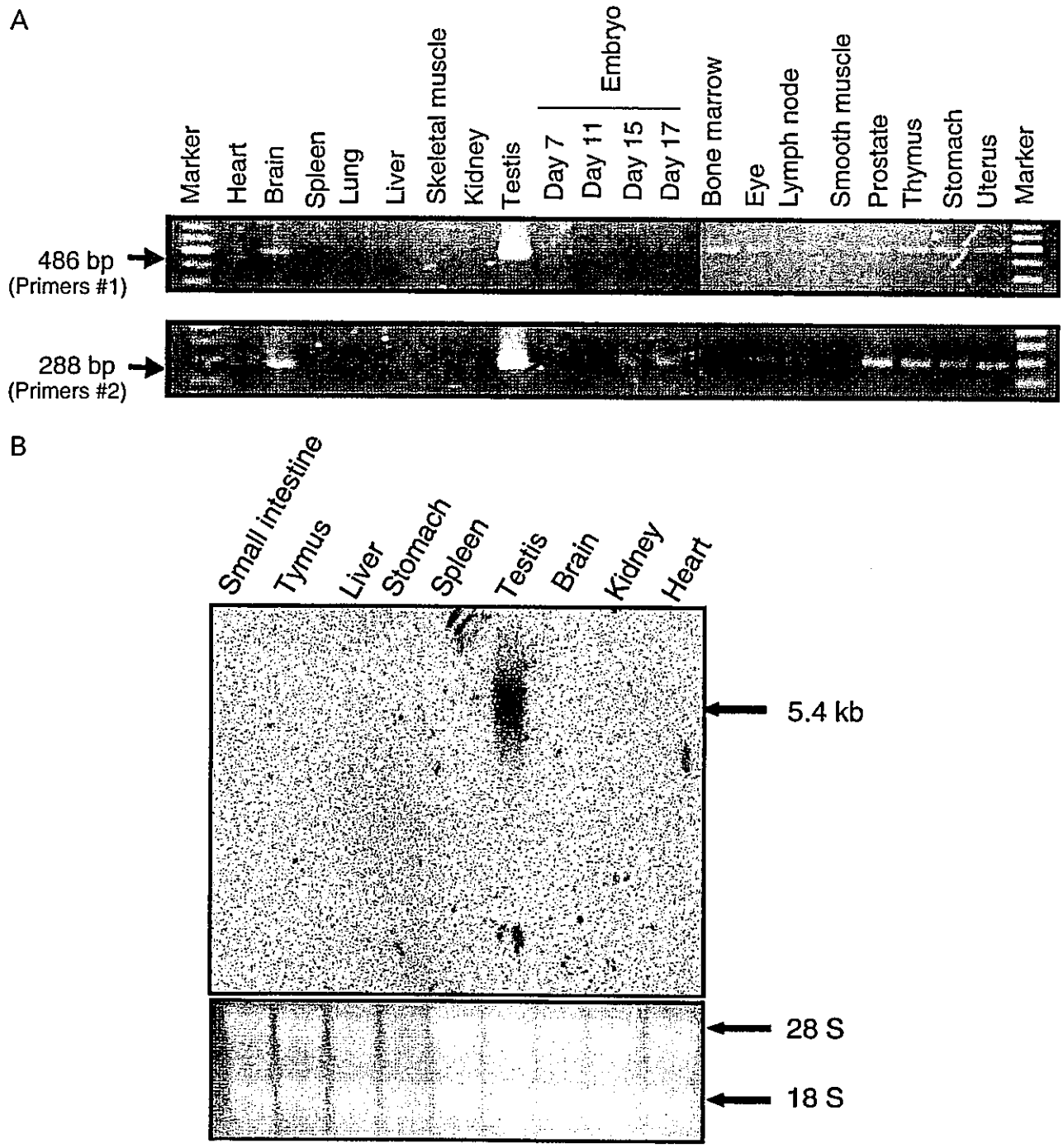

Fig. 5. Expression of the mouse *Abcc12* gene in different tissues. The Abcc12 transcript was detected by PCR (A) and by Northern hybridization (B) as described in Section 2. For the PCR detection (A), two sets of primers (#1 and #2; Fig. 1) were used. The resulting PCR products were 486 and 288 bp, as indicated by arrows. For the Northern hybridization (B), RNA (15 μg/lane) prepared from mouse tissues was fractionated by electrophoresis in 1.0% (w/v) agarose gels and visualized by ethidium bromide (bottom). 18S and 28S rRNAs are indicated by arrows. Northern hybridization (top) with a ^{32}P-labeled probe was carried out as described in Section 2. The detected Abcc12 mRNA (5.4 kb) is indicated by an arrow.

Abcc12 was observed in the testis. Relatively lower expression was detected in the brain, bone marrow, eye, lymph node, prostate, thymus, stomach, and uterus in the adult mouse. Northern blot hybridization (Fig. 5B) clearly demonstrates the predominant expression of mouse Abcc12 in the testis, being consistent with the results of RT–PCR (Fig. 5A). The transcript size of mouse Abcc 12 was about 5.4 kb.

3.5. Localization of mouse Abcc 12 in the testis

To elucidate the expression site of Abcc12 in the mouse testis, we have carried out laser-captured microdissection and RT–PCR. The seminiferous tubules and the interstitium were dissected, and RNA was extracted to prepare cDNA (see Section 2). PCR was carried out with the same primer

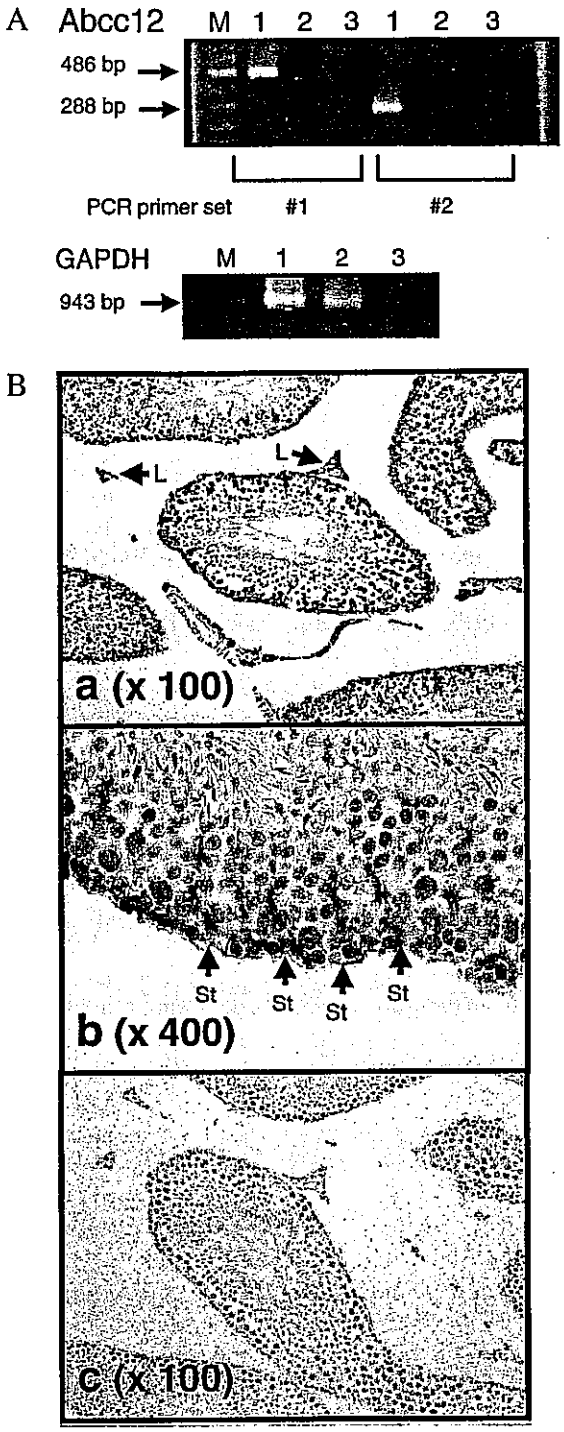

Fig. 6. Detection of the Abcc12 transcript in the mouse testis by means of laser-captured microdissection and RT–PCR (A) as well as by in situ hybridization (B). (A) Abcc12 and GAPDH transcripts were detected by PCR with RT reaction products prepared from micro-dissected samples. For the PCR detection, two sets of primers (#1 and #2; Fig. 1) were used, and the resulting PCR products were 486 and 288 bp, as indicated by arrows. The 943 bp product of GAPDH is the positive control for the PCR reaction. Lane M, DNA size markers; lane 1, seminiferous tubules; lane 2, stroma cells; lane 3, without RT reaction products. (B) The Abcc12 transcript in the mouse testis was detected by in situ hybridization as described in Section 2. Panels a and b show the results of hybridization with the anti-sense probe, whereas panel c shows the negative control, i.e. hybridization with the sense probe. Magnifications are indicated in parentheses. Arrows indicate Leydig (L) and Sertoli (St) cells.

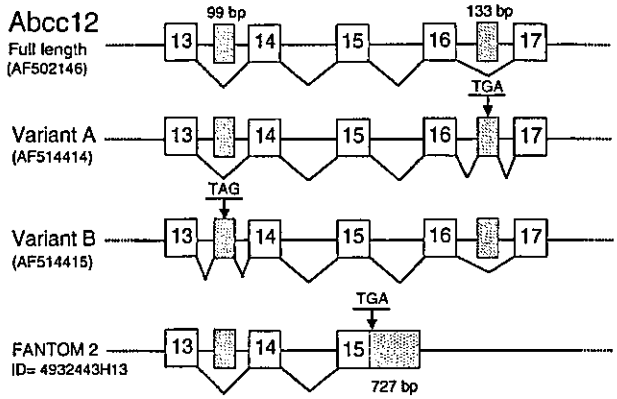

Fig. 7. Schematic illustration for alternative splicing of the *Abcc12* gene. Exons are numbered according to the sequence of the cloned Abcc12 cDNA. Variant A cDNA has one extra exon (133 bp) with a stop codon (TGA) between exons 16 and 17. Variant B cDNA has one extra exon (99 bp) with a stop codon (TAG) between exons 13 and 14. The exon 15 of the FANTOM 2 cDNA (ID = 4932443H13) has a 727 bp extension, as compared with the exon 15 of Abcc12 cDNA.

sets #1 and #2 as described above. As shown in Fig. 6A, Abcc12 expression was exclusively high in the seminiferous tubules, whereas little expression was detected in the interstitium. To gain further insight into cell type-specific expression of the *Abcc12* gene, we carried out in situ hybridization. Fig. 6B depicts the results of the in situ hybridization, demonstrating that the expression of Abcc12 was high in Sertoli cells of the seminiferous tubules (Fig. 6B, panel b). In addition, expression of Abcc12 was also detected in Leydig cells of the interstitium (Fig. 6B, panel a) under our hybridization conditions. No hybridization signal was observed with the sense probe, as the negative control (Fig. 6B, panel c).

3.6. Splicing variants of Abcc12

During the cloning of Abcc12 cDNA in the present study, we have discovered two variant forms of Abcc12 (GenBank accession numbers: AF514414 and AF514415 for variants A and B, respectively). Fig. 7 summarizes the configurations of those variants of the Abcc12 transcript together with the partial cDNA (ID = 4932443H13) reported in the FANTOM 2 database. The cDNAs of both variants A and B consist of 30 exons. As shown in Fig. 7, the variant A cDNA has an extra exon (133 bp) located between exons 16 and 17. Although the variant A cDNA consists of a total of 30 exons, the variant A is considered to encode a short peptide (775 amino acid residues), because the extra exon has a translation stop codon, TGA (Fig. 7). Likewise, the variant B cDNA has one extra exon (99 bp) with a stop codon (TAG) between exons 13 and 14 (Fig. 7), and, therefore, it also encodes a short peptide (687 amino acid residues). On the other hand, the FANTOM 2 cDNA (ID = 4932443H13) cloned by the 5′-oligo-cap method (Carninci et al., 1996) has an extension (121 bp) at the

5′-end of the cDNA, as compared with the cloned Abcc12 cDNA (data not shown). It is noteworthy that the exon 15 of the FANTOM 2 cDNA is different from that of the Abcc12 cDNA cloned in this study, although the other exons 2–14 are identical. Indeed, the exon 15 in the FANTOM 2 cDNA is 727 bp larger than the exon 15 of Abcc12 cDNA, but it encodes a translation stop codon (TGA) in the extended sequence (Fig. 7).

4. Discussion

4.1. Molecular characteristics of mouse Abcc12 cDNA

In the present study, we have cloned and characterized the cDNA of a new mouse ABC transporter, named Abcc12. The cloned cDNA was 4511 bp long and comprised a 4101 bp open reading frame. The deduced peptide consists of 1367 amino acid moieties, carrying two sets of Walker A, Walker B (Walker et al., 1982), and signature C (Higgins, 1992) motifs within the peptide (Fig. 2A). Based on the ATP binding cassettes and the putative trans-membrane spanning domains (Fig. 2B), Abcc12 is regarded as a 'full' ABC protein. The amino acid sequence of the Abcc12 protein deduced from the cloned cDNA exhibits the highest identity (84.5%) to human ABCC12 among all of the members of the ABCC subfamily hitherto identified in the human and the mouse (Table 1 and Fig. 3). Indeed, the hydropathy profile of mouse Abcc12 is virtually the same as that of human ABCC12 (Fig. 2B). From these results, it could be concluded that mouse Abcc12 is the orthologue of human ABCC12. In addition, our data suggest that the cDNA sequence of human ABCC12 (MRP9) recently reported by Bera et al. (2002) may be a splicing variant form, since exons 5 and 16 are missing in their sequence.

Based on the phylogenic relationship deduced from the amino acid sequence identities, the ABCC subfamily could be clustered into four classes (Fig. 3). For example, class A involves human ABCC1, ABCC2, ABCC3, and ABCC6, as well as mouse Abcc1, Abcc2, and Abcc6. These ABC transporters appear to function as conjugate transporters, e.g., GS-X pumps and/or multi-specific organic anion transporters (cMOAT) (Ishikawa, 1992; Borst and Oude Elferink, 2002). On the other hand, class B includes human ABCC8 (SUR1), ABCC9 (SUR2), and mouse Abcc9, which are sulfonylurea receptors coupled with potassium channels, i.e., Kir 6.1 or Kir 6.2. Human CFTR (ABCC7), ABCC10, mouse Abcc7 (mouse CFTR), and Abcc10 are involved in class C. Mutations in the *CFTR* gene are known to be the cause of cystic fibrosis, an autosomal recessive genetic disorder affecting a number of organs, including the lungs, airways, pancreas, and sweat glands (http://www.genet.sickkid.on.ca/cftr/). The physiological function of ABCC10 in this class is not known at the present time.

According to this clustering, the mouse Abcc12 belongs to class D, which involves human ABCC4, ABCC5, ABCC11, and ABCC12, as well as mouse Abcc4 and Abcc5 (Fig. 3). Recent studies demonstrated that human both ABCC4 and ABCC5 transport nucleotide analogues (Schuetz et al., 1999; Wijnholds et al., 2000; Jedlitschky et al., 2000; Chen et al., 2001). ABCC5 reportedly does not confer multidrug resistance when over-expressed in human embryonic kidney 293 cells (McAleer et al., 1999). Because of the similarity of the amino acid sequences, it is assumed that human ABCC11 and ABCC12 are functionally related to ABCC4 or ABCC5.

4.2. Mouse Abcc12 gene: an orthologue of human ABCC12 gene

Our conclusion that mouse Abcc12 is the orthologue of human ABCC12 is supported by similarities in the location and organization of those genes, as well. The present study provides evidence that the open reading frame in the mouse Abcc12 cDNA consists of 29 exons, as does the human ABCC12 cDNA (Yabuuchi et al., 2001; Tammur et al., 2001). In addition, the mouse *Abcc12* and human *ABCC12* genes (29 exons and introns) span 62 and 63 kb, respectively. The mouse *Abcc12* gene is located between two microsatellite markers, D8Mit347 and D8Mit348, on the chromosome 8D3 locus. This locus reportedly contains many conserved linkage homologies with human chromosome 16q12.1 (Serikawa et al., 1998), where the human *ABCC12* gene has recently been discovered (Yabuuchi et al., 2001; Tammur et al., 2001). Being consistent with this idea, the chromosomal location of the mouse *Siah 1* gene and its distance (167 kb) from the Abcc12 gene is conserved in the human chromosome 16q12.1 where both *SIAH 1* and *ABCC12* genes are located. The human *SIAH 1* gene (Hu et al., 1997) encodes a 282-amino-acid protein with 76% amino acid identity to the *Drosophila* SINA protein which is involved in the *ras* signaling pathway to mediate the R7 photoreceptor formation in the *Drosophila* eye (Carthew and Rubin, 1990). *Siah 1a* is one of the mouse orthologue genes and is mapped on the chromosome 8D3 locus (Holloway et al., 1997). Taken together, it is strongly suggested that the mouse *Abcc12* gene is closely related to the human *ABCC12* gene in terms of both the protein structure and the organization of the gene.

It is of importance, however, to note that in spite of the tandem location of both *ABCC11* and *ABCC12* genes on human chromosome 16q12.1, there was no mouse orthologue gene corresponding to the human *ABCC11* at that mouse chromosomal locus. In addition, there was no putative *Abcc11* gene detected even by an extensive search throughout the currently available mouse genome data. Thus, it appears that the *Abcc11* gene is absent from the mouse genome.

4.3. Tissue-specific expression of the mouse Abcc12 gene

We have previously reported that the expression of the

human *ABCC12* gene was widely distributed in various tissues, including testis, brain, liver, lung, kidney, thymus, prostate, ovary, colon, and leukocytes as well as in several fetal tissues (Yabuuchi et al., 2001). In contrast, the present study demonstrates that the mouse *Abcc12* gene is expressed at high levels exclusively in the testis (Fig. 5). The reason for such differences in organ-specific expression profiles between mouse Abcc12 and human ABCC12 is not known, but may be eventually explained by analysis of the promoter regions of those genes.

In the present study, by means of laser-captured microdissection combined with RT–PCR as well as in situ hybridization, the *Abcc12* transcript was detected in Sertoli cells of the seminiferous tubules in the mouse testis (Fig. 6A,B). In addition, in situ hybridization further revealed the expression of the *Abcc12* in Leydig cells, as well (Fig. 6B). Accumulating evidence suggests that the blood-testis barrier plays an important role in protecting the germ cells from harmful influences. To date it has been reported that ABCB1 (P-glycoprotein or MDR1) is expressed in luminal capillary endothelium and on the myoid-cell layer around the seminiferous tubule (Bart et al., 2002), whereas ABCC1 (MRP) is located basolaterally on both Sertoli and Leydig cells (Wijnholds et al., 1998). These ABC transporters are regarded as the first line players in the body's detoxification system. In this context, Abcc12 is also considered to play a role as a member of such a detoxification system, or it may be involved in the transport of endogenous substances in the testis. The physiological function and substrate specificity of Abcc12 remains to be elucidated.

4.4. Concluding remarks

Northern blot analysis revealed that mRNA with a size of 5.4 kb is the major transcript of mouse Abcc12 in the testis (Fig. 5B). In the present study, however, we have detected the existence of at least two splice variants for mouse Abcc12 (Fig. 7). In addition, the results of the FANTOM 2 project (The FANTOM Consortium, 2002) demonstrate that there is another splicing variant form that encodes a shorter peptide of Abcc12 (Fig. 7). These data suggests that mouse Abcc12 is transcribed into multiple forms by means of alternative splicing.

In the previous paper, we demonstrated that the human ABCC12 gene is transcribed into several splice variants (Yabuuchi et al., 2001). Recently, Bera et al. (2002) reported that the human ABCC12 (MRP9) is expressed as two major transcripts of 4.5 and 1.3, and that the 4.5 kb transcript is highly expressed in the epithelial cells of breast cancer. Transcript of the ABCC12 gene were detected in cell lines of carcinoma and adenocarcinoma originating from breast, lung, colon pancreas and prostate, as well (Yabuuchi et al., 2001), suggesting that expression of the ABCC12 gene may be up-regulated during carcinogenesis. Therefore, it is of great interest to study how alternative splicing is regulated in the expression of the human ABCC12 and mouse Abcc12 genes.

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Regular Article

Expression and Functional Characterization of Human ABC Transporter ABCG2 Variants in Insect Cells

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Summary: Hitherto three variant forms of ABCG2 have been documented on the basis of their amino acid moieties (i.e., Arg, Gly, and Thr) at the position 482. In the present study, we have generated those variants of ABCG2 by site-directed mutagenesis and expressed them in Sf9 insect cells. The apparent molecular weight of the expressed ABCG2 variants was 130,000 under non-reductive conditions, whereas it was reduced to 65,000 by treatment with mercaptoethanol. It is suggested that ABCG2 exists in the plasma membrane of Sf9 cells as a homodimer bound through cysteinyl disulfide bond(s). Both ATPase activity and drug transport of ABCG2 variants were examined by using plasma membrane fractions prepared from ABCG2-overexpressing Sf9 cells. The ATPase activity of the plasma membrane expressing ABCG2 (Gly-482) was significantly enhanced by prazosin. In contrast, ABCG2 (Arg-482) transports [^{3}H]methotrexate in an ATP-dependent manner; however, no transport activity was observed with the other variants (Gly-482 and Thr-482). It is strongly suggested that the amino acid moiety at the position of 482 is critical for the substrate specificity of ABCG2.

Key words: ABC transporter; ABCG2; BCRP; single nucleotide polymorphism; pharmacogenomics

Introduction

There is accumulating evidence that individual variations in response to a drug originate from different causes, such as genetic polymorphism and altered expression levels of drug target molecules (e.g., membrane receptors, nuclear receptors, and enzymes) as well as those of drug metabolizing enzymes and drug transporters.[1)] It is critically important that we understand the relationship between the genetic alterations and the molecular mechanisms underlying such variations in drug response. Pharmacogenetic and pharmacogenomic approaches are expected to significantly contribute to the realization of "personalized medicine" in the near future.

Cancer is one of the gene-associated diseases, with multiple factors involved in its cause and progression.[2)] Despite enormous costs and efforts spent on the development of cancer chemotherapies, anticancer drugs are often effective only in a relatively small proportion of cancer patients. It has long been recognized that the effectiveness of anticancer drugs can vary significantly among individual patients. Indeed, acquired and intrinsic drug resistance in cancer is the major obstacle to long-term, sustained patient response to chemotherapy. There is accumulating evidence that active export of anticancer drugs from cancer cells is one of the major mechanisms of drug resistance. Several ATP-binding cassette (ABC) transporters underlie multidrug resistance in cancer cells by actively extruding the clinically administered chemotherapeutic drugs. Two major ABC transporters, ABCB1 (P-glycoprotein or

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MDR1) and ABCC1 (MRP1), have been well studied in terms of their structure and function in cancer drug resistance.[3-8] In addition, a novel ABC transporter, breast cancer resistant protein (BCRP), has recently been discovered in doxorubicin-resistant breast cancer cells.[9] The same transporter has also been found in human placenta[10] as well as in drug-resistant cancer cell selected mitoxantrone and DNA topoisomerase I inhibitors.[11-18] The newly found ABC transporter protein is now named ABCG2 and is classified in the G-subfamily of human ABC transporter genes according to the new nomenclature. The G-subfamily is a so-called "half-transporter" bearing six transmembrane domains and one ATP-binding cassette.

Overexpression of ABCG2 reportedly confers cancer cell resistance to anticancer drugs, such as mitoxantrone, topotecan, and 7-ethyl-10-hydroxycamptothecin (SN-38: active metabolite of irinotecan). SN-38-selected PC-6/SN2-5H human lung carcinoma cells were shown to overexpress ABCG2 with the reduced intracellular accumulation of SN-38 and its glucuronide metabolite.[19] We have recently demonstrated that plasma membrane vesicles prepared from those cells ATP-dependently transported both SN-38 and SN-38-glucuronide, and our results strongly suggested that ABCG2 is involved in the active extrusion of SN-38 and its metabolite from cancer cells.[20]

To date, at least three variant forms of ABCG2 have been documented on the basis of amino acid moieties at position 482, which is located in the third transmembrane domain (**Fig. 1**). The wild type form of ABCG2 has an arginine (Arg) at that position,[10] whereas other variants cloned from cancer cell lines[9,13] have glycine (Gly) and threonine (Thr) at position 482. It is currently speculated that the substrate specificities of ABCG2 may differ among those variant forms.[18,21-23] To elucidate the role of amino acid moieties at position 482 in the transport function, we have expressed each variant form of ABCG2 in Sf9 insect cells and examined the activity of those variants. Using the insect cell system, we provide direct evidence that the human ABCG2 protein functions as a homodimer bound via a cysteinyl disulfide bond(s). Furthermore, in the present study, we demonstrate that Arg-482 is critically involved in the substrate specificity of ABCG2.

Methods

Cloning of human ABCG2 (Arg-482) cDNA: Human ABCG2 cDNA was cloned from mRNA of the MCF7/BCRP clone-8 cell line (Mitomo, H. *et al.*, unpublished work). RT-PCR was carried out by using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and the following specific primers: sense 5′-CTCTCCAGATGTCTTCCAGT-3′ and antisense 5′-ACAGTGTGATGGCAAGGGAAC-3′, where the primers were designed based on the ABCG2 cDNA sequences. The PCR reaction consisted of 30 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 2 min. The resulting PCR product was inserted into the pCR2.1 TOPO vector, and its sequences were analyzed by automated DNA sequencing (TOYOBO Gene Analysis, Tokyo, Japan). The open reading frame of the ABCG2 cDNA was identical to the ABCG2 wild type (Arg-482) originally named ABCP (GenBank accession number: AF103796). The ABCG2 (Arg-482) cDNA was removed from the pCR2.1 TOPO vector by *EcoRI* digestion. After the treatment with alkaline phosphatase, ABCG2 cDNA was ligated to the *EcoRI* site of the pFASTBAC1 Expression vector (Invitrogen) using the Rapid DNA ligation kit (Roche Diagnosis Co., Indianapolis, IN, USA).

Generation of variant forms by site-directed mutagenesis: The pFASTBAC1 Expression vector carrying the ABCG2 (Arg-482) cDNA was used as the template, and variant forms (Gly-482 and Thr-482) were created by the site-directed mutagenesis (**Fig. 1B**) using the QuickChange Site-directed Mutagensis Kit (Stratagene) and internal complementary PCR primers as follows: 5′-CTGATTTATTACCCATGGGGATGTTACCAAGTATT-3′ and 5′-AATACTTGGTAACATCCCCATGGGTAATAAATCAG-3′ (for the Gly-482 variant form) or 5′-CTGATTTATTACCCATGACGATGTTACCAAGTATT-3′ and 5′-AATACTTGGTAACATCGTCATGGGTAATAAATCAG-3′ (for the Thr-482 variant form). The PCR reaction consisted 16 cycles of 95°C for 30 sec, 55°C for 1 min and 68°C for 15 min, and *Pfu Turbo* DNA polymerase was used for the PCR reaction. The mutations were confirmed by sequencing the inserted fragments. The cDNAs of ABCG2 Gly-482 and Thr-482 variants, thus obtained, were separately inserted into the original pFASTBAC1 Expression vector (Invitrogen), as described above.

Expression of the human ABCG2 cDNA in Sf9 cells and preparation of cell membranes: Recombinant baculoviruses to express the above-mentioned variant forms of ABCG2 in insect cells were generated with the BAC-TO-BAC Baculovirus Expression Systems (Invitrogen) according to the manufacture's instruction. Insect *Spodoptera frugiperda* Sf9 cells (1×10^6 cell/mL) were infected with the recombinant baculoviruses and cultured in the EX-CELL™ 420 Insect serum-free medium (JRH Bioscience, Levexa, KS, USA) at 26°C with gentle shaking. 48 hours after the infection, cells were harvested by centrifugation. Cell membranes were prepared as described previously.[24] After the measurement of protein concentration by the BCA Protein Assay Kit (PIERCE, Rockford, IL, USA), the membrane preparation was stored at −80°C until used.

Immunological detection of ABCG2 protein: Expression of ABCG2 in Sf9 cell membranes was deter-

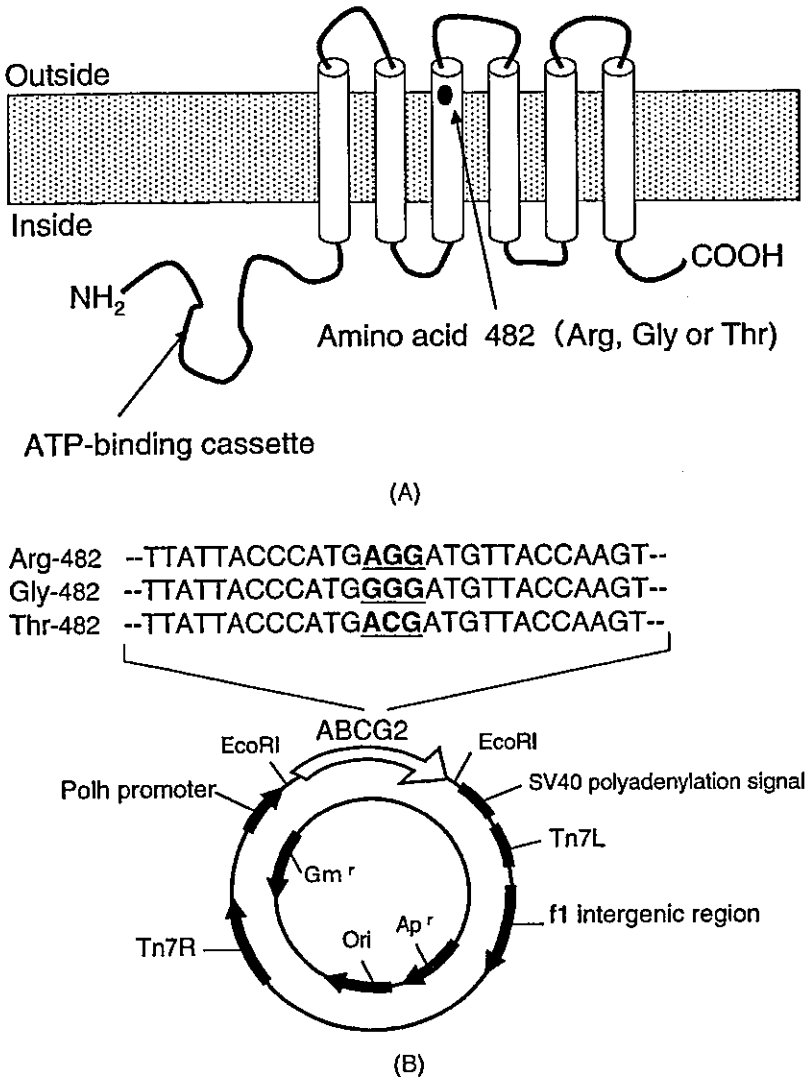

Fig. 1. Schematic illustrations of the molecular structure of ABCG2 (A) and the pFASTBAC vector for the expression of ABCG2 variants in Sf9 cells (B).
A: The molecular structure of ABCG2 was predicted from the cDNA sequence (AF103796) by using the SOSUI program (http://sosui.proteome.bio.tuat.ac.jp/sosuimenu0.html, and the amino acid 482 is indicated by an arrow. B: The partial cDNA sequences of three variants (Arg-482, Gly-482, and Thr-482) are shown in the recombinant expression vector pFASTBAC1 with the ABCG2 cDNA insert (B).

mined by immunoblotting with BXP-21 (SIGNET, Dedham, MA, USA), a specific antibody to human ABCG2 where membrane proteins were pre-treated with or without mercaptoethanol. Briefly, proteins of the isolated plasma membrane were separated by electrophoresis on 7.5% sodium dodecyl sulfate (SDS) polyacrylamide slab gels,[25] and the proteins were electroblotted onto Hy-bond ECL nitrocellulose membranes (Amersham, Buckinghamshire, UK). Immunoblotting was performed by using BXP-21 (1:250 dilution) as the first antibody and an anti-mouse IgG-horseradish peroxidase (HRP)-conjugate (Cell Signaling Technology, Beverly, MA, USA)(1:3000 dilution) as the secondary antibody. HRP-dependent luminescence was developed by using Western Lighting Chemiluminescent Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA) and detected by Lumino Imaging Analyzer FAS-1000 (TOYOBO, Osaka, Japan).

ATPase activity measurement: The ATPase activity of the isolated Sf9 cell membrane was determined by measuring inorganic phosphate liberation[26] according to the procedure reported by Sarkadi *et al.*[27] with some modifications. To adapt to our high throughput screening system with 96-well plates, we developed the standard procedure, as described previously.[24]

Detection of ATP-dependent transport of [^{3}H]methotrexate: The frozen stocked membrane was thawed quickly at 37°C, and vesicles were formed by passing the membrane suspension through a 27-gauge needle. The standard incubation medium contained

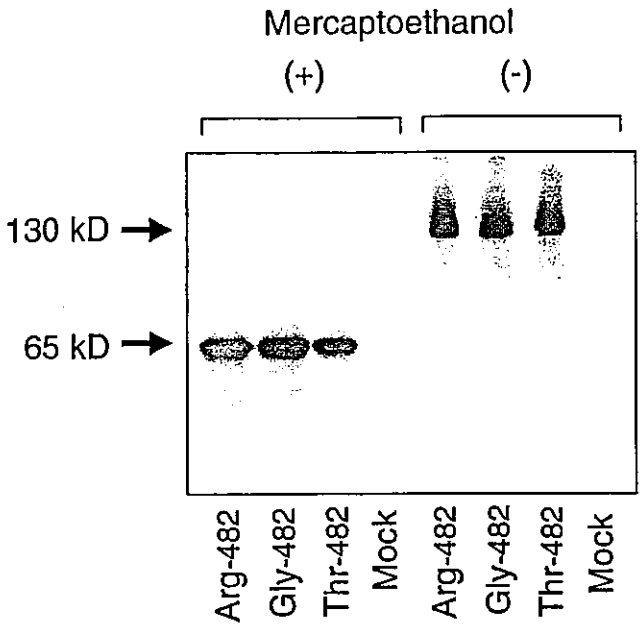

Fig. 2. Effect of mercaptoethanol on dimerization of ABCG2. The plasma membrane from ABCG2 (Arg-482)-transfected Sf9 cells was incubated with or without 100 mM mercaptoethanol at room temperature for 20 min. Thereafter, membrane proteins (2 μg for each lane) were separated by SDS-PAGE and ABCG2 was immunologically detected by Western blotting as described in Methods.

plasma membrane vesicles (40 or 80 μg of protein), 10 mM [3′,5′,7′-^{3}H]methotrexate (Amersham, Buckinghamshire, UK), 0.25 M sucrose, 10 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM creatine phosphate, and 100 μg/mL creatine kinase in a final volume of 110 μL. The reaction was started by adding [^{3}H]methotrexate to the incubation medium. The reaction was carried out at 37°C, and the amount of [^{3}H]methotrexate incorporated into the vesicles was measured by a rapid filtration technique as previously described.[28]

Results

Expression of ABCG2 variant forms in Sf9 cells: Three variant forms (i.e., Arg-482, Gly-482, and Thr-482) of human ABCG2 were generated by site-directed mutagenesis and expressed at high levels in Sf9 cells by infection with the baculoviruses derived from the recombinant pFASTBAC1 expression vectors (**Fig. 1B**). The expression of ABCG2 proteins in the plasma membrane of Sf9 cells was detected by Western blotting. As demonstrated in **Fig. 2**, the molecular size of ABCG2 expressed in Sf9 cells was 65,000 under reductive conditions (with mercaptoethanol), whereas its apparent molecular weight was 130,000 under non-reductive conditions (without mercaptoethanol) (**Fig. 2**). This phenomenon was observed equally for the Arg-482, Gly-482 and Thr-482 variants, and therefore it is strongly suggested that ABCG2 exists in the plasma membrane of Sf9 cells as a homodimer bound through cysteinyl disulfide bond(s).

Drug-stimulated ATPase activity in ABCG2-expressing cell membranes: Using membranes prepared from ABCG2-expressing Sf9 cells, we have measured ATPase activity in the presence of various drug compounds, i.e., prazosin, mitoxantron, and verapamil. As demonstrated in **Fig. 3A**, prazosin significantly enhanced the ATPase activity of the plasma membrane prepared from ABCG2 (Gly-482)-expressing Sf9 cells. The stimulation of ATPase activity was dependent on the concentration of prazosin, exhibiting a saturation curve with a half-maximal concentration of 5 μM. In addition, mitoxantron (50 or 100 μM) enhanced the ATPase activity in the membrane, however its extents were relatively small (**Fig. 3A**). On the other hand, in the membrane from ABCG2 (Thr-482)-expressing Sf9 cells, the ATPase activity was stimulated by prazosin to a certain extent, but not significantly by mitoxantron. Interestingly, no stimulation was observed with prazosin or mitoxantron in the membrane from ABCG2 (Arg-482)-expressing Sf9 cells. Verapamil, which is a substrate for ABCB1 (P-glycoprotein, MDR1) but not for ABCG2, had no effect on the ATPase activity of the membranes prepared from those three variant forms-expressing Sf9 cells.

Effect of vanadate on prazosin-stimulated ATPase activity: As shown in **Fig. 3B**, prazosin-stimulated ATPase activity in the ABCG2 (Gly-482)-expressing membrane was sensitive to vanadate, where ATPase activity was almost completely inhibited by 50 μM sodium vanadate to background levels. Likewise, in the ABCG2 (Thr-482)-expressing membrane, prazosin-stimulated ATPase activity was inhibited by vanadate in a similar dose-dependent manner. In the absence of prazosin, the basal ATPase activity was little affected by vanadate in the concentration range of up to 200 μM. This suggests that prazosin-stimulated ATPase activity can be distinguished from the basal ATPase activity by such vanadate effects.

ATP-dependent transport of methotrexate by ABCG2 (Arg-482): The transport function of Arg-482, Gly-482, and Thr-482 variants was examined by using plasma membrane vesicles prepared from Sf9 cells expressing those variants. **Figure 4A** depicts the time courses of methotrexate transport into plasma membrane vesicles in the presence or absence of ATP. The ATP concentration was maintained at constant levels for a period sufficient for measurement (at least 20 min) with the creatine phosphate and creatine kinase reaction system. It is important to note that ATP-dependent methotrexate transport was observed only in plasma membrane vesicles prepared from Sf9 cells expressing the Arg-482 variant. No significant transport

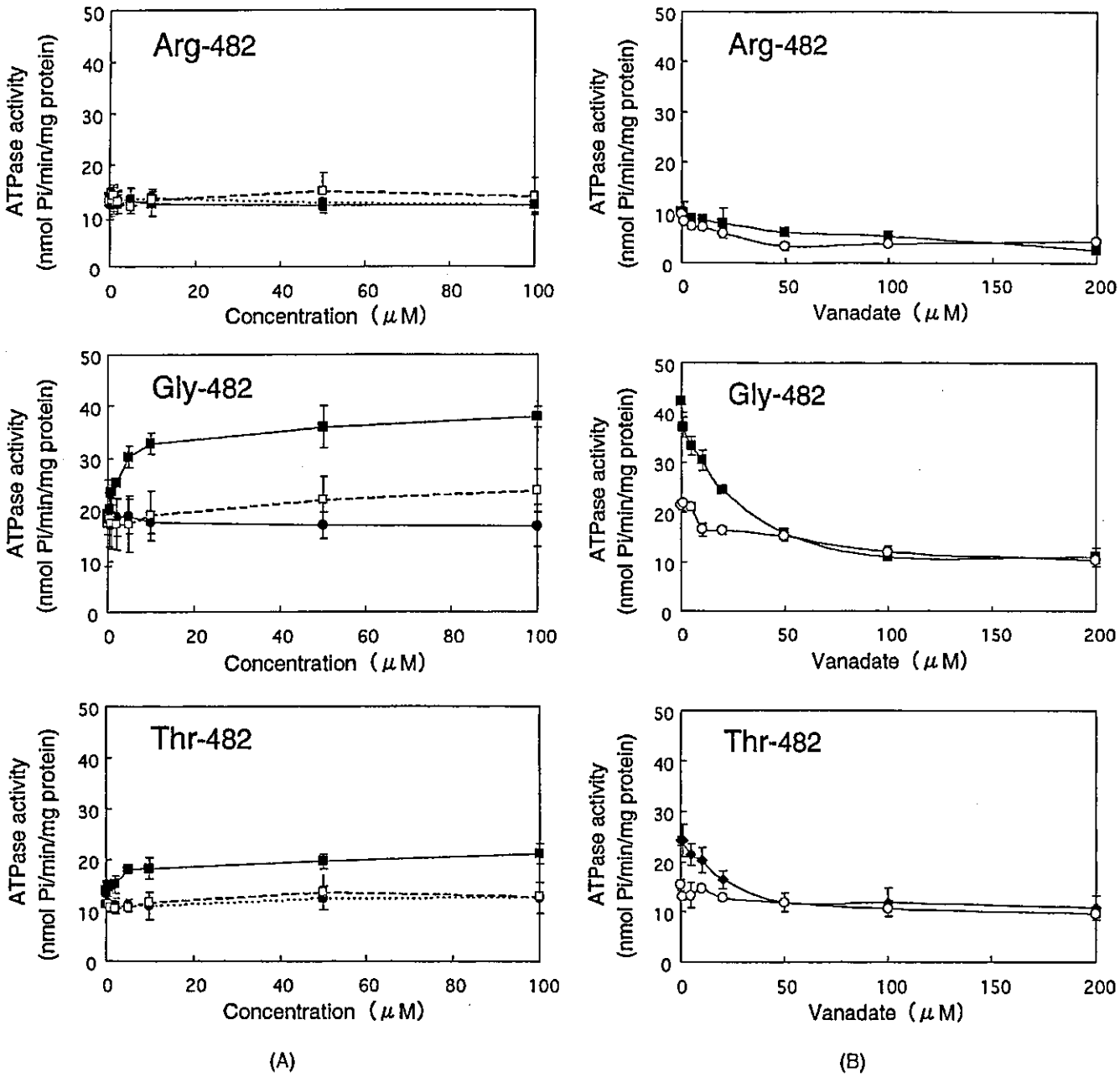

Fig. 3. Effect of prazosin, mitoxantron, and verapamil (A) as well as vanadate (B) on the ATPase activity of cell membranes prepared from Sf9 cells.

The ATPase activity was measured as described in the section of Methods. A: Drugs, i.e., prazosin (■), mitoxantron (□), and verapamil (●), were added to the reaction mixture at different concentrations as indicated. B: To test the inhibitory effect of vanadate, the ATPase activity was measured in the absence (○) and presence (■) of 50 μM prozosin. Results are expressed as mean values ± S.D. in triplicate measurements.

activity was detected from the Gly-482 or Thr-482 variant-expressing Sf9 cells. **Figure 4B** demonstrates a relationship between the methotrexate concentration and the rate of ATP-dependent transport by the Arg-482 variant. Based on the Lineweaver-Burk plot of this result, the apparent Km value for methotrexate was estimated to be 6 mM.

Discussion

Expression of human ABCG2 as a homodimer in insect cells: Since ABCG2 is an ABC half-transporter, it has been suspected that ABCG2 functions as a homodimer or heterodimer.[15] Most recently, using cross-linking reagents and specific antibodies, Litman *et al.* have provided evidence for the formation of an

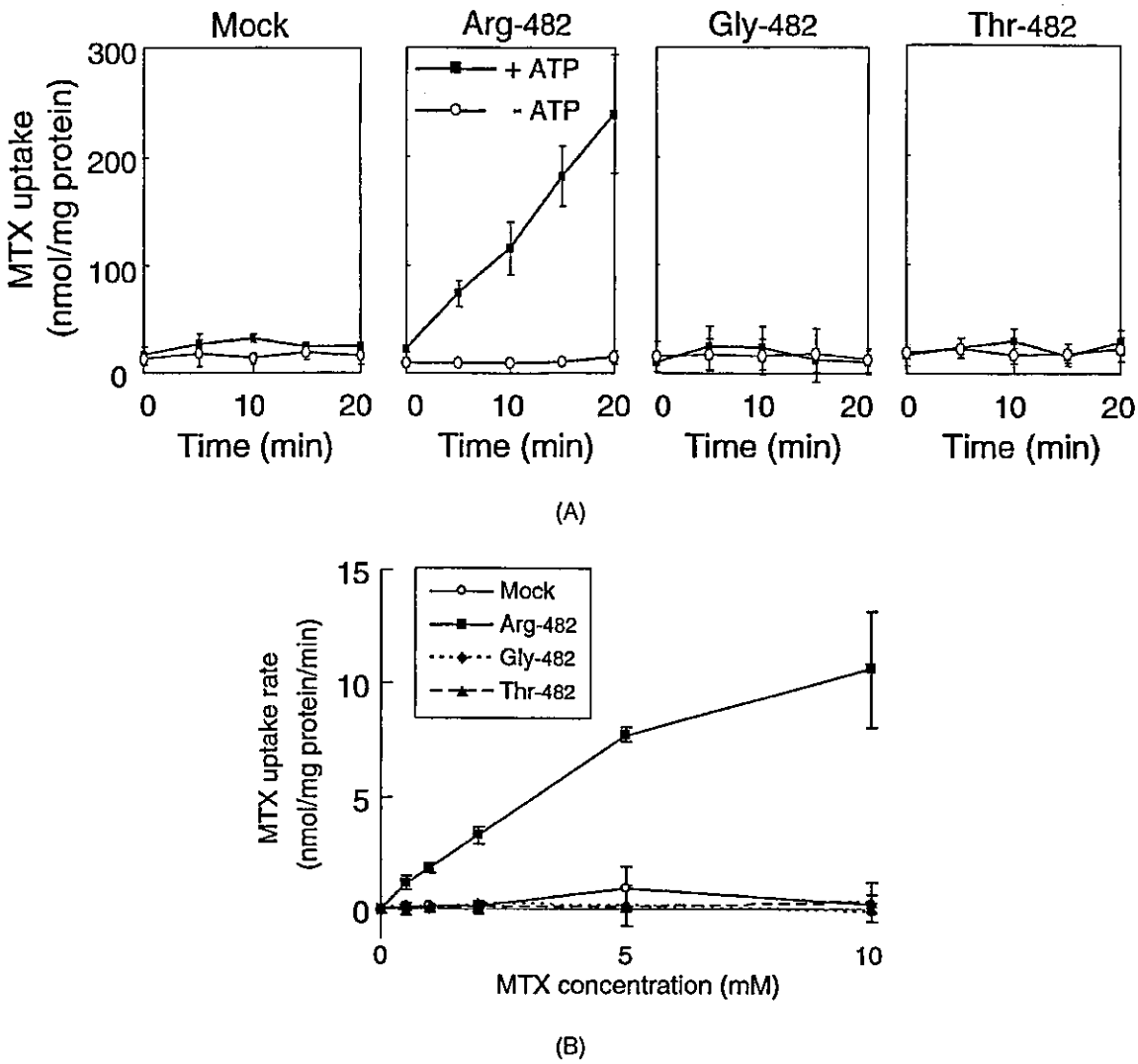

Fig. 4. ATP-dependent transport of methotrexate (MTX) in plasma membrane vesicles prepared from ABCG2-transfected Sf9 cells (A) and the effect of MTX concentration on the transport (B).

A: Plasma membrane vesicles were prepared from mock- and ABCG2 (Arg-482, Gly-482, or Thr-482)-expressing Sf9 insect cells as described in Experimental. Plasma membrane vesicles (80 μg of protein) were incubated with 10 mM [^{3}H]methotrexate (MTX) in the absence (○) or presence (■) of 1 mM ATP in the medium containing 0.25 M sucrose, 10 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM creatine phosphate, and 100 μg/mL creatine kinase. The incubation was carried out at 37°C and then stopped at different time points (0, 5, 10 15 and 20 min) as indicated in the figure. The amount of [^{3}H]methotrexate incorporated into the membrane vesicles was measured by a rapid filtration technique. Results are expressed as mean values ± SD in triplicate measurements. B: The effect of the MTX concentration on the initial rate of ATP-dependent MTX transport into membrane vesicles. The ATP-dependent MTX transport was measured as described above, and the initial rate was measured at 0 – 20 min in the incubation.

ABCG2 homodimer.[29] In addition, Kage *et al.* have recently established PA317 transfectants expressing Myc and HA epitope-tagged ABCG2 proteins, and they demonstrated that those hybrid proteins formed S-S homodimers.[30] In the present study, by expressing native ABCG2 (without tag) in insect cells, we could provide direct evidence that human ABCG2 exists in the plasma membrane as a homodimer bound through cysteinyl disulfide bond(s) (**Fig. 2**). Treatment with mercaptoethanol reduced the apparent molecular weight of ABCG2 from 130,000 to 65,000 (**Fig. 2**). The dimerization through cysteinyl disulfide bonds occurred in all three variant forms of human ABCG2 tested in this study. Similar results were obtained with human ABCG2 expressed in human embryonic kidney (HEK) 293 cells (Mitomo *et al.*, manuscript submitted). Therefore, it is strongly suggested that the human ABCG2 protein expressed in Sf9 insect cells operates as a homodimer as does the protein expressed in mammalian cells. This knowledge is important, since ABCG2 expressed in Sf9 cells may be widely used for the functional screening of ABCG2 with drug candidates. Based on

the cDNA sequence, a total of twelve cysteine residues exist in the ABCG2 peptide. From a biochemical point of view, it is of great interest to study which cysteine residues participate in the disulfide bond formation and how inter-peptide disulfide bonds are formed in the cell.

Drug-stimulated ATPase activity of ABCG2 variants: Several acquired mutations have hitherto been documented for ABCG2 cloned from drug resistant cell lines.[21,31] Drug resistance phenotypes vary among different cell lines expressing variant types of ABCG2. In fact, transfectants with the wild type (Arg-482) were not resistant to topotecan,[18] while overexpression of the Gly-482 and Thr-482 variants conferred resistance to mitoxantrone, doxorubicin, daunorubicin, and various camptothecin analogs including topotecan.[9,14-16] Furthermore, Gly-482 and Thr-482 variants mediated the efflux of rhodamine 123 and doxorubicin from cells; however, Arg-482 did not.[21,22] These findings indicate that Arg at position 482 plays a critical role in the substrate specificity of ABCG2. The identification of mutations at 482 in ABCG2 may explain some discrepancies observed in the cross-resistance profiles of human cancer cell lines. To examine the function of ABCG2 as a drug transporter, we have previously established ABCG2 (Arg-482)-overexpressing Sf9 insect cells and tried to measure drug-induced ATPase activity.[24] However, because of high levels of the basal ATPase activity present in the plasma membrane, we were not able to accurately measure the drug-induced ATPase activity of ABCG2.[24]

In the present study, prazosin was found to significantly enhance the ATPase activity in plasma membranes prepared from Gly-482 and Thr-482 variants-expressing Sf9 cells. However, such stimulation was not observed in the plasma membrane expressing the Arg-482 variant (**Fig. 3A**). The prazosin-stimulated ATPase activity was dose-dependently inhibited by vanadate ($IC_{50}=20\,\mu M$; **Fig. 3B**). Such inhibition by vanadate is characteristic for ABC transporters, including ABCB1 (P-glycoprotein/MDR1)[24] and ABCC1 (MRP1/GS-X pump).[28] Thus, it is suggested that Gly-482 and Thr-482 variants of human ABCG2 transport prazosin, but the Arg-482 variant does not or its transport activity is very low. In several cases, however, the substrate stimulation of the ABCG2-ATPase could not be directly correlated with the actual transport process, as suggested by Özvegy *et al.*[22] It would be important to examine the actual transport of drugs by ABCG2 variant forms.

ATP-dependent methotrexate transport by the ABCG2 (Arg-482): Recently, Volk *et al.* suggested that overexpression of the wild type (Arg-482) of ABCG2 mediates methotrexate resistance.[23] However, the mitoxantrone-selected MCF/MX cell line was used in their study, and therefore molecular mechanisms underlying the drug resistance should be rather complex. The actual involevement of ABCG2 (Arg-482) in the methotrexate resistance remained to be elucidated. In the present study using the plasma membrane vesicle system, we could clearly demonstrate that ABCG2 (Arg-482) transports methotrexate; but, the other variants Gly-482 and Thr-482 do not (**Fig. 4**). Our finding suggests that the presence of Arg-482 in the third transmembrane domain is a critical amino acid residue involved in the substrate specificity of ABCG2. Since the arginine residue is positively charged under the physiological condition, it is likely that its positive charge is a prerequisite for interactions between the active site of ABCG2 and anionic substrates, such as methotrexate.

Earlier works[32,33] have predicted the existence of energy-dependent transport systems for the methotrexate efflux from cells. Molecular cloning and functional expression of ABC transporters led to the identification of human ABCC1, ABCC2, ABCC3, and ABCC4 as methotrexate transporters,[34-36] and all of these transporters belong to the C-subfamily of human ABC transporters.[7,8] In this context, this is the first report that ABCG2, a member of the "G" sub-family, transports methotrexate. However, because of its high Km value of ABCG2 to methtrexate (6 mM; **Fig. 4B**), the contribution of this transporter to methotrexate resistance might be small at physiological concentrations. A relevant study on the relationship between ABCG2 variant forms expressed in HEK293 cells and their drug resistance profiles will be reported elsewhere.[37]

Concluding Remarks: Like human ABCG2, one mutation 'hot spot' was identified at the same amino acid position 482 in mouse Abcg2, where the Arg-482 is the wild type.[38] In the case of mouse Abcg2, variants of Arg, Ser, and Met significantly affect the drug resistance profile in cancer cell lines.[38] Recently, Jonker *et al.* demonstrated that mouse Abcg2 protects against a major chlorophyll-derived dietary phototoxin and protoporphyria.[39] In their study, the wild type (Arg-482) of mouse Abcg2 has been suggested to transport chlorophyll and porphyrin metaboites. Since those metabolites are organic anions like methotrexate, it is hypothesized from our findings that the Arg-482 moiety may be important for the transport of such anionic metabolites. With this respect, further studies should be needed to examine our hypothesis.

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Molecular identification and characterization of rat Abcc1 cDNA: existence of two splicing variants and species difference in drug-resistance profile

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The human *ABCC1* gene, a member of the ATP-binding cassette transporter super-family, plays a critical role in conferring cancer cell resistance to chemotherapeutic drugs. In the present study, we have cloned the full-length cDNA of rat Abcc1 and evaluated its significance in drug resistance. Analysis using the currently available genome database revealed that the rat *Abcc1* gene is located on rat chromosome 13 and consists of at least 30 exons. The rat Abcc1 cDNA cloned from the spleen was 4981-bp long, within which two additional splicing variants were discovered. The rat *Abcc1* gene is expressed in a wide variety of organs, with the highest expression being observed in the spleen. Human embryonic kidney 293 cells were transfected with the rat Abcc1/pcDNA3.1 vector to stably express rat Abcc1. Overexpression of rat Abcc1 elicited high resistance to etoposide. In contrast to the hitherto known drug-resistance profile of human ABCC1, rat Abcc1 did not significantly confer cellular resistance to anthracyclins or *Vinca* alkaloids. Our results strongly suggest that there is a significant species difference between human ABCC1 and rat Abcc1 in their contribution to the drug-resistance profile.

Keywords ABC transporter, multidrug resistance, splicing, species difference

INTRODUCTION

Multidrug resistance in human cancer is the major obstacle to long-term, sustained patient response to chemotherapy. It has been convincingly documented that several ATP-binding cassette (ABC) transporters cause multidrug resistance in cancer cells by actively extruding certain chemotherapeutic drugs. The ABC transporter gene family comprises about 50 gene members (http://gene.ucl.ac.uk/nomenclature/genefamily/abc.html). Based on the arrangement of molecular structural components, i.e., nucleotide-binding domains (NBDs) and topologies of transmembrane domains, human ABC transporter proteins are classified into seven different sub-families (A to G). Among them, ABCB1 (P-glycoprotein or MDR1), ABCC1 (multidrug resistance-associated protein 1 (MRP1)), ABCC2 (MRP2, cMOAT), and ABCG2 (BCRP) have been characterized in the greatest detail with respect to their structure and function (1,2).

In humans, the *ABCC1* gene was first identified by molecular cloning from multidrug-resistant lung carcinoma cells as MRP1 (3). ABCC1 acts as an organic anion transporter involved in the so-called "phase III" detoxification system. The importance of active efflux of phase II metabolites by the anion transporter was previously pointed out, and it was named the GS-X pump (4). ABCC1 is expressed not only in cancer cells but also on

The cDNA sequences of rat Abcc1 and its splicing variants have been submitted to GenBank under the following accession numbers: AF487549 (full length), AY174892 (variant A), and AY174893 (variant B).

the basolateral membrane of epithelial cells in a wide variety of tissues. Organic anions, such as folic acid, leukotriene C_4, glutathione disulfide (GSSG), and glucuronate and sulfate conjugates, are substrates for human ABCC1, whereas anthracyclines, *Vinca* alkaloids, and etoposide are reportedly transported together with glutathione (GSH) by human ABCC1 (5).

ABCC2, ABCC3, ABCC4, ABCC5, and ABCC6 are also known as organic anion transporters. ABCC2 (cMOAT/MRP2) and ABCC3 (MRP3) are highly expressed in liver, kidney, and gut (1,5), and thereby critically affect the pharmacokinetic profiles of a variety of drugs (6–9). ABCC2 and ABCC3 are localized to apical and basolateral membranes of epithelial cells, respectively. A genetic mutation in the *ABCC2* gene causes the conjugated hyperbilirubinemia called Dubin–Johnson syndrome.

Recently, our group and others have identified two novel genes, *ABCC11* and *ABCC12*, which are tandemly located on chromosome 16q12.1 (10–13). The deduced proteins of ABCC11 and ABCC12 show high homologies with ABCC5, and their substrate selectivities are considered to be related to those of ABCC5. Furthermore, our group has identified a novel ABCC family gene, *ABCC13* (14). This gene is located on chromosome 21q11.2 and consists of 14 exons. Tissue distribution analysis has revealed that the *ABCC13* gene is expressed in hematopoietic cells, including human fetal liver cells.

Based on the currently available knowledge as to the physiological function and substrate specificity of human ABC transporters, it is suggested that many members of the C-subfamily, e.g., ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, and ABCC6, are able to transport a wide variety of drugs and xenobiotics. In drug discovery research, experiments with animals are an important step in the pharmacological and toxicological validation processes of drug candidates. Drug responses, including side effects, can vary among different species, e.g., human, monkey, dog, and rat. From the pharmacological and toxicological points of view, species differences in the activity of ABC transporters appear to be critical.

Although more than 10 years have passed since the first discovery of human ABCC1, the full-length cDNA of the rat ortholog has not yet been cloned. The previously submitted sequence of rat Abcc1 cDNA (GenBank accession number: AJ277881) was a partial one, only 2969-bp long, lacking the translation start codon. Therefore, the aim of the present study was to clone the full length of rat Abcc1 cDNA and to characterize its pharmacological and toxicological properties by using in vitro cDNA expression systems. In this study, we identified the full-length rat Abcc1 cDNA as well as two splicing variants and examined their tissue-specific expression. To analyze the function, we have established rat Abcc1-expressing human embryonic kidney 293 (HEK 293) cell lines and examined their chemosensitivity to various drugs. There were notable differences in drug-resistance profiles between human ABCC1 and rat Abcc1.

MATERIALS AND METHODS

Molecular Cloning of Rat Abcc1 cDNA

To obtain the rat Abcc1 cDNA, we first amplified three fragments by the reverse transcriptase-polymerase chain reaction (RT-PCR) method (Figure 1A), with the first strand cDNA from rat spleen as the PCR template and TaKaRa Ex Taq™ (TaKaRa, Shiga, Japan). For this purpose, we have used the following three pairs of PCR primers: for the 5′ part, CGGTGACGCGAGCCAAC (rc1F-1) and CCTTCTTCCAGTTATTCACCAGCAC (rc1B-1); for the middle part, CATTGAATAAAGAGGACACGTCA (rc1F-2) and GGCTTTGACTCCTTCCCTAA (rc1B-2); and for the 3′ part, GCTACAAGGCGGTGATGGAAGC (rc1F-3) and GCTTGGCTCTATAAATGTGGCTGAATAGG (rc1B-3). The PCR reaction program was 95°C for 2 min, 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 3 min, which was finally followed by 72°C for 2 min. The resulting PCR products were cloned into pCR2.1TOPO vectors by using the TOPO TA cloning Kit (Invitrogen Corp., Carlsbad, CA), and the plasmids were isolated with the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). The sequence of the insert in the recombinant vectors was analyzed by TOYOBO Gene Analysis. Finally,

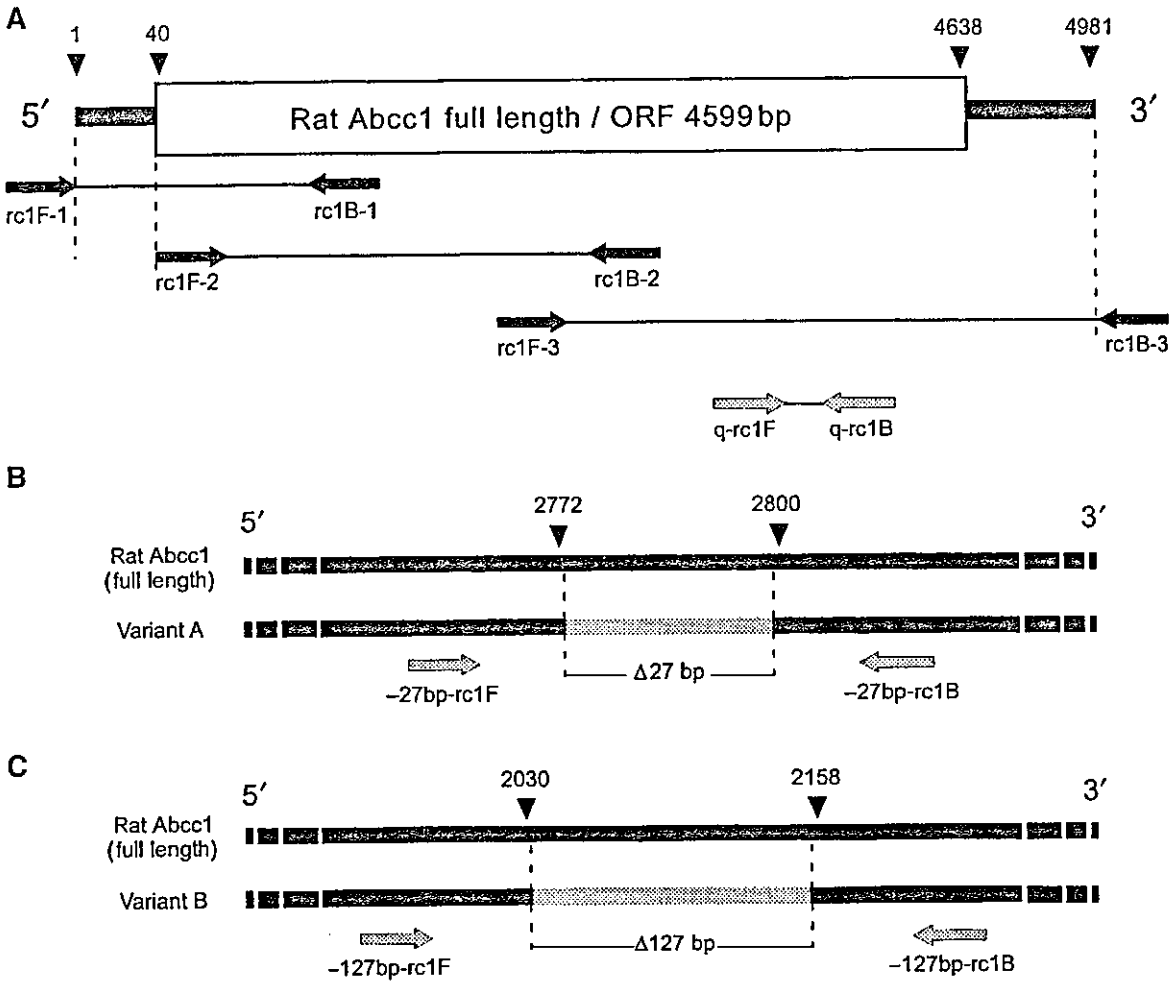

Figure 1. Strategy for the cloning of rat Abcc1 cDNA **(A)** and the detection of splicing variants A and B by polymerase chain reaction (PCR) **(B** and **C)**. The open reading frame (ORF) of rat Abcc1 is indicated by a box. As described in *Materials and methods*, the cDNA was cloned by PCR with three sets of primers: for the 5′ part, rc1F-1 and rc1B-1; for the middle part, rc1F-2 and rc1B-2; and for the 3′ part, rc1F-3 and rc1B-3. Specific primers, q-rc1F and q-rc1B, are used for the quantitative PCR detection, as indicated in panel **A**. Detection of splicing variants A and B by PCR was made with the following specific primers: –27bp-rc1F and –27bp-rc1B **(B)**; –127bp-rc1F and –127bp-rc1B **(C)**.

the rat Abcc1 cDNA was obtained by assembling the partial sequences.

Detection of Splicing Variants

For the detection of both the full-length fragment and that of the splicing variant A, the following PCR primers were used: sense primer sequence GAATGGTGTCAGTGGTTTAGGG (–27bp-rc1F), and anti-sense primer sequence GCTGGTTAGTAACCACACTCTG (–27bp-rc1B). The PCR reaction consisted of 38 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 15 s. The sizes of the resulting DNA fragments were 131 bp and 104 bp for the full-length rat Abcc1 and the splicing variant A, respectively (Figure 1B).

In addition, to detect both the full-length fragment and that of the splicing variant B, the following PCR primers were used: sense primer sequence TCACTGTGAAGAATGCAAC (–127bp-rc1F), and anti-sense primer sequence GGATTTCCAAATCCGGAAG (–127bp-rc1B). The PCR reaction consisted of 42 cycles of 95°C for 15 s, 52°C for 15 s, and 72°C for 15 s. The resulting DNA fragments were 327 bp and 200 bp for the full-length rat Abcc1 and the splicing variant B, respectively (Figure 1C).

Quantitative PCR Analysis

Expression levels of rat Abcc1 were determined by quantitative PCR by using the