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Molecular Cloning and Expression of cDNAs Encoding Rat Brain and Liver Cytosolic Long-Chain Acyl-CoA Hydrolases[1]

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cDNAs encoding the long-chain acyl-CoA hydrolases (ACHs) from rat brain and liver, referred to as rBACH and rLACH1, respectively, were isolated and sequenced. The rBACH cDNA contained an open reading frame encoding a 338-amino acid polypeptide with a calculated molecular weight of 37,559, of which the deduced amino acid sequence matched partial amino acid sequences directly determined for peptides generated by tryptic digestion or CNBr cleavage of purified rBACH. The rLACH1 cDNA contained an open reading frame encoding a 343-amino acid polypeptide with a molecular weight of 38,240. When expressed in *Escherichia coli,* these cDNAs produced palmitoyl-CoA hydrolase activity and 44-kDa proteins with molecular masses similar to those of purified rBACH and rLACH1 (43 kDa). These expressed proteins and enzyme activity were immunoblotted and neutralized, respectively, by anti-rBACH or anti-rLACH1 antibodies. rLACH1 cDNA had 84 and 94% identity with rBACH cDNA at the nucleotide and amino acid levels, respectively. However, the 5′-end of the former cDNA which contained the N-terminal coding region of rLACH1 was entirely different from the corresponding region of rBACH cDNA, suggesting that these enzymes may be generated by alternative use of exons of the same gene. Northern blot analysis showed that ACH mRNA was expressed constitutively in the rat brain and testis, whereas its expression in the liver was inducible by treatment with the peroxisome proliferator. This study demonstrated the molecular diversity of ACH and suggested the presence of tissue-specific mechanisms to regulate the ACH gene expression.

Long-chain acyl-CoA hydrolases (EC 3.1.2.2.) include a number of enzymes which catalyze the hydrolysis of fatty acyl-CoA thioesters to free fatty acids and CoASH, and are present in all living organisms (1). In view of the importance of acyl-CoA in lipid metabolism, the ability of these enzymes to regulate acyl-CoA concentration in the cell may provide a mechanism for the control of lipid metabolism. Long-chain acyl-CoAs also have a role in intracellular protein transport (2), activation of protein kinase (3) and functional regulation of thyroid hormone receptor (4), suggesting a role of the hydrolases in the regulation of these events. So far, the termination of chain elongation in fatty acid synthesis has been attributed to an acyl-CoA hydrolase/thioesterase in the mammary glands of certain species (5, 6) and the water fowl uropygial gland (7), as well as the thioesterase domain of fatty acid synthase. Moreover, an acyl-CoA hydrolyzing thioesterase was recently discovered, which is capable of hydrolyzing palmitoyl-protein thioesters of signal transduction molecules such as Ras and heterotrimeric G protein α-subunit (8). However, the physiological significance of the long-chain acyl-CoA hydrolases is not yet fully understood.

Previously, we purified long-chain acyl-CoA hydrolases from the cytosol of rat tissues; rLACH1 and rLACH2 isolated from the liver cytosol of rats treated with DEHP, a peroxisome proliferator (PP) (9), and rBACH (10) and rTACH (unpublished data) from the brain and testis, respectively. Our studies of the enzy-

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with Accession Nos. D88890 and D88891.

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Abbreviations used: DEHP, di(2-ethylhexyl)phthalate; PP, peroxisome proliferator; 5′-RACE, 5′-rapid amplification of cDNA ends; bp, base pair(s); kb, kilobase(s).

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matic properties of these enzymes suggested that, in addition to the mammary gland acyl-CoA hydrolase (6), there are at least two major groups of soluble long-chain acyl-CoA hydrolases in rat tissues (10); *i.e.* group-1 including rBACH, rLACH1 and rTACH, and group-2 which includes rLACH2. The former group of ACHs have very high long-chain acyl-CoA hydrolyzing activity, are oligomers of subunits with molecular masses of 43 kDa, are constitutively expressed at high levels in the brain and testis and are markedly induced in the liver after treatment of rats with PPs (9,10). On the other hand, rLACH2 is a monomer of 47 kDa and is expressed at much lower levels in untreated rats, although its expression is also markedly induced in the liver by PPs (9). The recent studies by Svensson *et al.* (11,12) also partially support this notion; they isolated and characterized two kinds of long-chain acyl-CoA hydrolases from rat liver mitochondrial matrix, MTE-I and II corresponding to groups-2 and 1, respectively. To date, many group-1 hydrolases have been purified. However, their reported catalytic properties such as chain length specificity as well as molecular masses are very similar, as can be seen with rLACH1 (9), rBACH (10), rTACH (our unpublished data), MTE-II (11), rat brain cytosolic ACT isolated by Broustas and Hajra (13), and the liver cytosolic enzyme isolated by Miyazawa *et al.* (14). Therefore, their identities are of interest in studies of soluble long-chain acyl-CoA hydrolases in terms of possible molecular diversity of these enzymes and the regulatory mechanisms of the individual enzymes in particular tissues and intracellular compartments where they are present.

To examine the molecular diversity of the group-1 ACHs and moreover to obtain information about possible functions of ACHs through analyzing their primary structures, we cloned cDNAs encoding rBACH and rLACH1.

MATERIALS AND METHODS

rBACH partial amino acid sequencing. rBACH was purified from the rat brain cytosol (10). Peptide fragments of purified rBACH were prepared by digestion with trypsin (100:1) in 0.1 M ammonium bicarbonate at 37°C for 24 h, or CNBr cleavage in 70% formic acid at 25°C for 24 h. Single peptides separated by HPLC using a VYDAC C_8 (0.46 × 25 cm) reverse-phase column were sequenced with an Applied Biosystems Model 477A protein sequencer.

cDNA cloning of rBACH. A cDNA library generated from rat brain (Sprague-Dawley) polyadenylated RNA in the λZap II vector (purchased from Stratagene) was screened with anti-rBACH antibody, essentially as described by Sambrook *et al.* (15). The insert cDNAs of positive clones were subcloned into pBluescript SK(−) by the *in vivo* excision method. The double-stranded cDNA was denatured with sodium hydroxide and sequenced by the dideoxy chain termination method of Sanger *et al.* (16) with BcaBEST DNA polymerase (Takara). Sequence homology was analyzed and alignments were confirmed using the BLAST search server at the National Center for Biotechnology Information.

cDNA cloning of rLACH1. A λgt 11 library was constructed from mRNA prepared from the liver of a male Wistar rat treated with DEHP. Screening was performed with an 834-bp rBACH cDNA corresponding to nucleotide 557-1,390 using the ECL direct nucleic acid labeling and detection system (Amersham). 5′-RACE was carried out using a Marathon cDNA amplification kit (Clontech). Double-stranded cDNA was synthesized using 1 μg of mRNA prepared from the liver of male Wistar rats fed a diet containing 2% DEHP for 2 weeks. PCR was carried out with an adapter primer and rLACH1 cDNA-specific antisense primer corresponding to nucleotide 600-620 (5′-CCTCCTGTTCCTGCCGTAAAT-3′). The inserts of positive clones and PCR products were subcloned into pBluescript II SK(+) (Stratagene) and sequenced.

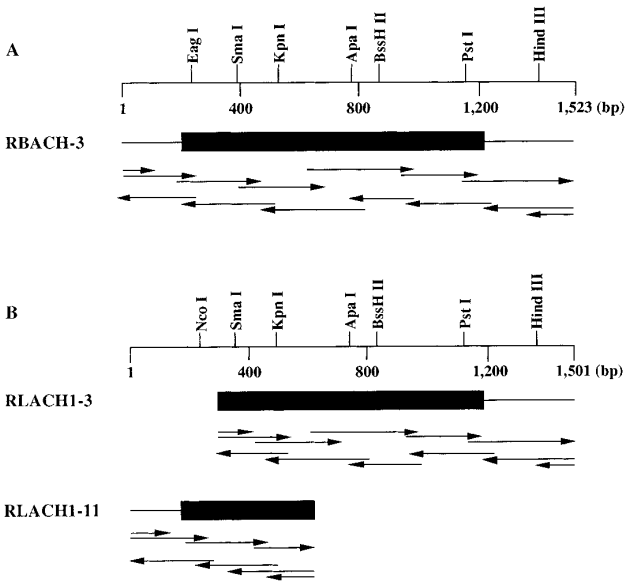

FIG. 1. Restriction maps and sequencing strategies for rBACH (A) and rLACH1 (B) cDNAs. The open reading frames are shown as solid bars. Arrows indicate the direction and extent of sequence determination.

Expression in E. coli. rBACH and rLACH1 cDNAs were directionally inserted into the prokaryotic expression vector pKK223-3 (Pharmacia) at EcoRI and HindIII sites and used to transform *E. coli* JM 105, following which expression of the ACHs was induced by isopropyl thiogalactoside. Bacterial extracts were prepared according to Sambrook *et al.* (15). The acyl-CoA hydrolase activity was measured by spectrophotometry as described previously (9). Expressed proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using anti-rBACH and anti-rLACH1 antibodies.

Other methods. Northern and Southern blot analyses were performed as described (15,17) using an 834-bp rBACH cDNA as a probe.

RESULTS AND DISCUSSION

cDNA cloning and sequence analysis of rBACH. Screening of the rat brain cDNA library with anti-rBACH antibody yielded 3 positive clones out of 3×10^5 independent recombinants. These clones, designated RBACH-1-3, contained cDNA inserts of 1.2-1.6 kb. All these clones were identical, except that RBACH-3 was 324 bp longer at the 5′-end. As shown in Figs. 1A and 2, the largest clone isolated, RBACH-3, spanned 1,523 bp not including the poly(A) tail and contained 207 nucleotides of 5′-noncoding sequence, an open

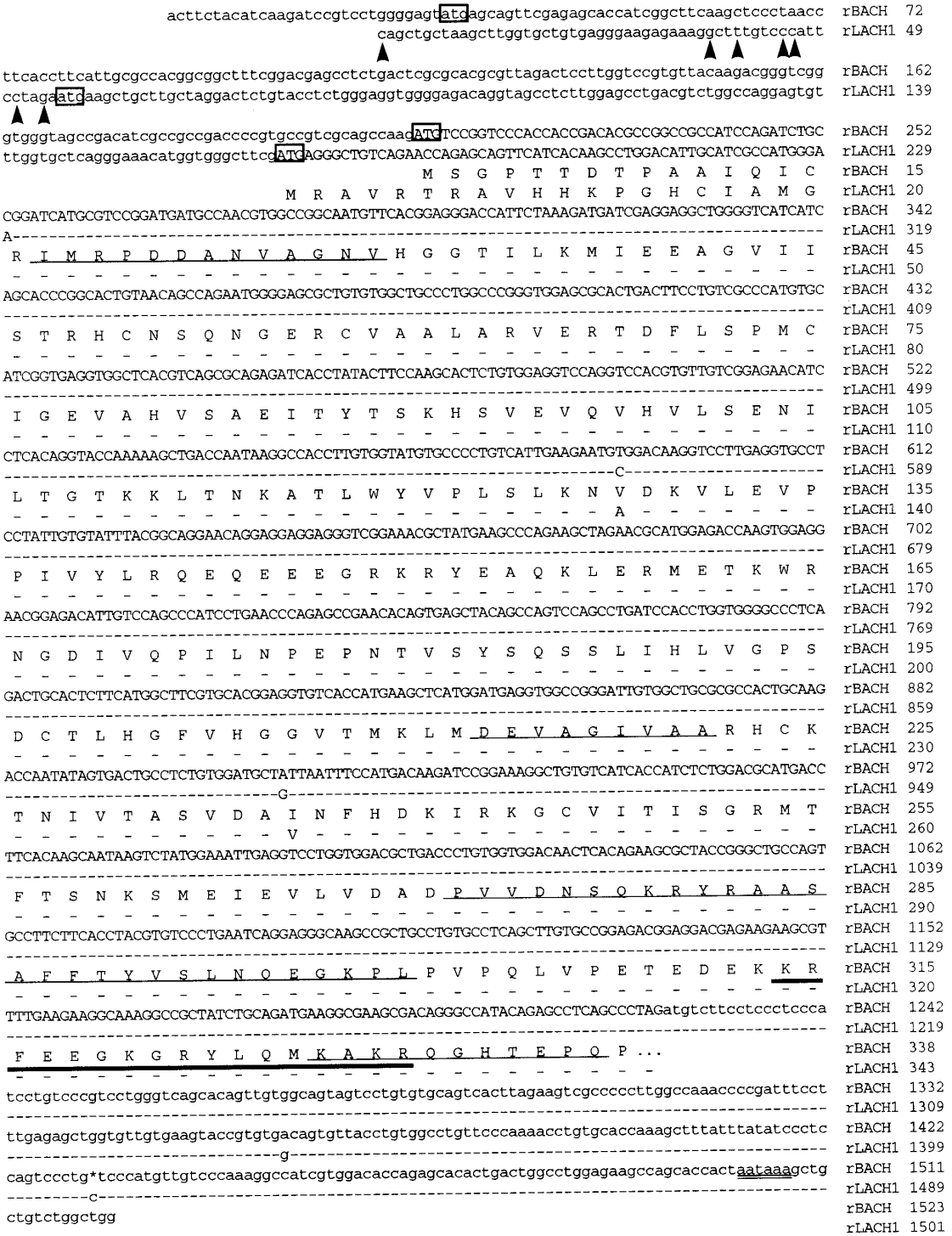

FIG. 2. Nucleotide and deduced amino acid sequences of rBACH and rLACH1 cDNAs. The sequences are aligned to obtain maximum identity between the two cDNAs. In the rLACH1 cDNA, the nucleotide and amino acid sequences matching those of rBACH are indicated with bars, and those lacking are indicated with asterisks. Amino acid sequences confirmed by Edman degradation of peptides generated by tryptic digestion or CNBr cleavage of purified rBACH are underlined. The possible initiation methionine codons (ATG) are boxed, and amino acids are numbered taking the second methionine as 1. The termination codon is dotted. The consensus polyadenylation signal in the 3′-noncoding region is double-underlined. The possible nuclear targeting signal is indicated by a bold-underline. 5′-Termini of 5′-RACE products for rLACH1 are indicated by arrowheads.

reading frame encoding a 338-amino acid polypeptide, followed by a termination codon (TAG), followed in turn by 302 nucleotides of 3′-noncoding sequence which contained a consensus polyadenylation signal (AATAAA) located 17 residues upstream of a poly(A) tail. The authenticity of the coding region of rBACH cDNA was supported by matching its deduced amino acid sequence with partial amino acid sequences directly determined for 5 peptides obtained by tryptic digestion or CNBr cleavage of purified rBACH (Fig. 2, underlines).

cDNA cloning and sequence analysis of rLACH1. Screening the DEHP-treated rat liver cDNA library with an 834-bp rBACH cDNA yielded 7 positive clones out of 4×10^5 independent recombinants. Five of these clones, designated RLACH1-1-5, contained cDNA inserts of about 1.2 kb. All these clones had the same sequence. As shown in Fig. 1B, the largest clone isolated, RLACH1-3, spanned 1,200 bp not including the poly(A) tail, corresponding to nucleotide 302-1,501, and contained an open reading frame encoding 299 amino acids. However, RLACH1-3 did not contain an in-frame initiation codon (ATG), suggesting that this cDNA lacked part of the 5′-end of the coding region. To obtain a cDNA corresponding to this missing region, we continued further screening by 5′-RACE as described in "Materials and Methods". As a result, 7 cDNAs of different lengths designated as RLACH1-6-12 were obtained (Fig. 2, arrowheads), the largest of which, RLACH1-11, was 620 bp in length and contained a 319-bp sequence that overlapped the 5′-end of RLACH1-3 and 301 bp of new sequence (Fig. 1B). The combined sequence of these two cDNAs spanned 1,501 bp and contained 169 nucleotides of 5′-noncoding sequence, an open reading frame encoding a 343-amino acid polypeptide, followed by a termination codon (TAG), followed in turn by 303 nucleotides of 3′-noncoding sequence which contained a consensus polyadenylation signal (AATAAA) located 17 residues upstream of a poly(A) tail (Fig. 2).

Expression in E. coli. To determine N-terminal sequences of rBACH and rLACH1, direct sequencing of the purified enzymes by Edman degradation was performed. However, we failed to identify N-terminal amino acid residues of these enzymes. Deblocking reactions using acylamino acid-releasing enzyme and pyroglutamate aminopeptidase were also not successful. Therefore, N-terminal amino acid sequences were estimated by comparing the molecular masses of these enzymes expressed in *E. coli* and those of purified preparations.

As shown in Fig. 2 (boxes), the cDNA sequences obtained contained two possible initiation methionine codons in frame. Therefore, to determine the N-terminus of rBACH, we subcloned rBACH cDNAs corresponding to nucleotide 31-1,221 (calculated molecular weight, 44,081) and nucleotide 208-1,221 (calculated molecular weight, 37,559) into pKK223-3 vectors, designated pKK-rBACH-M1 and pKK-rBACH-M2, respectively, and then used these constructs to transform *E. coli* JM 105. As shown in Fig. 3A, immunoblots of the extracts prepared from pKK-rBACH-M1- and pKK-rBACH-M2-transformed *E. coli* with anti-rBACH antibody gave protein bands which were approximately 8 and 1 kDa larger than purified rBACH (43 kDa), respectively. Similarly, we also subcloned rLACH1 cDNA corresponding to nucleotide 170-1,198 (calculated molecular weight, 38,240) into the same vector, designated pKK-rLACH1, and then detected a protein band that was approximately 1 kDa larger than purified rLACH1 (43 kDa) by immunoblotting of the extract from pKK-rLACH1-transformed *E. coli* with anti-rLACH1 antibody (Fig. 3B). These results strongly suggested that N-termini of rBACH and rLACH1 are located downstream of the methionines corresponding to nucleotide 208-210 and 170-172, respectively.

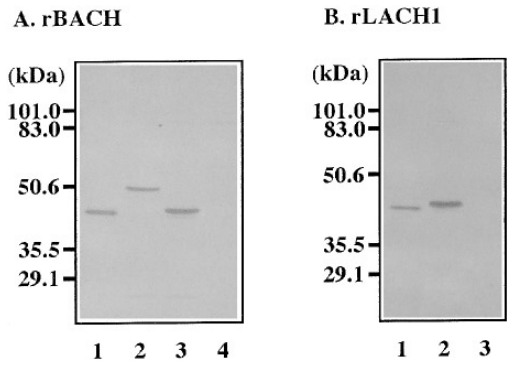

FIG. 3. Immunoblot analysis. (A) Immunoblot of the extracts prepared from *E. coli* transformed with rBACH cDNAs probed with anti-rBACH antibody. Lane 1, purified rBACH (0.05 μg); lanes 2, 3 and 4, extracts (5 μg) from *E. coli* transformed with pKK-rBACH-M1, pKK-rBACH-M2 and pKK223-3, respectively. (B) Immunoblot of the extract prepared from *E. coli* transformed with rLACH1 cDNA probed with anti-rLACH1 antibody. Lane 1, purified rLACH1 (0.05 μg); lanes 2 and 3, extracts (5 μg) from *E. coli* transformed with pKK-rLACH1 and pKK223-3, respectively.

Acyl-CoA hydrolase activities against 20 μM palmitoyl-CoA of the extracts prepared from *E. coli* transformed with pKK-rBACH-M2 (5.3 U/mg protein) and pKK-rLACH1 (6.3 U/mg protein) were two orders of magnitude higher than that of pKK223-3-transformed *E. coli* (0.04 U/mg protein). In addition, more than 90% of these induced hydrolase activities in the former two extracts were neutralized by anti-rBACH and anti-rLACH1 antibodies, respectively (data not shown). Therefore, we confirmed that both cDNAs encoded long-chain acyl-CoA hydrolases.

Homology search. The deduced amino acid sequence of rBACH was not identical with any reported sequences in the data base and shared weak homology of less than 30% with other hydrolyzing enzymes in-

cluding the thioesterase domain of fatty acid synthase (18-23). However, rBACH cDNA had 84 and 91% identity with a cDNA encoding the recently reported rat brain cytosolic acyl-CoA thioester hydrolase ACT (24) at the nucleotide and amino acid levels, respectively. However, the C-terminal amino acid sequence of rBACH (amino acid 329-338) was entirely different from that of ACT, and therefore the rBACH cDNA obtained in this study encoded a different enzyme. As is the case for ACT (24), rBACH and rLACH1 also had no serine as a catalytic center (Gly-x-Ser-x-Gly) which is commonly present in thioesterase domain of fatty acid synthase and carboxylesterases, suggesting that these enzymes are grouped into a novel class of acyl-CoA hydrolyzing enzyme. On the other hand, a possible nuclear targeting signal (28) was found in the rBACH and rLACH1 amino acid sequences (Fig. 2, bold-underline), although whether this signal is truly functional is not clear.

Nucleotide and deduced amino acid sequences of the 1,501-bp rLACH1 cDNA were 84 and 94% identical to those of rBACH cDNA, respectively. However, unmatched nucleotides were confined to the 5′-end (nucleotide 1-230) which contained the N-terminal coding region of rLACH1, and the sequences in this region of rLACH1 cDNA were entirely different from the corresponding region of rBACH cDNA, whereas its downstream sequences (nucleotide 231-1,501) were completely identical, with a few unmatched nucleotides which may be due to polymorphism. These findings suggest that rBACH and rLACH1 may be derived from the same gene and generated by alternative use of exons so that they have different N-termini, as demonstrated with several other genes expression of which is regulated in a tissue-specific manner (25-27). As shown in Fig. 4, the results of Southern blotting of rat genomic DNA also supported this hypothesis, in which only a single species of genom DNA EcoRI-digest (about 6 kbp) was detected using a common sequence to both rBACH and rLACH1 cDNAs as a probe.

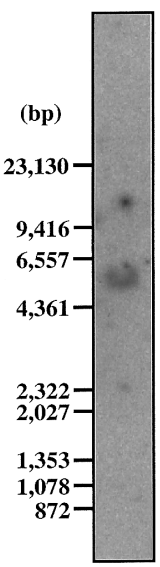

FIG. 4. Southern blot analysis of genomic DNA. Genomic DNA (10 μg) isolated from male Wistar rat liver was digested with EcoRI, and resolved by electrophoresis on agarose gels, capillary transferred onto nylon membranes, and hybridized with an 834-bp rBACH cDNA (nucleotide 557-1,390) labeled with [α-^{32}P]dCTP using the Megaprime DNA labeling system (Amersham). The hybridized signal on the blots was detected by radioluminography using a BAS 2000 II bioimaging analyzer (Fuji Photo Film, Tokyo, Japan). The positions of denatured λ/HindIII digest-ϕX174/HaeIII digest are indicated in the left margin of the blot.

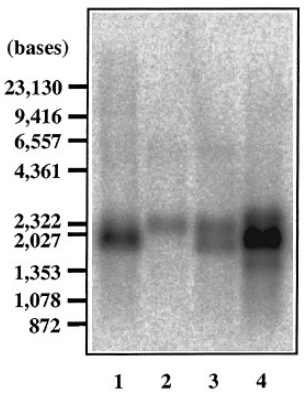

FIG. 5. Northern blot analysis of mRNAs extracted from the brain, liver and testis of rats. Lane 1, brain; lane 2, liver; lane 3, liver from DEHP-treated rats; lane 4, testis. mRNAs (2 μg) were resolved by electrophoresis on denaturing formaldehyde/agarose gels, capillary transferred onto nylon membranes, and hybridized with an 834-bp rBACH cDNA (nucleotide 557-1,390) labeled with [α-^{32}P]dCTP. λ/HindIII digest-ϕX174/HaeIII digest was denatured and used as a size marker.

Northern blot analysis. The distribution of mRNAs encoding ACHs in different rat tissues was studied by Northern blotting using an 834-bp rBACH cDNA as a probe (Fig. 5). Although mRNAs of several sizes (1.5-2.5 kb) hybridized with the probe in the tissues examined, only a single species of about 1.9 kb was detected in the brain (lane 1). This 1.9-kb transcript was also expressed in the testis (lane 4), but not in the liver (lane 2). After treatment of rats with DEHP, a similarly sized transcript was induced in the liver (lane 3), whereas there were no significant changes in the patterns of hybridization in the brain and testis (data not shown). The induction of this transcript correlated well with that of rLACH1 in DEHP-treated rat liver (9). Levels of expression of these transcripts detected in brain and liver were roughly correlated with specific contents of rBACH and rLACH1 (1.5 and 0.3 μg/mg cytosol protein in brain and liver after treatment with DEHP, respectively) determined by immunotitration with anti-ACH antibodies. However, this was not the case for that in the testis. Although the specific content

of rTACH (0.7 μg/mg cytosol protein in testis) was lower than that of rBACH, the level of this transcript in the testis was much higher than in the brain, suggesting that ACH expression may be regulated by an additional mechanism at the translational level in the testis.

In this study, we cloned and analyzed the sequences of rBACH and rLACH1 cDNAs. Our results demonstrated the molecular diversity of cytosolic long-chain acyl-CoA hydrolase in the rat, and suggested the presence of tissue-specific mechanisms to regulate the expression of ACHs in different tissues at the transcriptional or translational levels. Recent studies indicated that PPARs, members of a nuclear receptor superfamily, play an important role in the expression of lipid-metabolizing enzymes (29). It is likely that the induction of rLACH1 by PP (Fig. 4) is also mediated by these nuclear receptors, probably PPARα, in the liver. It will be of interest to identify the PPAR binding sequence (PP-response element) in the ACH gene and examine its role in regulation of ACH gene expression. For better understanding of the molecular mechanism for the tissue-specific expression and molecular diversity of ACHs, analysis of the organization as well as regulatory region of ACH genes will be important. The cDNA clones isolated and their primary structures presented in this study provide a useful tool for these studies, as well as studies of the structure-function relationship of these novel long-chain acyl-CoA hydrolases.

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