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Molecular identification of the liver- and the heart-type fatty acid-binding proteins in human and rat kidney

Use of the reverse transcriptase polymerase chain reaction

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The cDNAs of two types of fatty acid-binding protein (FABP) present in human kidney, previously described as types A and B, were isolated using reverse transcriptase-PCR (RT-PCR) with human kidney mRNA and various sets of primers. The cDNA fragments were cloned and sequenced. Renal FABP type A and B cDNAs appeared to be completely identical to human liver- and heart-type FABP cDNAs respectively. In the second part of this study we demonstrated the presence of liver-type FABP in rat kidney by chromatography, e.l.i.s.a. and immunocytochemistry. The ratio and cellular distribution of the two FABP types varies markedly in human and rat kidney. Using RT-PCR we were also able to prepare and identify liver- and heart-type FABP cDNAs with mRNA from both male and female rat kidney.

INTRODUCTION

In mammalian cells, fatty acids are important molecules for energy delivery and for synthesis of membrane lipids and lipid mediators. Fatty acids are metabolized in mitochondria, peroxisomes and on the endoplasmic reticulum. The transport of fatty acids from the plasma membrane to these cellular organelles is believed to be performed by fatty acid-binding proteins (FABPs) [1–3]. On the basis of their primary structure at least five FABPs, with sequence similarities of 25–65%, have been identified [3]. These FABPs have been named liver, heart, intestinal, adipocyte and myelin FABPs, after the tissue from which they were initially isolated. The existence of different FABP types suggests a type-specific function. The presence of more than one FABP type in a tissue, e.g. intestine [4], stomach [5] and kidney [6,7], supports this hypothesis. Since the first isolation of an FABP cDNA, the rat liver FABP cDNA [8], cDNAs of all five FABP types have been obtained [9–16].

In rat kidney two FABP types have been demonstrated. One was identified as heart-type FABP, based on biochemical and immunological characterization [8,17,18] and mRNA detection [11,19]. The second type was biochemically and immunologically different from heart- or liver-type FABP and was named renal-specific FABP [6,18,20]. Amino acid sequencing, however, demonstrated that this renal-type FABP was identical with α_{2U}-globulin [18,21]. The latter protein is a secretory protein of the liver and undergoes endocytotic uptake into the proximal tubules [22]. The α_{2U}-globulin molecule is structurally not an FABP, but is more closely related to the lipocalins such as lactoglobulin and serum retinol-binding protein [23,24]. Recently we isolated two FABP types from human kidney; one (type A) showed similarity with human liver FABP and the other (type B) was similar to human heart FABP [7]. However, cDNA analysis has yet to prove the exact identity of both human kidney FABPs.

In the present study we describe the preparation and identification of the two human renal FABP cDNA types by reverse transcriptase-PCR (RT-PCR). We also applied this PCR technique to investigate the FABP types of rat kidney, since we obtained evidence indicating the presence of liver-type FABP in this tissue.

MATERIALS AND METHODS

Materials

Moloney murine leukaemia virus (MMuLV) RNAase H$^-$ RT was obtained from Bethesda Research Laboratories, Life Technologies, Gaithersburg, MD, U.S.A.; recombinant *Taq* DNA polymerase Amplitaq was from Perkin–Elmer Cetus, Norwalk, CT, U.S.A.; Sequenase version 2.0 was from United States Biochemical, Cleveland, OH, U.S.A.; synthetic oligonucleotide primers were from Pharmacia, Uppsala, Sweden; wheat germ extract, L-[^{35}S]methionine, [α-^{35}S]dATP (15 mCi/ml) and [α-^{32}P]dATP (10 mCi/ml) were from Amersham; SP6-RNA polymerase, RNasin and pGEM-5 Zf[+] were from Promega Corporation, Madison, NY, U.S.A.; peroxidase-conjugated goat anti-(rabbit IgG) was from Tago, Burlingama, CA, U.S.A.; rabbit peroxidase–anti-peroxidase couples were from Dakopattis, Glostrup, Denmark; goat anti-(Tamm–Horsfall glycoprotein) was from Cappel, West Chester, PA, U.S.A.

Human kidney tissue was obtained after nephrectomy. Rat kidneys were from 12-week-old Wistar rats.

RNA isolation and blot hybridization

Total RNA was extracted with LiCl/urea by a modification of the procedure of Auffray & Rougeon [25]. Kidney tissue (1–2 g) was homogenized in a mixture of 3 M-LiCl/6 M-urea/20 mM-sodium acetate (pH 5.2)/heparin (2 mg/ml) and left overnight at 0 °C. The RNA precipitate was pelleted by centrifugation for 15 min at 16000 g and washed once with 4 M-LiCl/8 M-urea. After centrifugation for 15 min at 16000 g the pellet was dissolved in 50 mM-sodium acetate (pH 5.2)/0.2% SDS/2 mM-EDTA and extracted successively with phenol, phenol/chloroform and chloroform. Subsequently the RNA was precipitated with ethanol and stored at −20 °C. Poly(A)$^+$ RNA was selected on oligo(dT)–cellulose [26].

For Northern blot analysis, RNA was electrophoresed on 1% agarose gels in the presence of formaldehyde. Prior to electrophoresis, ethidium bromide was added to the RNA samples in order to allow visualization of the rRNAs in the gel. In this way it was ascertained that the amounts of RNA in the different lanes were approximately the same. Following electrophoresis the

Abbreviations used: FABP, fatty acid-binding protein; RT-PCR, reverse transcriptase PCR; MMuLV, Moloney murine leukaemia virus.
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Table 1. Primers used in RT-PCR

Primer	Sequence
1. Human heart FABP (forward)	GCCAGCATCACCATGGTGGACGCTTTC
2. Human heart FABP (reverse)	ATCACCAGTGGATCCAGGTCATGCCTC
3. Rat heart FABP (forward)	TCTCATTGCACCATGGCGGACGCCTTT
4. Rat heart FABP (reverse)	AGTGACGGGGGATCCAGGTCACGCCTCCTT
5. Human liver FABP (forward)	AAGGGGGTGTCGGAAATCGTG
6. Human liver FABP (forward)	ATTGCCCATATGAGTTTCTCCGGCAAGTAC
7. Human liver FABP (reverse)	GCGCTGCAGGGATCCGTCGAC$[T]_{17}$
8. Human liver FABP (reverse)	AATGGATCCTGTTTAAATTCTCTTGCTGATTCT
9. Rat liver FABP (forward)	GCCCATATGAACTTCTCCGGCAAGTAC
10. Rat liver FABP (reverse)	CTGGGATCCCTAAATTCTCTTGCTGACTCTCTT

RNA was transferred to nitrocellulose filters [26]. DNA probes were labelled by the random priming method [27]. The RNA blot was hybridized at 37 °C in 0.5 M-sodium phosphate as described by Church & Gilbert [28]. The blots were finally washed in 0.05 M-sodium phosphate at 65 °C, dried and subjected to autoradiography at −70 °C using intensifying screens.

RT-PCR procedure

Primers derived from the heart and liver FABP cDNA sequences were designed so as to amplify the coding region. The primers used are given in Table 1.

First-strand synthesis reactions were carried out with 50 μg of total RNA or 2 μg of poly(A)$^+$ RNA in a total volume of 20 μl containing 50 mM-Tris/HCl (pH 8.3)/75 mM-KCl/3 mM-$MgCl_2$/125 μM-dNTPs/40 units of RNasin/0.5 μg of primer and 400 units of MMuLV RT. A reverse primer was added to the RNA sample, heated for 10 min at 70 °C and quenched on ice. The reverse transcription reaction was carried out for 1 h at 37 °C and 30 min at 45 °C.

The PCR amplification reactions were carried out in a total volume of 100 μl containing 2 μl of the reverse transcription reaction, 0.5 μg each of the reverse and forward primers, 10 mM-Tris/HCl (pH 8.3)/50 mM-KCl/1.5 mM-$MgCl_2$/0.001 % gelatin/250 μM-dNTPs and 2.5 units of Amplitaq. The mixture was overlaid with mineral oil. After 8 min denaturation at 94 °C, 40 cycles of amplification were carried out by using a step programme (94 °C, 1 min; 50 °C, 1.5 min; 72 °C, 1.5 min) followed by a 2 min final extension at 72 °C.

Cloning and sequencing of the cDNAs

RT-PCR products were digested with restriction enzymes which recognize specific sites in the primers. The cDNA fragments were cloned in M13mp18 and PGEM 5Zf(+). Plasmids with cDNA inserts were single- and double-strand-sequenced in both directions with Sequenase, using the supplier's recommendations.

In vitro transcription/translation assay

The cDNA insert was ligated in PGEM 5Zf(+) as described above, grown in JM101 and afterwards linearized with *Bam*HI. RNA transcripts were synthesized *in vitro* from the *Bam*HI-digested clone in the presence of SP6 RNA polymerase and the dinucleotide primer G(5′)ppp(5′)G [29]. *In vitro* translation in a nuclease-treated wheat germ extract was performed for 60 min at 25 °C in 30 μl of a reaction mixture containing 1 mM amino acids, 1 mM-[^{35}S]methionine and 0.65 M-KCl.

Immunoprecipitation of newly synthesized FABP

Protein A-Sepharose CL-4B (200 μl) in phosphate-buffered saline (10 %, v/v) was incubated with 75 μl of anti-(human liver FABP) serum for 2 h at room temperature under rotation. Non-specifically bound proteins were removed by washing the gel suspension four times with 500 μl of buffer A (500 mM-NaCl/10 mM-Tris/HCl (pH 7.4)/0.05 % Nonidet P40). Subsequently the gel suspension was incubated with 25 μl of the translation assay mixture in 500 μl of 150 mM-NaCl/50 mM-Tris/HCl (pH 7.4)/0.05 % Nonidet P40 for 3 h at 4 °C, under rotation. Finally, the gel suspension was washed four times with buffer A and 50 μl of SDS gel electrophoresis loading buffer was added. SDS gel electrophoresis was performed on a 15 % polyacrylamide gel.

Immunocytochemistry

Kidney tissue from male and female Wistar rats (200 g) were fixed for 2 h in 2 % periodate–lysine–paraformaldehyde and embedded in paraffin. Deparaffinized sections were treated with 1 % (v/v) H_2O_2 in methanol in order to remove intrinsic peroxidase activity. After rehydration and treatment with 20 % (v/v) normal goat serum in Tris-buffered physiological saline for 30 min, sections were incubated with rabbit antisera raised against rat liver FABP or rat heart FABP, optimal dilutions being 1:25–1:50. Immunopositive sites were detected by subsequent incubations with goat anti-(rabbit IgG)–peroxidase and rabbit peroxidase–anti-peroxidase complex. Peroxidase activity was visualized using diaminobenzidine as substrate. Sections were counterstained for 1 min with Harris's haematoxylin and embedded in glycerol/gelatine mounting medium. Preimmune sera and sera raised against unrelated antigens were used as controls. In addition, immune sera were absorbed (18 h, 20 °C) with both FABPs, the optimal amount being 25 μg of FABP/100 μl of diluted serum. Antiserum against Tamm–Horsfall glycoprotein was used for the localization of distal tubules.

Other procedures

Preparation of rat kidney cytosol, Sephacryl S-200 gel filtration and DEAE-Sepharose chromatography were performed as described for human kidney [7]. The FABP contents of kidney cytosol were determined by e.l.i.s.a. as described [17]. Rat liver and heart FABPs were isolated as described [30,31] and antisera were raised in rabbits [17,32]. cDNA probes of human heart (muscle) FABP and of human liver FABP were prepared as described previously [15] or during this study respectively.

RESULTS AND DISCUSSION

Identification of two FABP transcripts in human kidney

Northern blot analysis of total RNA (50 μg) and poly(A)$^+$ mRNA (20 μg) from human kidney showed the presence of two transcripts using human liver and heart FABP cDNAs as probe.

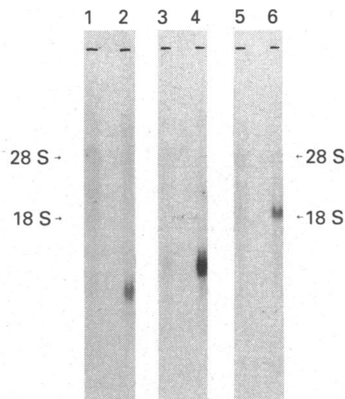

Fig. 1. Northern blot analysis of human renal FABP type A and B transcripts

Total RNA (50 μg) (lanes 1, 3, 5) and poly(A)$^+$ RNA (20 μg) (lanes 2, 4, 6) from human renal tissue was fractionated by electrophoresis on 1 % agarose–formaldehyde denaturing gels, transferred to nitrocellulose and hybridized with random-primed labelled human liver FABP cDNA (lanes 1 and 2), human heart FABP cDNA (lanes 3 and 4) or human actin cDNA (lanes 5 and 6).

The transcripts were 0.5 and 0.7 kb in length for the liver and heart types respectively (Fig. 1). From the autoradiogram it can be concluded that the mRNA concentration of both FABP types is rather low in kidney, since 50 μg of total RNA only gave a faint band at the position of the FABP transcripts.

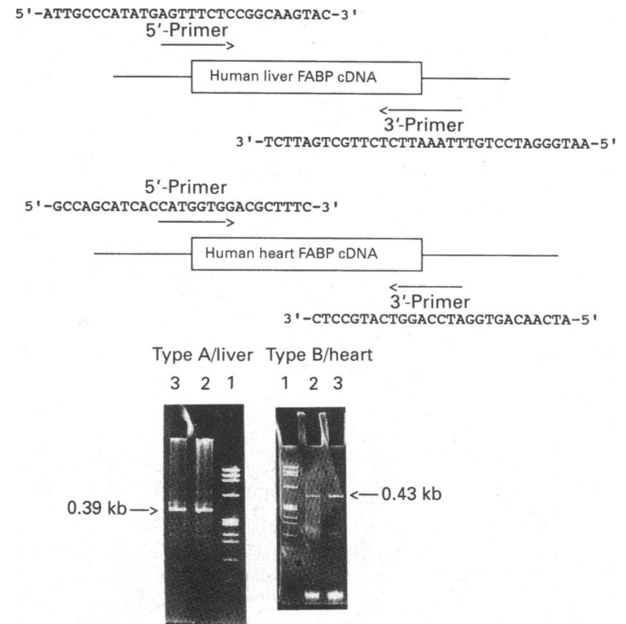

Fig. 2. RT-PCR isolation of human kidney type A and B FABP cDNAs

Poly(A)$^+$ RNA was reverse-transcribed with 3′-primers of human liver or heart FABP cDNA, amplified in PCR using 5′- and 3′-primers and subjected to polyacrylamide mini-gel electrophoresis. Lane 1 shows the ΦX174 × *Hae*III marker and lanes 2 and 3 contain the RT-PCR fragment prepared with 1 and 2 μg of poly(A)$^+$ RNA respectively.

Isolation of cDNAs of the two FABP types of human kidney

After the identification of two transcripts, we attempted the isolation of both cDNAs by RT-PCR using primers based on the cDNA sequences of human liver- and heart-type FABP cDNAs. The structural and immunological data concerning FABP type A from human kidney revealed many similarities but also some differences compared with human liver FABP [7]. The cDNA sequence was required to establish the structure. For the isolation of the FABP type A cDNA the RACE (rapid amplification of cDNA ends) protocol described by Frohman *et al.* [33] was followed. The reverse transcription primer (primer 7) was an oligonucleotide with 17 dT residues and an adaptor sequence containing three restriction enzyme recognition sites. The forward primer (primer 5) was derived from human liver FABP cDNA (nucleotides 106–126) and is located in a highly conserved region [10]. The DNA fragment isolated after RT-PCR was cloned in M13mp18 and sequenced. This 350 bp fragment appeared to be completely identical with human liver FABP cDNA. Subsequently RT-PCR was applied with a reverse and forward primer (primers 8 and 6) encoding the 3′- and 5′-ends respectively of the human liver FABP cDNA coding region (Fig. 2). The 403 bp fragment was isolated and cloned in pGEM-5 Zf(+) and sequenced. The sequence appeared to be identical with the liver FABP cDNA sequence described by Chan *et al.* [9].

Data on the protein have indicated that the type B FABP from human kidney is identical with the heart-type FABP [7]. To establish this, we carried out a reverse transcription with primer 2, which encoded the 3′-end of the coding region of the heart FABP cDNA. The reverse transcription mixture was amplified with the same reverse primer and primer 1, encoding the 5′-end of the heart FABP cDNA coding region (Fig. 2). A DNA fragment of 432 bp was isolated, digested with restriction enzymes, which recognize sites present in the primers, and subcloned in M13mp18. The sequence appeared to be fully identical with the heart- or muscle-type FABP cDNA sequence published by Peeters *et al.* [15].

Both total RNA and mRNA from both males and females could be used for the isolation of the renal FABP cDNAs. The FABP mRNA concentrations appeared to be similar when RT-PCR was applied with equal amounts of RNA of male or female kidney.

In vitro transcription/translation of human renal type A FABP cDNA

The identity of the cDNA fragment isolated with human liver FABP cDNA primers was confirmed by an *in vitro* transcription/translation assay followed by immunoprecipitation. pGEM-5 Zf(+), containing the human renal FABP type A cDNA, was linearized with *Bam*HI, which is located 5 bp downstream of the stop codon. After linearization, the pGEM-5Zf(+)-FABP type A vector was used in an *in vitro* transcription assay. The RNA product yielded in an *in vitro* translation assay the 14.3 kDa product (Fig. 3). The smaller products are due to a start of translation at ATG codons located further downstream. These ATG codons correspond to amino acid positions 19 and 74 [9,10]. Immunoprecipitation of the translation product with antibodies against human liver FABP gave the same result (Fig. 3).

Renal FABP type B cDNA was directly cloned in the expression vector pET8c and expressed in *Escherichia coli.* The protein was identical with heart (muscle) FABP on the basis of various biochemical and immunological tests (results not shown) [15].

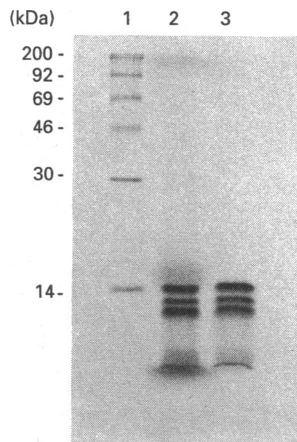

Fig. 3. SDS/PAGE and subsequent autoradiography of the products obtained after *in vitro* transcription/translation of human liver FABP cDNA

pGEM 5 Zf(+)-HL–FABP was *Bam*HI-digested, transcribed with SP6 RNA polymerase and translated with wheat germ extract. The translation product was immunoprecipitated with antibodies against human liver FABP. Lane 1, molecular-mass markers; lane 2, total translation mixture (4 μl); lane 3, an equal amount of total translation mixture after immunoprecipitation (15 μl).

Table 2. FABP content of kidney

Values (determined by e.l.i.s.a.) are means ± S.D. for five or six individuals.

	Content (nmol/mg of cytosolic protein)	
	Liver-type	Heart-type
Rat male	0.078 ± 0.0005	0.79 ± 0.10
Rat female	0.051 ± 0.0007	0.67 ± 0.04
Human male	0.63 ± 0.19	0.38 ± 0.26

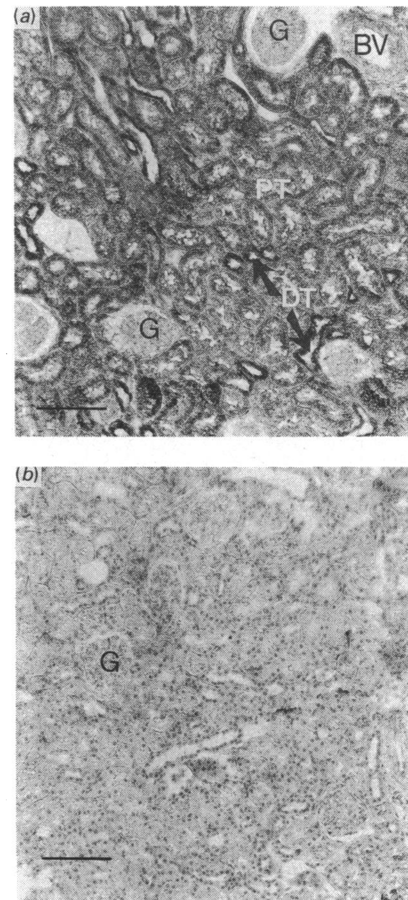

Fig. 4. Immunoperoxidase staining of rat renal tissue using antiserum against rat liver FABP before (*a*) and after (*b*) preabsorption with rat liver FABP

Proximal tubules (PT) and distal tubules (DT) are positive, the latter being more immunoreactive. Glomeruli (G) and blood vessels (BV) are negative. The bar represents 100 μM.

Identification of FABP types and FABP RNA transcripts in rat kidney

As already mentioned, male rat kidney contains the heart-type FABP and α_{2U}-globulin, but female kidney only contains the heart-type FABP [6,17,18,20]. The presence of liver FABP in rat kidney has never been demonstrated. We obtained evidence that the liver-type FABP is also present in rat kidney, as in human kidney. The low-molecular-mass fraction obtained by Sephacryl gel filtration of rat kidney cytosol revealed on DEAE-Sepharose chromatography two fractions with fatty acid-binding activity. The first fraction was not bound, like the liver-type FABP, whereas the second fraction was eluted from the column during the gradient, like the heart-type FABP. The first peak bound about 10% of the [^{14}C]oleic acid bound by the second peak.

E.l.i.s.a. showed low amounts of liver-type FABP in rat kidney cytosol (Table 2). The concentrations are much lower than those of the heart-type FABP, and the ratio of liver- and heart-type FABPs differs considerably from that in man.

Immunocytochemistry on rat kidney sections also established the presence of liver-type FABP. Using anti-(liver FABP) antiserum, proximal as well as distal tubules reacted positively, the latter being more immunoreactive (Fig. 4*a*). In addition, the collecting tubules and the papillary epithelium were also immunoreactive, but glomeruli did not stain. Preabsorption of immune serum with liver FABP resulted in a complete abolition of staining (Fig. 4*b*). No difference could be observed between male and female rat kidneys. Distal tubules were the only structures that were strongly positive when stained with anti-(heart FABP) antiserum (Fig. 5): proximal tubules were not distinctively positive and glomeruli were negative. Preabsorption of immune serum with liver FABP had no effect. Male and female rat kidneys reacted similarly. The cellular distribution of the heart-type FABP is similar in rat kidney to that previously found in human kidney [7]. The liver-type FABP, however, is restricted to the proximal convoluted and straight tubules in human kidney [7]. Like the kidney, the rat stomach also shows the presence of both heart- and liver-type FABPs with a specific cellular distribution [5].

With RT-PCR we also investigated the presence of the transcripts of liver- and heart-type FABPs in rat kidney. Poly(A)$^+$ RNA was isolated and reverse-transcribed with a reverse primer (primer 4 or 10), which partially overlapped the 3′-end of the coding region of the rat heart- or liver-type FABP cDNA respectively. PCR amplification was accomplished by using the reverse primer and the appropriate forward primer (primer 3 or 9), which partially overlapped the 5′-end of the coding region of the FABP cDNA. Subsequently the RT-PCR products were subjected to polyacrylamide mini-gel electrophoresis and electroblotting to Hybond N$^+$. The DNA blots were probed with human heart and liver FABP cDNA. As a control, RT-PCR was

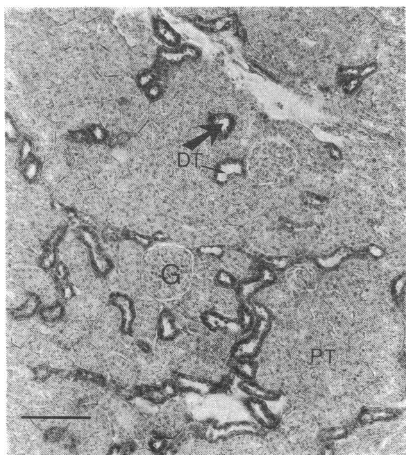

Fig. 5. Immunoperoxidase staining of rat renal tissue using antiserum against rat heart FABP

Distal tubules (DT) are strongly immunopositive. PT, proximal tubules; G, glomeruli. The bar represents 100 μM.

also applied to rat heart and liver mRNAs with both rat liver and heart FABP type cDNA primers.

The rat heart FABP cDNA could be demonstrated on the blot with the human muscle FABP cDNA probe to be present in rat heart and kidney mRNA, but not in rat liver mRNA (Fig. 6). The blot hybridized with human liver FABP cDNA showed the presence of liver FABP mRNA in both liver and kidney mRNA, but not in heart mRNA (Fig. 6). Bands at higher molecular masses may be due to reverse transcription of pre-mRNAs. Based on the RT-PCR and hybridization results, the content of the mRNAs of the liver and heart FABP types do not differ markedly in kidneys of male and female rats.

Studies on transgenic animals expressing reporter genes under the control of the liver FABP gene promoter have revealed that

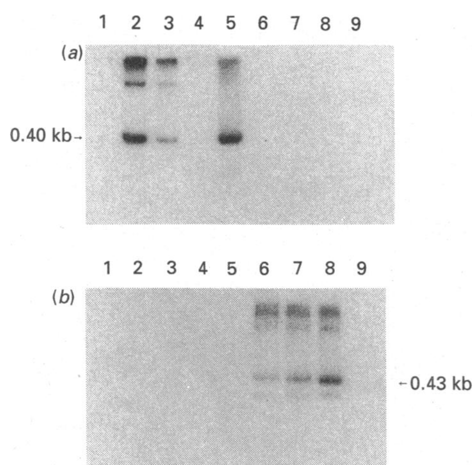

Fig. 6. Identification of FABP RNA transcripts in rat tissues

Poly(A)$^+$ RNA (2 μg) from rat tissues including male kidney (lanes 2 and 6), female kidney (lanes 3 and 7), heart (lanes 4 and 8) and liver (lanes 5 and 9) were reverse-transcribed with 3′-primers of rat liver-type FABP cDNA (lanes 2–5) or rat heart-type FABP cDNA (lanes 6–9). The reverse transcription mixture was amplified in PCR and subjected to polyacrylamide mini-gel electrophoresis, electroblotted to Hybond N$^+$ and probed with human liver (*a*) or heart (*b*) FABP cDNA. Lane 1 shows the ΦX × *Hae*III marker.

expression of liver-type FABP is possible in mouse kidney [34,35]. The heart-type FABP mRNA has been demonstrated in rat kidney by others [11,19]. Its content shows marked variations from fetal to adult life [19]. We can only speculate on the physiological relevance of the two FABP types in kidney. The liver-type FABP binds various ligands and may be involved in the renal excretion of exogenous and endogenous metabolites. The liver-type FABP also binds some drugs [2,3], and may in this way prevent nephrotoxicity. The heart-type FABP only binds fatty acids and seems to be involved in lipid metabolism.

In conclusion, we have isolated the cDNAs of both human renal FABP types by RT-PCR. Human renal FABP type A and B cDNAs appeared to be fully identical with human liver and heart FABP cDNAs respectively. The cDNA analysis gave the conclusive evidence for the presence of liver- and heart-type FABPs in human kidney, in agreement with most or all of the physiochemical and immunochemical data on the protein [7]. Furthermore, we also demonstrated the presence of liver- and heart FABPs and their mRNAs in kidneys from both male and female rats. The significance of the occurrence in kidney of two FABP types with different ligand specificities and cellular distributions requires further investigation. The availability of cDNAs and FABP preparations of both types from human and rat kidney will allow future work in this area.

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