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Functional characterization of the mouse organic-anion-transporting polypeptide 2

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Abstract

We have isolated and functionally characterized an additional murine member of the organic-anion-transporting polypeptide (Oatp) family of membrane transport proteins from mouse liver. The 3.6 kb cDNA insert contains an open reading frame of 2010 bp coding for a 670 amino acid protein. Based on its amino acid identity of 88% to the rat Oatp2, it is considered the mouse Oatp2 orthologue. Functional expression in *Xenopus laevis* oocytes demonstrated that mouse Oatp2 transports several general Oatp substrates such as estrone-3-sulfate, dehydroepiandrosterone sulfate (DHEAS), ouabain and BQ-123 but hardly any taurocholate nor rocuronium or deltorphin II. The high-affinity rat Oatp2 substrate digoxin is transported with a rather low affinity with an apparent K_m value of 5.7 μM. Bromosulfophthalein (BSP), a substrate not transported by the rat Oatp2, is transported very well by mouse Oatp2. Northern blot analysis demonstrated a predominant expression in the liver with additional signals in kidney and brain. Using fluorescence *in situ* hybridization, the Oatp2 gene (gene symbol *Slc21a5*) was mapped to chromosome 6G1–G3. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Mouse; Transport protein; Organic anion

1. Introduction

The liver plays an essential role in the elimination of a wide variety of endogenous and exogenous compounds. The first step in this hepatic elimination is uptake from the portal blood across the sinusoidal membrane of hepatocytes. Many uptake carriers have been cloned from rat and human liver, among them several members of the organic anion transporting polypeptide (rodent: Oatps; human: OATPs) family of membrane transporters [1–8]. Oatps/OATPs, which are classified within the SLC21A family of solute transporters, mediate Na^+-independent transport of bile salts, bromosulfophthalein (BSP), steroid hormones and their conjugates, thyroid hormones, cardiac glycosides, linear and cyclic peptides, bulky organic cations and various drugs [6,9]. In general, the substrate specificities of the individual Oatps/OATPs overlap considerably, but unique features such as a preference of Oatp1 (*Slc21a1*) for organic anions, of Oatp2 (*Slc21a5*) for the cardiac glycoside digoxin and of OATP-A (*SLC21A3*) for organic cations were demonstrated [2,6,10]. Most Oatps are broadly expressed in different organs such as liver, kidney and brain with the exception of Oatp4 (*Slc21a10*), OATP-C (*SLC21A6*) and OATP8 (*SLC21A8*), the expression of which seems to be restricted to the liver [4,8,11]. Differences in substrate specificity and tissue distribution make it difficult to assign orthologous rat and human Oatps/OATPs [6]. Therefore, it is important to clone the mouse Oatps and to identify their chromosomal localization as the human–mouse synteny is well established and thus possible human orthologues of rodent Oatps can more easily be identified in the mouse. In addition, the mouse offers the possibility to knock out individual *Oatp* genes in order to assess the physiological importance of these carriers.

Abbreviations: Oatp/OATP, organic anion transporting polypeptide; BSP, bromosulfophthalein; SSC, standard saline citrate (0.15 M NaCl/0.015 M sodium citrate); FISH, fluorescence in situ hybridization; DHEAS, dehydroepiandrosterone sulfate; STS, sequence tagged site; RH, radiation hybrid

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The first mouse orthologue to be cloned and characterized was Oatp1 [12]. Mouse Oatp1 is likely to be the orthologue of rat Oatp1 because of similar substrate specificities and tissue distributions, but its chromosomal localization on the X chromosome is not syntenic with the localization of any cloned human OATP [12]. In contrast, mouse Oatp3 is localized on chromosome 6 [3], which is syntenic with human chromosome 12p12 where several human OATPs are located [8,13]. The chromosomal localization of additional mouse Oatps has not been studied yet, but this information is needed to clarify the relationship between rodent and human Oatps/OATPs.

Recently, the cloning and molecular characterization of mouse Oatp4 (lst-1) [14], mouse Oatp5 (*Slc21a13*) [15] and mouse Oatp2 [16] were described by the same group. Unfortunately, no functional data were reported so far for any of the isolated proteins. Without demonstration of functional transport, only speculations based on knowledge derived from other species can be made about the potential physiological role of the protein. However, with the option of knockout mice, it is crucial to understand mouse physiology and thus also to functionally characterize newly cloned mouse Oatps. Therefore, we report here the isolation of a functional mouse Oatp2, its molecular characterization, and its chromosomal localization.

2. Materials and methods:

2.1. Animals

Female *Xenopus laevis* were purchased from the African *Xenopus* facility c.c., Noordoek, R. South Africa.

2.2. Materials

[^{3}H]Digoxin (703 GBq/mmol), [^{3}H]oestrone-3-sulfate (1.96 TBq/mmol), [^{3}H]dehydroepiandrosterone sulfate (DHEAS, 2.22 TBq/mmol), [^{3}H]taurocholic acid (111 GBq/mmol), [^{3}H]ouabain (610.5 GBq/mmol) and [^{3}H] deltorphin II (1.52 TBq/mmol) were obtained from NEN Life Science Products AG (Boston, MA, USA). [^{3}H] BQ-123 (1.33 TBq/mmol) and deoxycytidine 5′ [α-^{32}]triphosphate (111 TBq/mmol) were obtained from Amersham Pharmacia Biotech AB, Uppsala, Sweden. [^{14}C]Rocuronium (54mCi/mmol) was the kind gift of Organon International BV (Oss, The Netherlands). [^{35}S]-BSP (2.5 Ci/mmol) was synthesized as described [17].

2.3. Isolation of the mouse Oatp2 cDNA clone and its functional characterization in X. laevis oocytes

To isolate the mouse orthologues of the human OATP-A [5], a hybridization probe was generated by RT-PCR using mouse liver mRNA and OATP-A specific primers [12]. The amplified product of 685 bp was 93% identical to the rat Oatp2 cDNA [2] and successfully used to isolate the mouse Oatp1 [12]. Primers derived from this cDNA (forward:5′-CAGGAATGACCATTGGCCC-3′; reverse: 5′-CCTTGATTTTTTTCTCTGTGC-3′) were then used to screen a mouse liver Rapid-Screen cDNA library panel (OriGene Technologies, Rockville, MD, USA) by PCR. This resulted in the isolation of a single cDNA clone that represented mouse Oatp2 but was missing the first 70 amino acids. Using 5′ RACE, the missing cDNA was obtained, cloned and sequenced. To obtain the full-length mouse Oatp2 cDNA, the two overlapping fragments were individually PCR amplified using the proofreading enzyme TurboPfu (Stratagene, La Jolla, CA, USA). After gel purification, these two fragments were used as a template for another PCR with the flanking primers and one long fragment was obtained and cloned into the pUC19 vector. Sequencing revealed that the resulting cDNA insert had a unique *Not*I site at its 3′ end (from the original pCMV6-XL4 vector) and a unique *Sal*I site at its 5′ end (from the pUC19 vector) and therefore could be directionally sub-cloned into the *Not*I/*Sal*I cut pSPORT1. Given the low error rate of TurboPfu, we only worked with one independently isolated clone that was sequenced on both strands using an ALFexpress (Amersham Pharmacia Biotech) and used for all subsequent experiments.

For in vitro synthesis of cRNA, the cDNA clone was linearized with *Not*I and capped cRNA was synthesized using the mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX, USA). *X. laevis* oocytes were prepared and handled as described previously [18] and after an overnight incubation at 18 °C, healthy oocytes were injected with 5 ng of Oatp2 cRNA. After 2–3 days in culture, uptake of the indicated substrates was measured at 25 °C in a medium containing 100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes/Tris, pH 7.5, as described previously [19].

2.4. Northern blot analysis

A commercially available Northern Blot (ClonTech, Palo Alto, CA, USA) was prehybridized for 30 min at 55 °C in ExpressHyb hybridization solution and then hybridized for 60 min at 55 °C with a PCR amplified ^{32}P-labelled mouse Oatp2 cDNA probe (nts 611–1276 of AB031814). The blot was washed twice for 5 min at room temperature with 2× standard saline citrate (SSC)/0.1% SDS, followed by 15 min at 45 °C with 0.1× SSC/0.1% SDS. The blot was then exposed to autoradiography film at −70 °C overnight. To confirm equal loading, the blot was hybridized with a human β-actin probe as a control.

2.5. Fluorescence in situ hybridization analysis (FISH)

FISH was performed as described previously [12]. The *Oatp2* containing BAC clone was labelled using the Gibco Nick Translation System with digoxigenin-dUTP (Roche

Diagnostics, Germany), while the chromosome 6 painting-probe (Cambio, Cambridge) was labelled with biotin-16-dUTP.

3. Results and discussion

3.1. Isolation and characterization of cDNA and amino acid sequence

To functionally characterize the mouse orthologue of the rat Oatp2 [2], we isolated a cDNA clone of 3634 bp including a poly(A) tail of 28 nucleotides. This cDNA insert consisted of 67 bp of 5′ untranslated sequence followed by 2010 bp of open reading frame and 1557 bp of 3′ untranslated sequence. A closer inspection of the short 5′ untranslated region revealed that it started almost at the end of exon 3 and contained, similar to one of the published mouse Oatp2 clones [16], an additional 4-bp sequence (ACAG) between exon 3 and exon 4. The open reading frame encoded a protein of 670 amino acids with a calculated molecular mass of 73899 Da (Fig. 1). The coding region of this cDNA clone isolated form a Swiss Webster mouse liver library was nearly identical to the cDNA isolated from a BALB/c library (GenBank accession number AB031814 [16]). There were 12 nucleotide changes that resulted in eight amino acid replacements: I301V (AB031814 amino acid residue listed first), G391A, E398D, P428S, D438N, D501N, E505D and T552A. These differences are most likely due to the different mouse strains used to isolate the cDNAs. Interestingly, five of the eight amino acid residues of the BALB/c sequence are identical to the mouse Oatp1 amino acid sequence. However, since there is no functional data available for the BALB/c sequence [16], we only can speculate that such amino acid changes would not change the substrate specificity of the transport protein. Comparisons of the available amino acid sequences revealed 80%, 98%, 83%, 40% and 44% identity to mouse Oatp1 (AF148218, gene symbol *Slc21a1*), Oatp2 (AB031814, *Slc21a5*), Oatp3 (AF240694, *Slc21a7*), Oatp4 (AJ271682, *Slc21a10*) and Oatp14 (also called organic anion transporter 2, AY007379, *Slc21a14*). Highest amino acid sequence identities to human OATPs were found to be 72%, 43%, 43% and 44% to Oatp-A (U21943, *SLC21A3*), OATP-C (AB026257, *SLC21A6*), OATP8 (AJ251506, *SLC21A8*) and OATP-F (AF260704, *SLC21A14*), respectively.

The alignment of the amino acid sequences of mouse Oatp2 with its rat orthologue Oatp2 and its closest mouse relatives Oatp1 and Oatp3 is shown in Fig. 1. Two major differences exist between mouse and rat Oatp2. In contrast to rat Oatp2, the mouse protein does not have an amino acid deletion at position 117 and the C terminal end of mouse Oatp2 corresponds more to the sequences of Oapt1 and Oatp3. Thus, all mouse clones are composed of 670 amino acids, whereas rat Oatp2 contains only 661 amino acids. Whether these findings could have implications for the substrate specificity of mouse, Oatp2 was tested with a series of typical Oatp substrates in *X. laevis* oocytes.

3.2. Functional characterization of mouse Oatp2 in X. laevis oocytes

Although the members of the Oatp family have broad and overlapping substrate specificities, organic anions such as BSP and the opioid peptide deltorphin II were identified as preferred substrates of rat Oatp1, whereas the cardiac glycoside digoxin and bulky organic cations such as rocuronium are more specific for rat Oatp2 [2,10,20,21]. In a substrate comparison, mouse Oatp1 showed the same substrate specificity as rat Oatp1 [12]. Table 1 summarizes the transport rates of various substrates determined in mouse Oatp2 cRNA-injected oocytes. Thereby, digoxin and BQ-123 showed the highest transport rates. Ouabain, BSP, estrone-3-sulfate and DHEAS were also identified as substrates for mouse Oatp2, while taurocholate, deltorphin II and rocuronium were hardly or not transported at all. Rat and mouse Oatp2 share the unique feature of digoxin transport, but surprisingly mouse Oatp2 also transported BSP, which is not a substrate of rat Oatp2 [1]. Furthermore, mouse Oatp2 did not transport rocuronium, which is an additional difference between rat and mouse Oatp2 [21]. These differences in the substrate specificities of rat and mouse Oatp2 emphasize the importance of the functional characterization of orthologous gene products since functional predictions that are based on protein homologies could be misleading.

Since the binding sites of rat and mouse Oatp2 have not been identified yet, it is tempting to speculate that the additional amino acid at position 117 and/or the different C terminal end compared to rat Oatp2 might be involved in BSP transport and the absence of rocuronium uptake by mouse Oatp2.

As differences in the substrate specificity between rat and mouse Oatp2 were found, transport of the typical Oatp2 substrate digoxin was further investigated. As depicted in Fig. 2, digoxin uptake by mouse Oatp2 was saturable and yielded an apparent K_m value of 5.7 μM. The affinity of mouse Oatp2 for digoxin is much lower than the affinity of rat Oatp2 for which an apparent K_m value of 0.24 μM was reported [2]. Thus, rat and mouse Oatp2 differ not only in their substrate specificity, but also in their affinity for digoxin.

3.3. Tissue distribution of mouse Oatp2

The tissue distribution of mouse Oatp2 was determined by Northern blot analysis using a cDNA probe derived from the open reading frame of the transporter. As shown in Fig. 3, the strongest signal was obtained in liver and weaker signals in kidney and brain. Several transcript sizes were detected in liver and kidney, whereas in brain a single band of 4.4 kb was found. The signals in the liver and brain are in

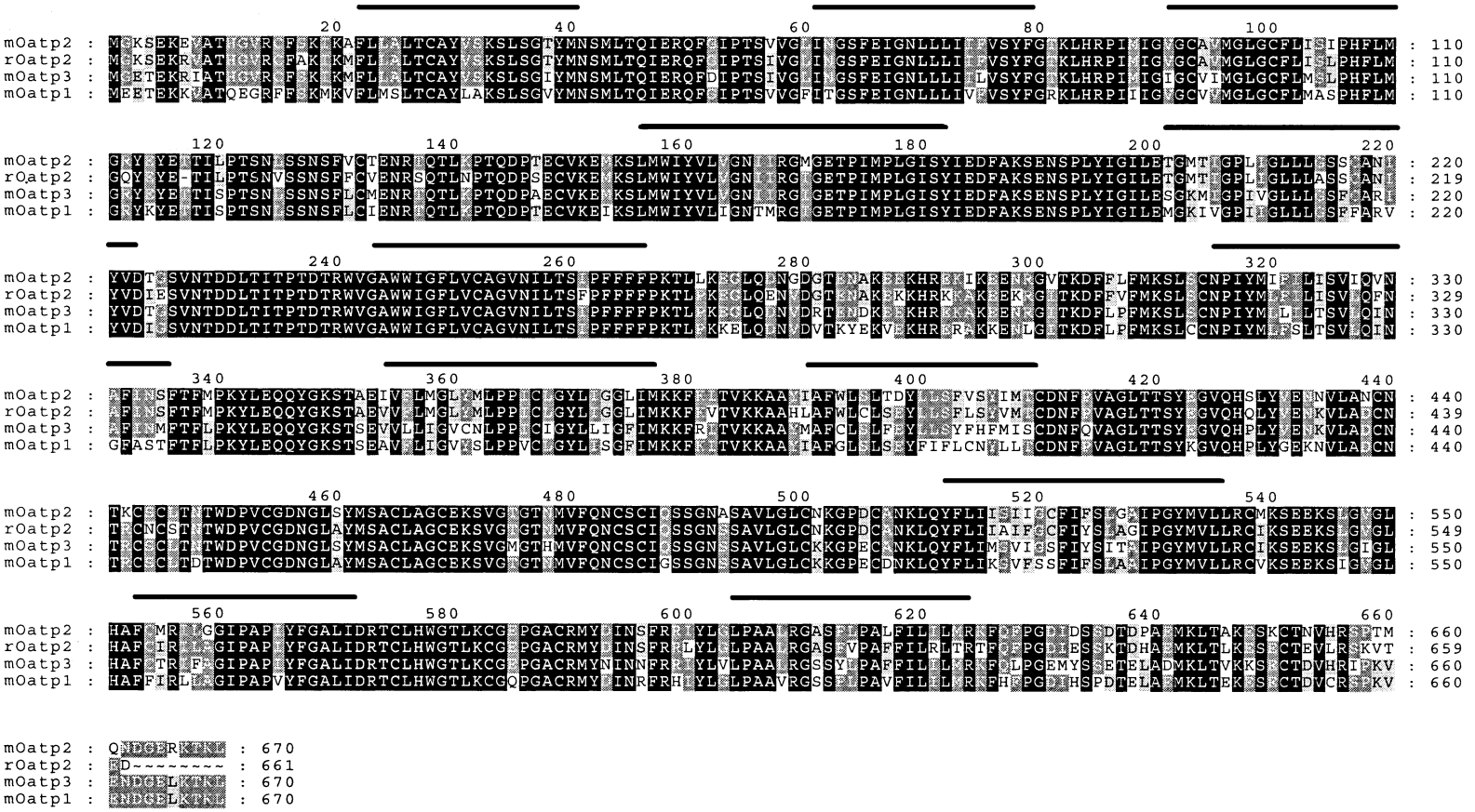

Fig. 1. Amino acid alignment of mouse Oatp2 (mOatp2), rat Oatp2 (rOatp2) mouse Oatp3 (mOatp3) and mouse Oatp1 (mOatp1). Amino acids identical in all four proteins are boxed in black, amino acids identical in three proteins are boxed in dark grey while amino acids identical in only two of the proteins are boxed in light grey. The twelve putative transmembrane domains are indicated with lines.

Table 1
Substrate specificity of mouse Oatp2 in *X. laevis* oocytes

Substrate	Uptake	
	mOatp2	Ratio
Digoxin (0.5 μM)	246 ± 56	13.7
BQ-123 (0.67 μM)	3 ± 0.8	10
BSP (2 μM)	645 ± 128	4.6
DHEAS (0.2 μM)	14 ± 2.4	3.7
Estrone-3-sulfate (0.5 μM)	43 ± 7	3.3
Ouabain (2.4 μM)	40 ± 6	2.7
Taurocholate (5 μM)	188 ± 60	1.5
Rocuronium (10 μM)	1120 ± 180	1.3
Deltorphin II (0.2 μM)	11 ± 1.4	0.9

Values represent means ± S.D. of 9 to 12 determinations and are given in femtomoles per oocyte. Ratios correspond to uptake in cRNA-injected oocytes divided by uptake in water-injected control oocytes. *X. laevis* oocytes were injected with 5 ng of mouse Oatp2 cRNA, and after 3 days in culture, uptake of the indicated substrates was measured for 30 min.

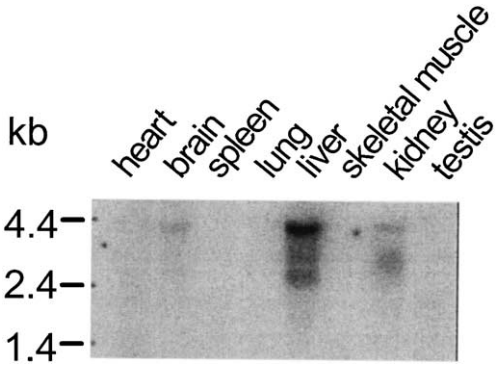

Fig. 3. Northern blot analysis of the tissue distribution of Oatp2. A commercially available blot (ClonTech) containing 2 μg of mRNA from different tissues per lane was hybridized with an Oatp2-specific cDNA probe as described in Materials and methods. Molecular size standards are indicated on the left.

accordance with the results of Ogura et all. [16]. However, since the hybridization probe that was used in this study has a high identity to other Oatps (e.g. Oatp1, Oatp3 and Oatp5), the signal obtained for the kidney could be due to cross-hybridization with an Oatp that is highly expressed in this tissue. Therefore, real-time PCR experiments with Oatp2-specific primers were performed and demonstrated that Oatp2 levels in the kidney were less than 0.5% of the expression in the liver (data not shown). Thus, the kidney signal obtained on the Northern blot might represent expression of another Oatp. In contrast to Ogura et al. [16] who detected a mouse Oatp2 transcript also in heart and lung, even after a 48 h exposure of the blot, no signal was detected in heart, spleen, lung, skeletal muscle and testis in this study (not shown). While Ogura et al. [16] used a probe derived from exon 3 located in the 5′ untranslated region, we used a probe derived from the protein coding region of Oatp2. As only the exon 3 probe gave a signal in heart and lung, it is not very likely that mouse Oatp2 plays a role in digoxin transport in the heart. The mRNA distribution of mouse Oatp2 is therefore in accordance with the mRNA distribution of rat Oatp2, which was also found to be expressed in liver, kidney and brain [2].

3.4. Chromosomal localization of mouse Oatp2

Finally, the chromosomal localization of mouse Oatp2 was determined by FISH. As demonstrated in Fig. 4, the mouse *Oatp2* gene was localized to chromosome 6 at locus 6G1–G3. In addition, searching NCBIs UniSTS, a resource that reports information about markers or sequence tagged sites (STS), we could locate the mouse Oatp2 gene with the radiation hybrid (RH) marker 617897 close to the location of mouse Oatp3 (*Slc21a7*) (RH D6Mit58) [3], mouse Oatp5

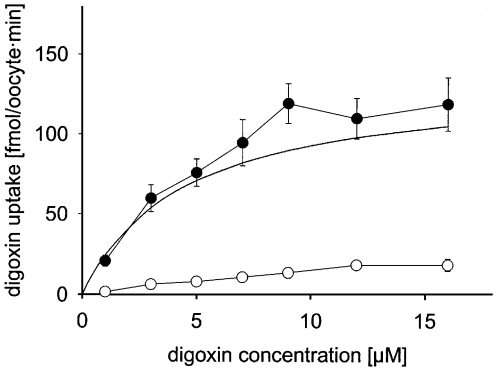

Fig. 2. Kinetics of Oatp2-mediated digoxin uptake into *X. laevis* oocytes. Oocytes were injected with 5 ng of Oatp2-cRNA (●) or with 50 nl of water (○) and after 3 days in culture, initial (10 min) uptake of [^{3}H]digoxin uptake was performed in the presence of increasing digoxin concentrations at 25 °C. The values represent means ± S.E. of 9–12 determinations. After subtracting the values of the water-injected oocytes, the data were fitted by nonlinear regression analysis to the Michaelis–Menten equation and plotted as a line.

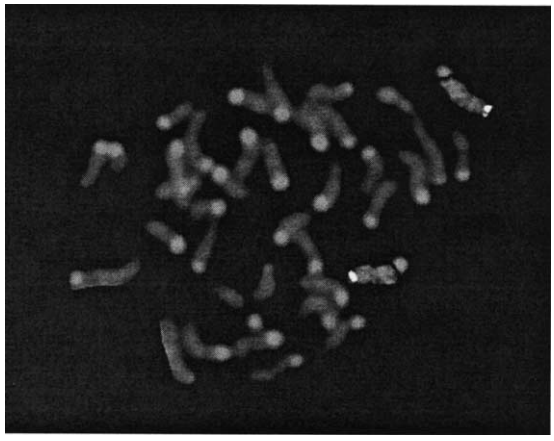

Fig. 4. Chromosomal mapping of the mouse *Oatp2* gene to chromosome 6G1–G3. A two-colour FISH analysis was performed with the Oatp2 probe (green signal) and a chromosome 6 painting probe (red signal). Where the two signals are superimposed, a yellow signal is observed and the green signal is no longer visible.

(*Slc21a13*, RH 622831) and Oatp14, the mouse orthologue of human OATP-F (close to RH 662222). This region is syntenic with human chromosome 12p12 where a cluster of human OATP (*SLC21A*) genes is located, which includes *OATP-A*, *OATP-C*, *OATP8* [8,13] and *OATP-F* (*SLC21A14*) [22]. Thus, similar to this human OATP gene subfamily, there seems to exist a mouse subfamily, which, given its clustered occurrence on chromosome 6, most likely arose through several gene duplications. The mouse *Oatp1* gene is so far the only Oatp gene in this closely related group of membrane transporters that is not located on chromosome 6 but localized to XA3–A5 [12]. Based on the amino acid identity, OATP-A is the most closely related human OATP on chromosome 12 for all, mouse Oatp1, Oatp2, Oatp3 and Oatp5. This could be explained by gene duplications in rodents after they diverged from primates, and makes it impossible to determine the true orthologues transport systems in rodents for human OATP-A. These findings stress once more that for certain gene products, the interspecies correlations are far from evident and that it is absolutely necessary to functionally characterize transport proteins before conclusions from animal models are adopted for other species.

In conclusion, we have isolated and functionally characterized mouse Oatp2. It is very likely the orthologue of rat Oatp2 because of its high amino acid identity and its similar substrate specificity and tissue distribution. However, some differences between mouse and rat Oatp2 were found such as, e.g. the ability to mediate BSP transport and a much lower digoxin affinity of mouse Oatp2, which can be explained by subtle differences in the amino acid sequence (e.g. an amino acid deletion in rat Oatp2 at position 117 or the different C terminal ends). The mouse Oatp2 gene was localized to chromosome 6, which is syntenic with human chromosome 12. The identification of the gene cluster on mouse chromosome 6 explains that several rodent Oatps have a high amino acid identity to one single human OATP, OATP-A, if the gene duplication in rodents occurred after speciation.

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