# Expression cloning of a rat liver Na⁺-independent organic anion transporter

(hepatocytes/sulfobromophthalein/bile acid transport)

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**ABSTRACT** Using expression cloning in *Xenopus laevis* oocytes, we have isolated a cDNA encoding a rat liver organic anion-transporting polypeptide (oatp). The cloned oatp mediated Na⁺-independent uptake of sulfobromophthalein (BSP) which was Cl⁻-dependent in the presence of bovine serum albumin (BSA) at low BSP concentrations (e.g., 2 μM). Addition of increasing amounts of BSA had no effects on the maximal velocity of initial BSP uptake, but it increased the $K_m$ value from 1.5 μM (no BSA) to 24 μM (BSA/BSP molar ratio, 3.7) and 35 μM (BSA/BSP ratio, 18.4). In addition to BSP, the cloned oatp also mediated Na⁺-independent uptake of conjugated (taurocholate) and unconjugated (cholate) bile acids. Sequence analysis of the cDNA revealed an open reading frame of 2010 nucleotides coding for a protein of 670 amino acids (calculated molecular mass, 74 kDa) with four possible N-linked glycosylation sites and 10 putative transmembrane domains. Translation experiments *in vitro* indicated that the transporter was indeed glycosylated and that its polypeptide backbone had an apparent molecular mass of 59 kDa. Northern blot analysis with the cloned probe revealed crossreactivity with several mRNA species from rat liver, kidney, brain, lung, skeletal muscle, and proximal colon as well as from liver tissues of mouse and rabbit, but not of skate (*Raja erinacea*) and human.

The liver efficiently extracts a wide variety of amphipathic organic anions, including bile acids, bilirubin, and sulfobromophthalein (BSP), from sinusoidal blood plasma (1). This hepatic organic anion uptake is mediated by Na⁺-dependent and Na⁺-independent basolateral (i.e., sinusoidal and lateral) membrane transport systems (2, 3). A Na⁺-dependent taurocholate-cotransporting polypeptide (Ntcp) has been cloned (4) and characterized in mammalian liver (5). Furthermore, a hepatocellular Cl⁻-dependent BSP uptake system has been functionally expressed in *Xenopus laevis* oocytes (6). Interestingly, the expressed BSP uptake activity was inhibited not only by non-bile acid organic anions such as bilirubin and indocyanine green but also by the bile acids taurocholate and cholate, suggesting that this transport system exhibits a broad substrate "specificity" for a variety of amphipathic organic anions (6). In the present study we have cloned and sequenced¶ this Na⁺-independent basolateral organic anion-transporting polypeptide (oatp) of rat liver. The results demonstrate that a single glycoprotein with an apparent molecular mass of 71 kDa (*in vitro* translation) is sufficient to account for carrier-mediated Na⁺-independent uptake of multiple organic anions in mammalian liver.

## MATERIALS AND METHODS

**Frogs.** Mature *X. laevis* females were purchased from H. Kähler (Hamburg, F.R.G.) and kept under standard conditions (7).

**Transport Assays.** [³⁵S]BSP was prepared at a specific activity of 3–4 Ci/mmol (1 Ci = 37 GBq) as described (8). [G-³H]taurocholic acid (2.1 Ci/mmol) and (2,4-³H)cholic acid (25 Ci/mmol) were obtained from New England Nuclear. Prior to study, frog oocytes were washed twice at 25°C in Cl⁻-containing or Cl⁻-free uptake medium. Tracer uptake was studied as indicated in the corresponding figure legends. After the indicated times, uptake was stopped by washing the oocytes with ice-cold NaCl-containing uptake medium, which in the case of BSP (but not the bile acids) was also supplemented with 5% bovine serum albumin (BSA), and the oocyte-associated radioactivity was determined (4, 6).

**Construction and Screening of a cDNA Library.** Rat liver mRNA was prepared and size-fractionated as described (6). cDNA was synthesized from a functionally active size class of mRNA of 2.0–3.5 kb (6) by using the Superscript kit (BRL) and ligated unidirectionally into the *Sal* I and *Not* I sites of plasmid pSPORT1 (BRL). Recombinant plasmids were introduced into *Escherichia coli* WM1100 by electroporation (Gene Pulser; Bio-Rad), resulting in a library of 2.2 × 10⁵ clones. Pools of 500 colonies of the initial unamplified cDNA library were then grown overnight on agar plates in the presence of ampicillin. The plates were replicated onto nylon filters (Hybond N, Amersham). After 4 hr of incubation at 37°C the bacteria were scraped from the filters and amplified in culture overnight. For *in vitro* synthesis of mRNA, plasmid DNA was isolated from each filter pool by using the Qiagen plasmid kit (Diagen Gmbh, Düsseldorf, F.R.G.). After linearization with *Not* I, capped mRNA was synthesized with T7 RNA polymerase (Promega) as described (9). Unincorporated nucleotides were removed with Quick Spin columns (Boehringer Mannheim), and the synthesized cRNA was recovered by ethanol precipitation and resuspended in water for injection into oocytes. Routinely 50 ng of cRNA was injected per oocyte, and 3 days later Cl⁻-dependent BSP uptake was determined as described (6). Once a positive filter had been identified, the colonies were further subdivided and screened (10) until a single positive clone was eventually isolated.

**Sequence Analysis of oatp cDNA.** Double-stranded cDNA was sequenced in both directions with a T7Sequencing kit (Pharmacia). oatp cDNA was sequenced by using either unidirectionally deleted clones (Erase-a-Base; Promega) or specially synthesized oligonucleotide primers. Nucleotide

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and amino acid sequence analyses were performed with the DNA and protein sequence analysis program DNASIS/PROSIS (Pharmacia). Putative membrane-spanning domains were determined according to Klein *et al.* (11).

**Northern Blotting.** Samples of mRNA (5–20 $\mu$g) from various rat tissues and from livers of various species were electrophoresed in a 1% agarose/formaldehyde gel, transferred to a nylon membrane (Hybond N), and the separated RNAs were UV crosslinked by using the autocrosslink mode (Stratalinker; Stratagene). The blots were hybridized in a hybridization oven (Appligene, Illkirch, France) with three different cDNA probes which were labeled with [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol; Amersham) by the random prime labeling kit (Boehringer Mannheim). Sequences related to oatp were detected either with a *Pfl*MI fragment (nucleotides 712–1447) or a *Nco* I–*Eco*NI fragment (nucleotides 86–2120). Glyceraldehyde-3-phosphate dehydrogenase was detected with a human cDNA probe (Clontech). For high-stringency conditions the blots were hybridized at 42°C in 50% formamide/5× SSC (1× is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0)/5× Denhardt's solution/0.5% SDS containing denatured salmon sperm DNA at 200 $\mu$g/ml. After hybridization the filters were washed twice at room temperature for 5 min in 2× SSC/0.1% SDS, followed by 15 min at 58°C in 0.1× SSC/0.1% SDS. For low-stringency conditions the hybridization was performed at 42°C in 30% formamide/5× SSC, 5× Denhardt's solution/ 0.5% SDS containing denatured salmon sperm DNA at 200 $\mu$g/ml. The filters were then washed twice at room temperature for 5 minutes in 2× SSC/0.1% SDS, followed by 15 min at 50°C in 2× SSC/0.1% SDS.

## RESULTS

From the cDNA library, a single positive clone was isolated, which, when transcribed and expressed in oocytes, conferred Na$^+$-independent BSP uptake. Injection of oatp cRNA into oocytes stimulated BSP uptake to a markedly higher degree than injection of total rat liver mRNA (Fig. 1). Similar to observations in cultured hepatocytes (12, 13) and in oocytes injected with total rat liver mRNA (6), oatp cRNA-injected oocytes also expressed Cl$^-$-dependent BSP uptake in the presence of 2 $\mu$M BSP and a 3.7-fold excess of BSA. This Cl$^-$-dependent portion of BSP uptake was saturable with increasing substrate concentration (data not shown). The apparent $K_m$ value was 5.6 ± 2.9 $\mu$M (mean ± SD), which is identical to the value obtained with total rat liver mRNA (6). This observation strongly suggests that the cloned BSP transporter corresponds to an endogenous organic anion uptake system of rat hepatocytes.

To more definitively determine the transport properties resulting from oatp expression in *X. laevis* oocytes, we studied the effects of BSA on BSP uptake as well as the substrate specificity of the cloned transporter. As summarized in Table 1, the apparent Cl$^-$ dependence of low-dose (2 $\mu$M) BSP uptake was not seen in the absence of BSA. In addition, omission of BSA increased BSP uptake ≈20-fold. At a BSP concentration of 100 $\mu$M, BSP uptake was further increased by a factor of 6–10 (Table 1). This high-dose BSP uptake could not be stimulated by Cl$^-$ in the presence of BSA, nor had BSA any inhibitory effect on overall BSP uptake (Table 1). These results differ in part from recent studies in cultured rat hepatocytes where Cl$^-$-dependent BSP uptake was seen even at high concentrations of BSA and BSP (unpublished observations). The mechanism for this differential Cl$^-$ dependence of BSP uptake in cultured hepatocytes as compared to oatp-expressing frog oocytes is not known. Its elucidation may require further studies in stably transfected cell lines. BSP uptake into oatp cRNA-injected oocytes was also saturable in the absence of Cl$^-$ (Fig. 2). Without addition of BSA, the apparent $K_m$ for BSP was 1.5
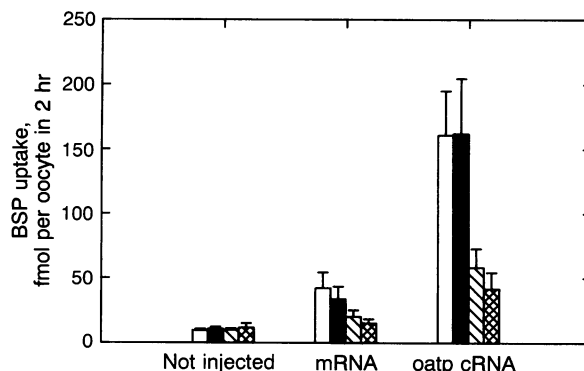


FIG. 1.    Functional expression of oatp cRNA in oocytes. Oocytes were either not injected or injected with 25 ng of total rat liver mRNA or 0.5 ng of oatp cRNA. The cells were cultured for 3 days and 2-hr [$^{35}$S] BSP (2 $\mu$M) uptake values were determined at 25°C in the presence of either 100 mM NaCl (open bars) or 100 mM choline chloride (filled bars) and 1.2 mM MgCl$_2$, 0.8 mM MgSO$_4$, 2.5 mM CaCl$_2$, and 25 mM Hepes/Tris (pH 7.2) or in the presence of 100 mM sodium gluconate (hatched bars) or 200 mM sucrose (cross-hatched bars) and 1.2 mM magnesium gluconate, 0.8 mM MgSO$_4$, 2.5 mM calcium gluconate, and 25 mM Hepes/Tris (pH 7.2). In addition, 7.4 $\mu$M BSA (fraction V, Sigma) was also present in all uptake media (BSA/BSP molar ratio, 3.7). Values represent means ± SD of 12–24 determinations in two separate oocyte preparations.

± 0.3 $\mu$M (mean ± SD; 30 uptake measurements in three separate oocyte preparations). It increased to 24 ± 11 $\mu$M and to 35 ± 12 $\mu$M at BSA/BSP concentration ratios of 3.7 and 18.4, respectively. These results are consistent with previous studies in rat hepatocytes, where BSA caused an ≈10-fold reduction in affinity of BSP for the hepatocyte surface in the absence of Cl$^-$ (12).

In addition to BSP uptake, expression of the cloned oatp in frog oocytes also conferred Na$^+$-independent bile acid uptake. Uptakes of both taurocholate (Fig. 3A) and cholate (Fig. 3B) were stimulated to similar degrees. Interestingly, oatp-induced bile acid uptake was significantly ($P < 0.05$; Student

Table 1.    Effect of BSA on BSP uptake in oatp cRNA-injected oocytes

| Conditions | BSP uptake, pmol per oocyte in 2 hr | |
| --- | --- | --- |
|  | Not injected | cRNA-injected |
| **2 $\mu$M BSP** | | |
|   7.4 $\mu$M BSA | | |
|     NaCl | 0.014 ± 0.008 | 0.14 ± 0.04 |
|     Choline chloride | 0.012 ± 0.008 | 0.13 ± 0.03 |
|     Sodium gluconate | 0.011 ± 0.003 | 0.06 ± 0.02 |
|   No BSA | | |
|     NaCl | 0.096 ± 0.036 | 3.50 ± 0.94 |
|     Choline chloride | 0.044 ± 0.017 | 2.75 ± 1.10 |
|     Sodium gluconate | 0.318 ± 0.258 | 4.90 ± 2.22 |
| **100 $\mu$M BSP** | | |
|   370 $\mu$M BSA | | |
|     NaCl | 0.854 ± 0.230 | 31.5 ± 11.1 |
|     Choline chloride | 0.814 ± 0.285 | 28.0 ± 7.7 |
|     Sodium gluconate | 1.304 ± 0.334 | 30.0 ± 8.7 |
|   No BSA | | |
|     NaCl | 2.17 ± 0.82 | 25.0 ± 9.7 |
|     Choline chloride | 1.70 ± 0.62 | 26.4 ± 8.8 |
|     Sodium gluconate | 1.94 ± 0.21 | 28.3 ± 8.1 |

Oocytes were either not injected or injected with 0.5 ng of oatp cRNA. After 3 days in culture, uptake of the indicated concentration of BSP was measured in the presence or absence of BSA. Values represent means ± SD of 15–22 determinations.

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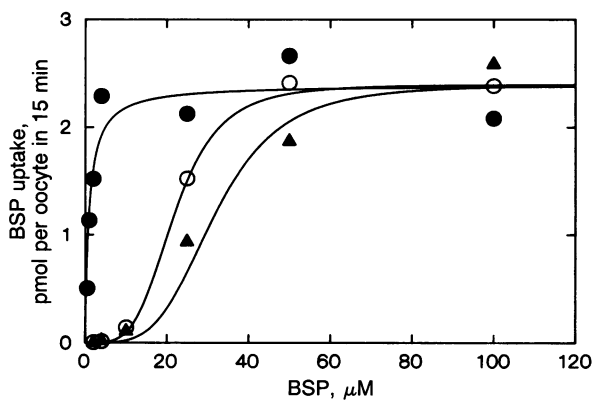*Proc. Natl. Acad. Sci. USA 91 (1994)* 135



FIG. 2. Effect of BSA on the kinetics of BSP transport in oatp cRNA-injected oocytes. Oocytes were injected with 0.5 ng of oatp cRNA. After 3 days in culture, uptake of [$^{35}$S] BSP was measured at 25°C in 0.25–100 $\mu$M BSP in a medium containing sodium gluconate. Uptake time points were 15 min, since in separate experiments linear uptake was demonstrated during 20 min at all substrate concentrations. BSA was either not present ($\bullet$) or added at BSA/BSP ratios of 3.7 (o) and 18.4 ($\blacktriangle$) to the incubation medium. Nonspecific BSP uptake by uninjected oocytes was subtracted from all uptake values. Data represent means of 10 determinations in one representative out of three oocyte preparations. The curves were fitted by nonlinear regression analysis.

*t* test) higher in Cl$^-$-containing medium than in Cl$^-$-free medium, suggesting that Cl$^-$ exerts a stimulatory effect on oatp-mediated Na$^+$-independent bile acid uptake as well. The marked stimulation of both BSP and bile acid uptake by injection of oatp cRNA into frog oocytes indicates that the cloned hepatocellular oatp can mediate Na$^+$-independent uptake of a variety of structurally dissimilar amphipathic organic compounds.

The DNA sequence and the deduced amino acid sequence of the Na$^+$-independent "multispecific" oatp are shown in Fig. 4. The total cDNA insert consists of 2759 nucleotides without and of 2814 nucleotides with the cloned poly(A) tail. Based on the Kozak consensus sequence (14), we assigned the initiation site to the first ATG codon at position 87. Consequently, the open reading frame extends over 2010 nucleotides, thus predicting a polypeptide of 670 amino acids with a calculated molecular mass of 74 kDa. Computer-aided analysis of the cloned protein revealed four potential N-linked glycosylation sites and 10 putative membrane-spanning domains (11). A search of the available data bases (January 1993) revealed 35.7% amino acid sequence identity with rat matrin F/G (15) but no significant (i.e., >20%) homology with any other cloned membrane transport proteins.

In separate *in vitro* translation experiments (performed as described in ref. 4), the protein product encoded by the cloned cDNA was identified as a polypeptide with an apparent molecular mass of 59 kDa. The reason for this lower apparent molecular mass of the *in vitro* translated protein product as compared to the theoretical value calculated from the cloned cDNA is unknown. However, a similar discrepancy has been observed for other cloned membrane transport proteins (4) and is most probably due to the complex migratory behavior of membrane proteins in SDS/polyacrylamide gels. When translation was performed in the presence of canine pancreatic microsomes (4), the apparent molecular mass increased to 71 kDa because of glycosylation of the translation product (data not shown). This difference of ≈12 kDa in SDS/polyacrylamide gels suggests glycosylation of at least three if not of all four potential N-linked glycosylation sites (Fig. 4).

Finally, we investigated the tissue and species distribution of the cloned hepatocellular oatp by Northern blot analysis
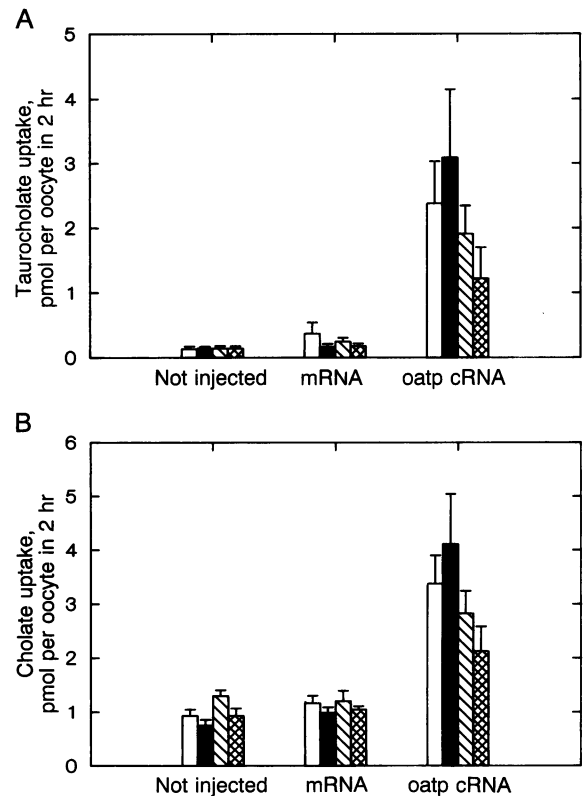


FIG. 3. oatp-mediated bile acid uptake in cRNA-injected oocytes. Oocytes were either not injected or injected with 25 ng of total rat liver mRNA or 0.5 ng of oatp cRNA. After 3 days in culture 2-hr [$^3$H] taurocholate (17 $\mu$M) (*A*) or [$^3$H] cholate (17 $\mu$M) (*B*) uptake values were determined at 25°C in the absence of BSA in 100 mM NaCl (open bars) or 100 mM choline chloride (filled bars) and 1.2 mM MgCl$_2$, 0.8 mM MgSO$_4$, 2.5 mM CaCl$_2$, and 25 mM Hepes/Tris (pH 7.2) or 100 mM sodium gluconate (hatched bars) or 200 mM sucrose (cross-hatched bars) and 1.2 mM magnesium gluconate, 0.8 mM MgSO$_4$, 2.5 mM calcium gluconate, and 25 mM Hepes/Tris (pH 7.2). Values represent means ± SD of 12–24 determinations in two separate oocyte preparations.

(Fig. 5). Under high-stringency conditions (see *Materials and Methods*), a *Pfl*MI fragment derived from the coding region of the cloned cDNA (nucleotides 712–1447) hybridized with several mRNAs from rat liver (4.3, 3.3, 2.5, and 1.4 kb). Similar reactive mRNAs were also found in the kidney (4.3, 3.1, 2.4, and 1.4 kb). In contrast, much weaker signals were obtained with mRNAs isolated from brain (4.0 and 2.8 kb), lung (3.2 kb), skeletal muscle (4.1 and 3.5 kb), and proximal colon (3.1 and 1.8 kb). No hybridization signals were obtained with mRNAs isolated from heart or distal colon (Fig. 5) or from ileum or duodenum (data not shown). If the stringency conditions were further increased by washing the blots at 65°C instead of 58°C in 0.1× SSC/0.1% SDS, positive hybridization with bands at 4.3 and 3.3 kb was observed in liver and kidney, whereas no positive reactions were obtained with samples from the other organs (data not shown). Low-stringency hybridization with both oatp cDNA probes also resulted in the detection of liver mRNAs in mouse (4.2 and 2.4 kb) and rabbit (3.1 kb), while no positive reactions were found with mRNAs from livers of human, skate (*Raja erinacea*), frog, chicken, and turtle (data not shown) although glyceraldehyde-3-phosphate dehydrogenase mRNA could be detected under the same stringency conditions after the filters were stripped (data not shown). These data indicate that multiple forms of oatp with similar homology may be present in the liver and kidney of rat, mouse, and probably also of rabbits. Furthermore, closely related transport polypeptides

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   1   CA TGA GTG TAC TTC TCT CTT GGC TAA GAA AGA CAG CAA AGC AAA GAC TTT TAA AGC GAA GAA GCT GGA AAC AAT CAG AAG AAC    83

   1      M   E   E   T   E   K   K   I   A   T   Q   E   G   R   L   F   S   K   M   K   V   F   L   L   S   L   T     27
  84  ACC ATG GAA GAA ACA GAG AAA AAG ATT GCA ACA CAA GAG GGT AGA TTG TTT TCC AAG ATG AAG GTG TTT CTG CTG TCA TTA ACA   167

  28      C   A   C   L   T   K   S   L   S   G   V   Y   M   N   S   M   L   T   Q   I   E   R   Q   F   D   I   S   T    55
 168  TGT GCA TGT TTA ACC AAA TCA CTA TCA GGA GTA TAT ATG AAT TCC ATG CTC ACA CAA ATA GAG AGA CAA TTT GAT ATC TCC ACA   251
                       *
  56      S   V   A   G   L   I   N   G   S   F   E   I   G   N   L   F   F   I   V   F   V   S   Y   F   G   T   K   L    83
 252  TCT GTA GCT GGA TTA ATC AAT GGG AGC TTT GAG ATT GGA AAC CTT TTT TTC ATT GTA TTT GTG AGT TAT TTT GGA ACA AAA CTG   335

  84      H   R   P   V   V   I   G   I   G   C   V   I   M   G   L   G   C   L   L   M   S   L   P   H   F   F   M   G   111
 336  CAC AGA CCT GTG GTG ATT GGT ATT GGA TGT GTG ATT ATG GGC CTG GGG TGT CTC TTA ATG TCA TTA CCT CAT TTC TTC ATG GGC   419
                                                    *                                              *
 112      R   Y   E   Y   E   T   T   I   S   P   T   G   N   L   S   S   N   S   F   L   C   M   E   N   R   T   Q   T   139
 420  AGA TAC GAA TAT GAA ACA ACG ATC TCA CCT ACA GGC AAC TTG TCC TCA AAC AGC TTC TTG TGT ATG GAG AAC CGA ACA CAG ACC   503

 140      L   K   P   T   Q   D   P   A   E   C   V   K   E   M   K   S   L   M   W   I   C   V   M   V   G   N   I   I   167
 504  TTA AAG CCA ACG CAA GAC CCA GCA GAA TGT GTG AAA GAA ATG AAA TCA TTA ATG TGG ATA TGT GTA ATG GTA GGA AAT ATT ATA   587

 168      R   G   I   G   E   T   P   I   V   P   L   G   I   S   Y   I   E   D   F   A   K   S   E   N   S   P   L   Y   195
 588  CGT GGA ATT GGT GAA ACT CCT ATT GTG CCC TTG GGT ATC TCT TAC ATC GAA GAC TTT GCC AAA TCA GAA AAC TCT CCT TTA TAC   671

 196      I   G   I   L   E   M   G   K   V   A   G   P   I   F   G   L   L   L   G   S   Y   C   A   Q   I   Y   V   D   223
 672  ATT GGG ATT TTA GAA ATG GGG AAG GTT GCT GGC CCA ATT TTT GGA CTT TTG TTG GGA TCT TAC TGT GCA CAA ATT TAT GTA GAC   755

 224      I   G   S   V   N   T   D   D   L   T   I   T   P   S   D   T   R   W   V   G   A   W   W   I   G   F   L   V   251
 756  ATT GGG TCT GTG AAT ACA GAT GAC CTG ACC ATA ACA CCC AGT GAT ACA CGC TGG GTT GGT GCT TGG TGG ATT GGC TTT TTG GTC   839

 252      C   A   G   V   N   I   L   T   S   I   P   F   F   F   L   P   K   A   L   P   K   K   G   Q   Q   E   N   V   279
 840  TGT GCA GGA GTG AAT ATC CTG ACC AGC ATC CCC TTT TTC TTT TTG CCG AAA GCA CTT CCA AAG AAA GGA CAA CAG GAG AAT GTT   923

 280      A   V   T   K   D   G   K   V   E   K   Y   G   G   Q   A   R   E   E   N   L   G   I   T   K   D   F   L   T   307
 924  GCT GTG ACT AAA GAT GGC AAA GTT GAG AAA TAT GGA GGA CAA GCC AGA GAG GAA AAT CTT GGA ATC ACT AAA GAT TTC TTG ACG  1007

 308      F   M   K   R   L   F   C   N   P   I   Y   M   L   F   I   L   T   S   V   L   Q   V   N   G   F   I   N   K   335
1008  TTC ATG AAG AGG CTC TTC TGC AAT CCG ATT TAC ATG CTT TTC ATC CTT ACA AGT GTG CTC CAG GTG AAT GGC TTT ATT AAT AAA  1091

 336      F   T   F   L   P   K   Y   L   E   Q   Q   Y   G   K   S   T   A   E   A   I   F   L   I   G   V   Y   S   L   363
1092  TTT ACG TTC TTG CCC AAA TAC CTG GAA CAG CAG TAT GGA AAA TCT ACT GCA GAG GCA ATA TTC CTC ATA GGT GTT TAT AGC TTA  1175

 364      P   P   I   C   L   G   Y   L   I   G   G   F   I   M   K   K   F   K   I   T   V   K   K   A   A   Y   L   A   391
1176  CCT CCC ATA TGC CTC GGG TAT CTA ATT GGT GGC TTT ATT ATG AAG AAG TTT AAG ATT ACT GTC AAG AAA GCT GCA TAC CTA GCA  1259

 392      F   C   L   S   V   F   E   Y   L   L   F   L   C   H   F   M   L   T   C   D   N   A   A   V   A   G   L   T   419
1260  TTT TGC CTA TCG GTG TTT GAA TAT CTT CTT TTT CTT TGT CAC TTT ATG TTG ACC TGT GAC AAT GCA GCA GTT GCT GGA TTA ACC  1343

 420      T   S   Y   K   G   V   Q   H   Q   L   H   V   E   S   K   V   L   A   D   C   N   T   R   C   S   C   S   T   447
1344  ACC TCT TAC AAA GGA GTT CAG CAC CAA CTA CAT GTG GAG AGC AAG GTC CTT GCT GAC TGC AAC ACA AGG TGT AGC TGC TCA ACG  1427

 448      N   T   W   D   P   V   C   G   D   N   G   V   A   Y   M   S   A   C   L   A   G   C   N   F   G   V   G   T   475
1428  AAC ACA TGG GAT CCA GTG TGT GGA GAC AAT GGT GTA GCA TAC ATG TCA GCT TGC CTT GCG GGC TGT AAG AAG TTT GTT GGA ACT  1511
                                                                   *
 476      G   T   N   M   V   F   Q   D   C   S   C   I   Q   S   L   G   N   S   S   A   V   L   G   L   C   K   K   G   503
1512  GGA ACC AAC ATG GTG TTT CAG GAT TGC AGC TGC ATT CAG TCA TTA GGA AAC TCG TCT GCA GTC CTG GGT CTG TGT AAA AAA GGT  1595

 504      P   E   C   A   N   R   L   Q   Y   F   L   I   L   T   I   I   I   S   F   I   Y   S   L   T   A   I   P   G   531
1596  CCT GAG TGT GCC AAC AGG CTG CAG TAC TTT TTA ATC TTA ACA ATA ATT ATC AGT TTC ATC TAC TCA CTT ACA GCC ATA CCT GGG  1679

 532      Y   M   V   F   L   R   C   V   K   S   E   E   K   S   L   G   V   G   L   H   T   F   C   I   R   V   F   A   559
1680  TAC ATG GTT TTT TTG AGG TGT GTC AAG TCT GAA GAG AAG TCA CTT GGA GTT GGA TTA CAT ACA TTT TGC ATA AGA GTA TTT GCT  1763

 560      G   I   P   A   P   V   Y   F   G   A   L   I   D   R   T   C   L   H   W   G   T   L   C   K   G   Q   R   G   587
1764  GGT ATT CCC GCA CCT GTT TAC TTT GGC GCT TTG ATA GAC AGA ACC TGT TTA CAT TGG GGA ACC CTG AAA TGT GGT CAG CGA GGG  1847

 588      A   C   R   M   Y   D   I   N   S   F   R   H   I   Y   L   G   L   P   I   A   L   R   G   S   S   Y   L   P   615
1848  GCA TGC AGG ATG TAT GAC ATA AAC AGT TTC AGG CAC ATT TAC CTG GGG TTG CCT ATA GCA CTA AGA GGA TCA AGC TAT CTG CCT  1931

 616      A   F   F   I   L   I   L   M   R   K   F   Q   F   P   G   D   I   D   S   S   A   T   D   H   T   E   M   M   643
1932  GCC TTC TTC ATT CTG ATA CTT ATG AGG AAA TTC CAG TTC CCC GGG GAC ATT GAC TCT TCA GCA ACT GAT CAT ACA GAG ATG ATG  2015

 644      L   G   E   K   E   S   E   H   T   D   V   H   G   S   P   Q   V   E   N   D   G   E   L   K   T   K   L   670
2016  CTC GGA GAG AAG GAA AGC GAG CAC ACA GAT GTG CAT GGA AGT CCT CAG GTC GAG AAT GAC GGA GAA CTG AAA ACG AAG CTG TAA  2099

2100  TGA GTT TTC TAC TGC CCT GTT CAA GGC CAT GAA CAG AAT GCA CAC TTC CCT TCC TCG GAA TCC TGA GAG ATA CAA TAG GAA CCT  2183
2184  TCT CTT TAA GGA CCT CAA CAA TTG TTT TTC TCA CTA TAA AAA TAA TTG CTG ATA TCA TTT TTA TAG TGA AAA CAA TAA AAA      2267
2268  TAA TTG CTG ATA TCA TTT TCA GAA TTT CAG GTA ATT TTT AAG ATT TCC CTG GGG AAG ACT TTT ATG GTG ACC CCC ACA CTA CAC  2351
2352  TTT AAA GCT TCC TTC ATT TTC ACA TAA CAT TTT CTC TTA ACT CAA TCA AGG GAA ATA TGT GTT TCC CAC ACA TCT TTA AAT GAG  2435
2436  TAT AAA ACT TTC CTA TTC CTG AAA CAG TAT TTA ATT TCA TTG AAT TTA TGG TTC ACA GTT GGA AGT ATT TAG AGC TGA AAA GAT  2519
2520  ACA TTC TCA TTA AGC CAC ATT GAA AAG TTA ACT TTT CTA TGT TCA GGA TGT CTC CAT ATG TCT ATA TGT AGG AGT TCT CAA ATA  2603
2604  TTA TTA TTA TTG TAG ACA TGA TGC TCC TAG ATT AGA CTT CTC ACT CCT GTG TCA TTT CCT GTT TCT AGT CCC TTC TTT CAT TAC  2687
2688  TCC TGT CAC ACA GTT GAA TGT TTT GTT TAG AGC ACT GAC AAA CTT AAT CAG GTT ATT AAA AAT CAC ATT AGA                  2759
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FIG. 4. Nucleotide and predicted amino acid sequence of the oatp cDNA. Putative membrane-spanning domains (11) are underlined. Potential N-linked glycosylation sites are marked by asterisks.

are probably also present in other organs such as brain, lung, skeletal muscle, and proximal colon.

## DISCUSSION

Organic anions such as bilirubin and BSP are avidly bound to albumin in the circulation. They are rapidly and efficiently extracted from this carrier by hepatocytes. A number of putative hepatocyte membrane surface organic anion transporters have been suggested (1–3), but a role for any of these candidate membrane proteins in organic anion transport has been elusive. Using a functional-expression cloning strategy (4), we have isolated and characterized a cDNA encoding a basolateral $Na^+$-independent oatp of rat liver. The strategy for cloning this cDNA depended upon an assay for high-affinity organic anion transport adapted from previous studies performed with short-term cultures of rat hepatocytes (12, 13). Although the organic anion transporter cloned in this study mediates $Cl^-$-dependent BSP uptake similar to that described previously in hepatocytes (Fig. 1; Table 1; refs. 12 and 13), omission of BSA from the incubation medium and/or increasing the substrate (BSP) concentration completely abolished the stimulatory effects of $Cl^-$ (Table 1). The maximal BSP uptake velocity was similar in the presence and absence of BSA (Fig. 2). These data indicate that neither $Cl^-$ nor BSA is required for oatp-mediated BSP uptake when oatp
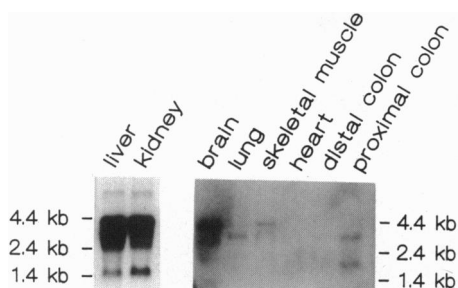
FIG. 5. Northern blot analysis for oatp mRNA in various rat tissues. mRNA (5 μg) was isolated from the indicated organs, denatured, and electrophoresed in a 1% agarose/formaldehyde gel. After overnight transfer to Hybond N, hybridization was performed as described in *Materials and Methods* for high-stringency conditions. Autoradiography was at −70°C for 16 hr (liver and kidney samples) or 7 days (other organ samples).

is expressed in *X. laevis* oocytes. This cloned hepatocellular transporter thus combines several organic anion transport features ($Cl^-$-dependent extraction of BSP from albumin; $Cl^-$ and BSA-independent BSP uptake) that have been thought to be mediated by distinct membrane transport proteins (1–3). In addition, the cloned oatp seems to exhibit a broad substrate specificity, since it can also mediate $Na^+$-independent uptake of bile acids such as cholate and taurocholate (Fig. 3).

Surprisingly, cell-free translation of the cloned oatp resulted in a protein of higher molecular weight than what was expected based upon previous studies in intact hepatocytes and isolated membrane vesicles. In those studies several distinct proteins were identified as candidate BSP transporters, including the so-called BSP/bilirubin-binding protein ($\approx$55 kDa) (2), the organic anion-binding protein ($\approx$55 kDa) (16, 17), and bilitranslocase ($\approx$37 kDa) (3). It is important to realize, however, that none of these proposed BSP-transporting polypeptides has been fully characterized on the molecular level, nor have their structure–function relationships been established. This study now demonstrates that a single polypeptide with a calculated molecular mass of 74 kDa (Fig. 4) is sufficient to account for $Na^+$-independent uptake of multiple organic anions in rat liver. After *in vitro* translation the unglycosylated oatp exhibited an apparent molecular mass of 59 kDa in SDS/polyacrylamide gels. Its apparent size increased to 71 kDa after *in vitro* translation under conditions permitting glycosylation. These calculated and apparent molecular masses are higher than the 37- to 55-kDa values previously proposed as apparent molecular masses for the basolateral rat liver $Na^+$-independent organic anion uptake system(s), indicating that the cloned oatp may represent a novel organic anion transport protein. The absence of any significant sequence homology with other cloned transport proteins suggests that the cloned oatp might belong to a distinct family of membrane transporters. However, the extent of posttranslational processing of this cloned protein in intact hepatocytes remains unknown. Thus, determination of the exact size of mature oatp in liver and its possible association with other membrane polypeptides

awaits further studies and the availability of specific antibodies.

The cloned cDNA hybridized with several mRNA species of variable sizes from rat liver, kidney, brain, lung, skeletal muscle, and proximal colon (Fig. 5). Although with liver and kidney samples the strongest hybridization signal was obtained with mRNA of 4.3 and 3.3 kb, additional but weaker hybridization signals were obtained after prolonged film exposure with mRNAs from various tissues (Fig. 5). These data indicate that there exist several additional mRNAs coding for what may be closely related organic anion transport proteins in various organs (brain, lung, skeletal muscle, and proximal colon) and that all of these proteins may belong to the same gene family. These results also support the concept of involvement of multiple proteins in $Na^+$-independent basolateral uptake of various organic anions in rat liver (1–3).

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