P-glycoproteins and hepatobiliary secretion
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CHAPTER 2

CLONING OF THE cDNA ENCODING THE RAT MULTIDRUG RESISTANCE (MDR) 1A P-GLYCOPROTEIN

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In this chapter we report on the cloning, tissue distribution and functional characterization of the cDNA encoding rat *Mdr1a* P-glycoprotein. Surprisingly, despite the many functional studies performed in rats, and despite the isolation of the related *Mdr1b* and human *MDR1* genes, the rat *Mdr1a* gene has not been cloned yet. The rat *Mdr1a* cDNA was isolated using a combination of screening of a liver cDNA library and polymerase chain reaction. This sequence was 4927 nucleotides long with an open reading frame of 3819 nucleotides, encoding a protein of 1272 amino acids. *Mdr1a* gene transcripts were most abundantly expressed in ileum and ovary, and to a lesser extent in liver. No expression was observed in lung, thymus and 10-12 day embryos. We observed a complementary tissue expression pattern of *Mdr1a* and *Mdr1b* transcripts. We expect that, analogous to mice, *Mdr1a* may be a major determinant of intestinal and biliary clearance of substances in rats.
CHAPTER 2: CLONING OF RAT \textit{Mdr1a} cDNA

\textbf{INTRODUCTION}

P-glycoproteins (Pgps) are integral plasma membrane glycoproteins that belong to the ATP-binding cassette (ABC) subfamily B of the ABC transporter protein superfamily (reviewed in Klein \textit{et al.} \textsuperscript{1}). P-glycoproteins utilize the energy released by ATP hydrolysis to mediate transport of its substrates across membranes, and initially were identified because some members of this subfamily confer multidrug resistance (MDR) in tumor cells (reviewed in Gottesman and Pastan\textsuperscript{2} and Ambudkar \textit{et al.} \textsuperscript{3}). More recently, an additional member of the ABC subfamily B was identified in pig liver\textsuperscript{4}. Mammalian Pgps are encoded by a family of highly conserved genes, consisting of three members in humans (\textit{ABCB1}, \textit{ABCB4}, and \textit{ABCB11}) and four members in rodents (\textit{Abcb1a}, \textit{Abcb1b}, \textit{Abcb4}, and \textit{Abcb11}). An up-to-date overview of the nomenclature and classification of Pgp isoforms is given in Table 1. However, for clarity, the nomenclature introduced by Hsu \textit{et al.}\textsuperscript{5} (i.e. the \textit{Mdr1a}/1b and \textit{Mdr2} classification) will be used throughout this paper. Members of the ABC subfamily B are highly homologous. Each protein is a tandemly repeated molecule with each half containing six (putative) transmembrane segments and a nucleotide binding fold\textsuperscript{1}. Pgps show a high degree of inter- and intraspecies sequence homology, but despite their great sequence similarity, striking functional differences have been observed between individual Pgp isoforms in transfection experiments. Pgps, encoded by human \textit{MDR1}, and mouse \textit{Mdr1a}/1b cDNAs, can confer resistance to a broad spectrum of natural product drugs on otherwise drug sensitive cells\textsuperscript{6-8}, but human \textit{MDR3}, its rodent orthologue \textit{Mdr2}, and rodent \textit{Bsep} gene products apparently cannot\textsuperscript{9-12}. The latter proteins function as phosphatidylcholine translocase and bile salt export pump, respectively\textsuperscript{13,14}. Interestingly, qualitative and quantitative differences in the drug resistance phenotypes imposed by human \textit{MDR1} and mouse \textit{Mdr1a}/1b have also been reported\textsuperscript{8,15}, and single amino acid substitutions have been shown to modulate the specificity of human and mouse drug transporting Pgps (summarized in Ambudkar \textit{et al.}\textsuperscript{3}). The two mouse \textit{Mdr} genes differ in their patterns of relative expression in normal tissues. The mouse \textit{Mdr1a} gene is expressed at a high level in the intestinal epithelium and in the capillaries of the brain and testis, while the \textit{Mdr1b} gene is expressed preferentially in the adrenal gland, pregnant uterus, and ovaries. In this species significant levels of both \textit{Mdr1a} and \textit{Mdr1b} mRNA are present in many other tissues, including liver, kidney, lung, heart, and spleen\textsuperscript{16,17}. The tissue distribution of human \textit{MDR1} roughly overlaps with that of mouse \textit{Mdr1a}, \textit{Mdr1b}, or both genes\textsuperscript{18,19}. A systematic study on the tissue distribution of the rat \textit{Mdr} genes has currently not been performed.

The tissue distribution pattern and subcellular localization of drug transporting Pgps, combined with the ability to transport a wide range of substrates, are compatible with the hypothesis that \textit{Mdr1a}/1b and \textit{MDR1} are involved in the transport of both endogenous and exogenous compounds. In order to characterize the normal physiological functions of the drug transporting Pgps, mice with a disruption of the \textit{Mdr1} genes have been generated\textsuperscript{20,21}. \textit{Mdr1} gene knockout mice grow and develop normally, are fertile, and do not exhibit obvious abnormalities. Thus, under laboratory conditions drug transporting Pgps are not essential for the basic physiological functioning of the organism. However, these mice are very sensitive to xenobiotics and display drastic alterations in the pharmacological handling of drugs (reviewed in Schinkel\textsuperscript{22}). These effects are due to (a combination of) profoundly reduced clearance of substances in excretory organs such as liver, kidney, and intestine, altered metabolism and body distribution, and increased intestinal absorption of (orally) administered drugs. Collectively, these processes may

\textsuperscript{i} According to the new system of nomenclature for ABC transporter genes
affect the bioavailability, peak plasma concentration and other pharmacokinetic parameters of Pgp substrates. Moreover, these data also underline the potential role of drug transporting Pgps in clinically significant drug-drug interactions, as has been recently recognized.

Presently, neither the full-length rat Mdr1a cDNA nor its encoding protein have been isolated and characterized (see Table 1). In this study we aimed to clone the rat Mdr1a cDNA. In addition, we compared the tissue distribution of members of the rat ABC subfamily B.

**EXPERIMENTAL PROCEDURES**

**MATERIALS**

All chemicals were of analytical grade and readily available from commercial sources. Molecular biology reagents were obtained from Promega (Leiden, The Netherlands) or Roche Molecular Biochemicals (Almere, The Netherlands). Oligonucleotide primers were obtained from Life Technologies (Breda, The Netherlands).

**cDNA cloning of rat liver Mdr1a**

Sense (RMDR1S, 5'-GAT GGA ATT GAT AAT GTG GAC A-3') and antisense (RMDR2A, 5'-AAG GAT CAG GAA CAA TAA A-3') oligonucleotide primers were designed based upon a partial rat Mdr1a cDNA sequence (GenBank/EMBL database accession number S66618). Total RNA was extracted from male Wistar rat liver using TRIzol Reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. Subsequently, mRNA was isolated using the Oligotex mRNA mini-kit (Qiagen GmbH, Hilden, Germany). Single-stranded complementary DNA (cDNA) was synthesized from 1 µg mRNA using oligo-(dT)15 primers and AMV Reverse Transcriptase (Promega) at 42 °C for 60 min, followed by 95 °C for 5 min to inactivate the enzyme. A cDNA probe spanning 351 base pairs (bp) of the rat Mdr1a sequence was constructed by polymerase chain reaction (PCR) using the primer pair RMDR1S/2A and rat liver cDNA as template. The corresponding PCR product was isolated from an agarose gel and subcloned into the pGEM-T vector (Promega). Sequence analysis identified the PCR product as part of the Mdr1a gene.

Table 1:

<table>
<thead>
<tr>
<th>official gene symbolb</th>
<th>ABCB1</th>
<th>drug transporter</th>
<th>PC translocase</th>
<th>BS export pump</th>
</tr>
</thead>
<tbody>
<tr>
<td>human MDR1 (PGY1)32</td>
<td>n/a</td>
<td>n/a</td>
<td>MDR3 (MDR2)33</td>
<td>BSEP (SPGP)b4</td>
</tr>
<tr>
<td>mouse Mdr1a (Mdr3)b7</td>
<td>Mdr1b (Mdr1)b5</td>
<td>Mdr1b (Mdr1)b5</td>
<td>Mdr2b26</td>
<td>Bsep (Sppl)b22,37</td>
</tr>
<tr>
<td>rat Mdr1a (Pg1, Pgp1)b25,30</td>
<td>Mdr1b (Pg2, Pgp2)b30</td>
<td>Mdr2 (Pg3, Pgp3)b30</td>
<td>Bsep (Sppl)b11,14</td>
<td></td>
</tr>
<tr>
<td>hamster Mdr1a (Pg1)b31</td>
<td>Mdr1b (Pg2)b41</td>
<td>Mdr2 (Pg3)b41</td>
<td>Bsepd</td>
<td></td>
</tr>
<tr>
<td>former classificationc</td>
<td>class I</td>
<td>class II</td>
<td>class III</td>
<td>n/a</td>
</tr>
</tbody>
</table>

This table is adapted from Borst and Schinkel31 and based on the nomenclature introduced by Hsu et al.5. Other designations are indicated in parenthesis.

Abbreviations used: PC: phosphatidylcholine; BS: bile salt; n/a: not applicable.

a: based on the new system of nomenclature for ABC transporter genes (for more information please see http://www.gene.ucl.ac.uk/users/hester/abc.html or http://www.med.rug.nl/mdl/humanabc.htm).
b: since SPGP genes encode for bile salt export pumps (BSEP), these are preferably named BSEP genes.
c: presently only limited sequence information available.
d: presently no sequence information available.
cDNA sequence. This fragment was labeled with \([^{32}\text{P}]\text{dCTP}\) using the High Prime DNA Labeling kit (Roche) and used as a probe to screen a rat liver cDNA library constructed from size-fractionated rat liver poly(A\(^+\)) RNA\(^{14}\). Hybridization was carried out at 60 °C for 20 h according to standard procedures\(^{26}\). Positive clones were plaque-purified by secondary and tertiary screening rounds. This resulted in the isolation of two \(\text{Mdr1a}\) cDNA clones, the longest one (clone JW4) contained an insert of 4.0 kb. Sequence analysis of both ends of clone JW4 revealed that approximately 900 bp of the 5'-part of the \(\text{Mdr1a}\) cDNA was lacking, as judged by comparison of clone JW4 with a mouse \(\text{Mdr1a}\) cDNA sequence (GenBank/EMBL accession number M33581)\(^{27}\). A longer \(\text{Mdr1a}\) clone could not be isolated in a second hybridization round using a more 5'-located probe. Therefore, the 5'-end of the \(\text{Mdr1a}\) cDNA was isolated by PCR.

The database of expressed sequence tags (dbEST) was searched for rat sequences that are closely related to a 212 bp 5'-terminal fragment of mouse \(\text{Mdr1a}\) cDNA (nucleotides -137 to +75 relative to the translation start site of M33581). This resulted in the identification of 2 rat ESTs that had been deposited under accession numbers A1045408 and A1045221. Both ESTs contained 83 bp of the supposed coding region, and comprised 250 and 291 bp, respectively, of the 5'-untranslated region (UTR) of the \(\text{Mdr1a}\) cDNA. Based upon the 5'-end sequence of these ESTs a sense oligonucleotide primer was designed (EST-1, 5'-AAC TCA GAG CGC CGC TAG AGG TG-3'). Together with an antisense oligonucleotide primer (4.5AS, 5'-ATT TGG AGA TGC CTG CCC AAC ACT G-3') that was based upon the 5'-end sequence of clone JW4, primer EST-1 was used to amplify an approximately 1.2 kb rat liver cDNA fragment. PCR reactions were performed with the Expand High Fidelity PCR system (Roche). The PCR product was isolated from agarose gel and subcloned into the pGEM-T vector. The resulting plasmid was designated pGEM-GH11. Sequence analysis revealed that the 5'-end of pGEM-GH11 overlapped with both ESTs whereas the 3'-end overlapped with clone JW4. Moreover, the 1.2 kb fragment was 92% and 83% identical with its corresponding region of mouse \(\text{Mdr1a}\) and rat \(\text{Mdr1b}\) cDNA, respectively. Based on these data we concluded that pGEM-GH11 contained the 5'-end of the rat \(\text{Mdr1a}\) sequence. To minimize the possibility of experimental artifacts, four additional 1.2 kb clones from 4 independent PCR reactions, each using cDNA isolated from different animals as template, were sequenced. All clones yielded the same sequence.

**GENERATION OF FULL-LENGTH RAT \(\text{Mdr1a}\) cDNA**

The full-length rat \(\text{Mdr1a}\) cDNA was amplified from liver cDNA using oligonucleotide primers FL1A-3S (5'-ACG CGT CGA CCG GAA AGT AGA GAC ACG TGA GGT CG-3') and FL1A-4A (5'-ATT TGC GGC CGC CGC TTA ACA TCT CGC ATG GTC ACA G-3'). An additional \(\text{Sal}\) or \(\text{Not}\) site (underlined) was introduced in the sense and antisense primers, respectively, in order to facilitate possible downstream subcloning reactions. The resulting plasmid was designated pGEM-GH11 and ligated into the \(\text{Sal}/\text{Not}\) sites of the pGEM9zf(-) vector (Promega) to create pGEM9-Mdr1a. The cDNA insert was sequenced in its entirety and was found to be identical to pGEM-GH11 and clone JW4, except for two ‘silent’ base pair mutations at positions 1299 (G->A) and 3741 (C->A).

**REVERSE TRANSCRIPTASE-PCR ANALYSES OF \(\text{Mdr}\) GENE EXPRESSION**

Total RNA samples, isolated from several rat tissues, were obtained from Ambion (Austin, TX, USA), except for ileum and colon samples that were isolated in our laboratory. Reverse transcription was performed on 5 μg total RNA using random primers, and subjected to relative PCR as described by our laboratory\(^{28}\). For every PCR reaction the level of 18S ribosomal RNA served as internal control, whose quantity was related to internal competitor fragments. The primer sequences were 5'-CTA TTG CGC CGC TAG AGG TG-3' (sense) and 5'-CTG AAC GCC ACT TGT CCC TC-3' (antisense) for 18S, amplifying a 525 bp product, whereas those for \(\text{Mdr1a}\), \(\text{Mdr1b}\), \(\text{Mdr2}\), and \(\text{Bsep}\) fragments have been described previously\(^{28}\). An internal competitor fragment for 18S was made according to Celi et al.\(^{29}\) using the above-mentioned sense primer combined with an antisense primer (5'-CTG AAC GCC ACT TGT CCC TAC GAA TCG CTG CAC CAA C-3'). Ten microliters of PCR product was loaded on a 2.5% agarose gel and stained with ethidium bromide. Images were taken using a charge-coupled device video camera of the ImageMaster VDS system (Pharmacia, Uppsala, Sweden).
RESULTS

CLONING OF MDR1A CDNA FROM RAT LIVER

The full-length rat Mdr1a cDNA was cloned from rat liver by a combination of screening of a liver cDNA library and PCR. Following an initial screening round 8 positive plaques were identified (designated clones JW1-8), after two consecutive hybridization rounds 5 of which were successfully purified and their DNA was isolated. Partial sequence data were obtained for all 5 bacteriophage inserts. The sequences of 2 clones (JW3 and JW7) were identical to the rat Mdr1b cDNA, the sequences of JW1 and JW4 were similar to the mouse Mdr1a gene, while clone JW6 was not related to any mdr sequence. Southern blotting analysis revealed that only clone JW1 and JW4 hybridized with a specific Mdr1a probe (data not shown). The largest rat Mdr1a insert (clone JW4) was approximately 4.0 kb and was characterized by comparison to the mouse Mdr1a cDNA. Clone JW4 begins approximately 900 bp 3’ to the start of translation. Since a second library screening round using a more 5’-located probe did not result in isolation of a longer Mdr1a clone, we obtained the remaining 5’-region of the cDNA by PCR.

The complete sequence of the rat Mdr1a cDNA is deposited in the GenBank database under accession number AF257746. This sequence is 4927 nucleotides long with an open reading frame of 3812 nucleotides (including termination codon). The open reading frame encodes a protein of 1272 amino acids with 12 transmembrane-spanning segments, two potential N-glycosylation sites in the first extracellular loop, and the typical structural features of ATP-binding cassette transport proteins (Figure 1). A comparison of the amino acid sequence of the Mdr1a protein with other members of the ABC transporter superfamily is given in Table 2.

**Figure 1. DEDUCED AMINO ACID SEQUENCE OF RAT MDR1A.** Potential transmembrane spanning segments (TMS1-TMS12) were assigned by comparison to hamster and mouse Mdr1a proteins as well as using the TopPred2 program44. TMS are bold-underlined. The Walker A and B motifs45 are indicated in bold-italics, whereas the ABC transporter signatures46 are indicated in italics. Two potential extracellular N-glycosylation sites are marked with an asterisk. Binding sequences of the monoclonal antibody C219 are indicated in bold (C219)47.
Chapter 2: Cloning of Rat Mdr1a cDNA

Tissue distribution of members of the ABC subfamily B in rats

RT-PCR analysis was used to study the tissue distribution of gene transcripts in rats since this assay is more sensitive, specific and quick than Northern analysis. Ileum and ovary had the highest level of Mdr1a mRNA expression (Figure 2). Expression of Mdr1a mRNA was also easily detected in brain, testicle, kidney, liver and colon, whereas low expression was observed in heart and spleen. No Mdr1a mRNA transcripts were detected in lung, thymus and 10-12 day embryos. In rats, Mdr1b expression differs from Mdr1a expression in that the tissues with the highest Mdr1b levels are, apart from ovary, lung (when corrected for low 18S rRNA levels) and heart, with much lower levels detected in spleen, thymus, embryo, liver, and ileum. No Mdr1b expression was observed in brain, testicle, kidney, and colon. High Mdr2 mRNA levels were found in ovary and liver, whereas much lower Mdr2 expression was detected in heart and spleen. This agrees with previous observations30. In accordance with Gerloff et al.14, we found Bsep mRNA expression exclusively in liver.

Discussion

In the present study we report on the cloning and tissue distribution of rat Mdr1a cDNA. It is remarkable that the Mdr1a protein consists of 1272 amino acids, whereas the length of other mammalian P-glycoproteins ranges from 1276 to 1280 amino acids. Compared with mouse Mdr1a, rat Mdr1a lacks four consecutive amino acids in the first extracellular loop. Since five independent PCR clones comprising the corresponding cDNA region yielded the same sequence, we conclude this is not due to a possible cloning artefact. Moreover, when the amino acid sequences of other drug transporting P-glycoproteins are compared, it is clear that the most divergent segments are the amino terminus and the short so-called linker domain joining the two homologous halves2.

Rat Mdr1a gene expression is not restricted to only a few tissues, although it is notably different from the expression of other Mdr genes (Figure 2), and it resembles that of mouse Mdr1a16,17. Except for liver and ovary, we observed a complementary expression of the Mdr1a and Mdr1b genes: tissues with high Mdr1a expression displayed low Mdrb expression (e.g. brain, testis, intestine, kidney), and vice versa (e.g. lung, heart, thymus, embryo). This is consistent with the protective function assigned to both Mdr1a and Mdr1b. On the other hand, the patterns of differential expression in rat tissue may suggest that the two drug transporting Pgp isoforms may play alternative roles in selectively regulating the intracellular levels of (yet unidentified) endogenous substrates in
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the various tissues. Mdr2 and Bsep are preferentially, if not exclusively, expressed in liver, being compatible with their specialized functions in hepatobiliary transport, and previous reports.

Currently we cannot conclude whether functional similarities or differences exist between the human MDR1, rat Mdr1a and rat Mdr1b. This information may be of interest not only from a biochemical, but also from a pharmacological perspective, since it has been demonstrated that drug transporting Pgp isoforms play an important role in the absorption, distribution, and elimination of drugs. During drug development, detailed knowledge of the functional properties of each individual transporter may therefore eventually result into a higher predictive value with regard to elimination, bioavailability and safety of Pgp substrates.

In conclusion, we have isolated the rat Mdr1a cDNA. Tissue distribution studies revealed that Mdr1a gene transcripts are abundantly expressed in ileum and ovary, and to a lesser extent in liver. We expect that, analogous to mice, Mdr1a may be a major determinant of intestinal and biliary clearance of substances in rats.

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