Molecular Cloning and Expression of cDNA Encoding Rat Brain Cytosolic Acyl-Coenzyme A Thioester Hydrolase*

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The cDNA encoding rat brain cytosolic acyl-CoA thioester hydrolase (ACT) has been cloned and sequenced, and the primary structure of the enzyme has been deduced. A partial amino acid sequence (36 amino acids) of the enzyme was determined using the peptides generated after CNBr digestion of the purified enzyme. Primers synthesized on the basis of this information were used to isolate two cDNA clones, each encoding the full length of the enzyme. The nucleotide sequences of these clones contained an open reading frame encoding a 358-amino acid polypeptide with a calculated molecular mass of 39.7 kDa, similar to that determined for the purified enzyme (40.9 kDa). The deduced ACT sequence showed no homology to the known sequences of any other thioesterases nor to any other known protein sequence. However, there was a strong homology to a number of expressed sequence tag human brain cDNA clones. The identity of the ACT cDNA was confirmed by the expression of ACT activity in Escherichia coli. There was a 10–15-fold increase in ACT-specific activity in the bacterial extracts after induction with isopropyl thiogalactoside, and the properties of the expressed enzyme (fusion protein) were the same as those of the purified rat brain ACT. Northern blot analysis showed that a 1.65-kilobase ACT transcript was present in rat brain and testis but not in any other rat tissues examined. However, the ACT mRNA was induced in the liver of rats that were fed Wy-14,643, a peroxisome proliferator and inducer of rodent liver cytosolic acyl-CoA thioesterase. These results indicate that the induced rat liver ACT is homologous to the constitutive rat brain ACT.

Long chain acyl coenzyme A thioester hydrolase (E.C. 3.1.2.2) is present in all living organisms (Waku, 1992). Isoforms of this acyl-CoA thioesterase (ACT)° are present in a membrane-bound form in various subcellular organelles (Berge and Farstad, 1979; Berge et al., 1981) and in soluble form inside mitochondria (Svensson et al., 1995a), peroxisomes (Svensson et al., 1995b), and cytosol (Srere et al., 1959). The best characterized of these enzymes are those that are associated with the fatty acid-synthesizing enzyme, where ACT specificity determines the chain length of the synthesized fatty acids (Naggert et al., 1991a; Tai et al., 1993; Witkowski et al., 1991). Likewise, the estrogen-induced thioesterase in duck uropygial gland has also been shown to regulate the chain lengths of fatty acid end products (Hwang and Kolattukudy, 1993). The mitochondrial and microsomal membrane-bound enzymes may have a similar function in controlling the chain length of the fatty acid products during the enzymatic chain elongation process (Berge, 1979). Some of these enzymes may also act as acyltransferases (Lehner and Kuksis, 1993); however, their physiological function in many systems is not known (Anderson and Erwin, 1971; Cho and Cronan, 1993; Dormann et al., 1994; Hwang and Kolattukudy, 1993; Loader et al., 1993; Naggert et al., 1991a, 1991b). The properties, physiological regulation, and catalytic mechanisms of the purified and cloned enzymes have been extensively studied (Naggert et al., 1991a, 1991b; Cho and Cronan, 1993; Tai et al., 1993; Witkowski et al., 1991, Hwang and Kolattukudy, 1993).

Mammalian tissues contain a cytosolic acyl-CoA thioesterase not associated with fatty acid synthase that has high activity in brain and testis (Anderson and Erwin, 1971; Broustas and Hajra, 1995; Kurooka et al., 1972, Smith and Sun, 1981, Srere et al., 1959). Although the activity of this cytosolic thioesterase is normally very low in mammalian liver, the enzyme is highly induced in rodent livers when peroxisome-proliferating agents are administered to the animals (Kawashima et al., 1981; Miyazawa et al., 1981). Homologous soluble enzymes have also been reported to be induced inside liver mitochondria and peroxisomes (Svensson et al., 1995a; Wilcke and Alexson, 1994).

The mammalian soluble ACT has been purified to homogeneity from heart cytosol (Gross, 1983), and from liver cytosol (Miyazawa et al., 1981; Yamada et al., 1994), mitochondrial matrix (Svensson et al., 1995a), and peroxisomal matrix (Wilcke and Alexson, 1994) after induction of the enzyme by peroxisome-proliferating agents. Miyazawa et al. (1981) reported that the antibodies raised against the induced rat liver cytosolic thioesterase precipitated rat brain cytosolic thioesterase. Recently, Yamada et al. (1994) reported that two ACTs are induced in liver by peroxisome-proliferators, and the antibodies raised against the higher specific activity liver enzyme (ACH1) cross-reacted on a Western blot with a 36-kDa brain protein.

The rat brain cytosolic ACT has been partially purified, and its properties have been studied in different laboratories (Anderson and Erwin, 1971; Lin et al., 1984; Srere et al., 1959). We have recently purified this enzyme by 3500-fold to homogeneity, which showed a single band on SDS-polyacrylamide gel electrophoresis with a molecular mass of 40.9 kDa (Broustas and Hajra, 1995). The enzyme had a very broad

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank* and EMBL Data Bank with accession number(s) U49694.

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The abbreviations used are: ACT, acyl-CoA thioesterase; EST, expressed sequence tag; IPTG, isopropyl thiogalactoside; kb, kilobase(s); bp, base pair(s); PCR, polymerase chain reaction.

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substrate specificity with respect to the acyl-CoA chain length and a very high specific activity (turnover number $= 7 \times 10^4 \text{ min}^{-1}$) compared with other purified thioesterases. A partial amino acid sequence of the enzyme was determined by gas phase sequencing of peptides generated by CNBr digestion of the purified enzyme. From this information, we have cloned the rat brain cDNA encoding the cytosolic ACT. The validity of the sequence and the utility of the cDNA clone have been demonstrated by expressing the thioesterase activity in Escherichia coli.

**EXPERIMENTAL PROCEDURES**

Materials—Oligonucleotide primers were synthesized by the Biomedical Core Facility of the University of Michigan. Taq DNA polymerase, deoxynucleoside triphosphates, lysozyme, ampicillin, and tetra-cycline were from Boehringer Mannheim. DNA polymerase (Klenow fragment), restriction enzymes, and the 1-kb DNA ladder electrophoresis standard were from Life Technologies Inc. DNA sequencing reagents (Sequenase 2.0) were from U.S. Biochemical Corp. (alpha-32P)dATP and (alpha-32P)UTP were obtained from ICN Radiochemicals (Irving, CA). T4 DNA ligase and pGEM-T vector were from Promega (Madison, WI). The multiple tissue RNA blot and rat brain 5’ rapid amplification of cDNA ends-ready cDNA library were purchased from Clontech (Palo Alto, CA). Lambda ZAP phage (Roche) helper phage and rat brain cDNA library in lambda ZAP vector were from Stratagene (San Diego, CA). Sephakiss G-75, ethidium bromide, cesium chloride, DNAse I, polynucleotide phosphorylase, and Ficoll were purchased from Sigma. Agarose and NuSieve were purchased from MRC (Rockland, ME). The x-ray film was from Eastman Kodak. Sequences were analyzed using the Genetics Computer Group sequence analysis program. Partial Sequencing of the Enzyme—Amino acid sequencing was performed at the Protein Sequencing Facility of the University of Michigan by an automated Edman degradation method using a gas-phase sequenator (Applied Biosystems). The native enzyme was resistant to Edman degradation and was therefore digested with CNBr in formic acid (30 min, 25°C); and the products were separated by SDS-polyacrylamide gel electrophoresis separation, followed by blotting onto polycvinylpyrrolidone-free membrane (Immobilon P, Millipore). Two peptide bands, one approximately 8,000 Da and another 11,000 Da, were used directly for the gas-phase amino acid sequencing. Synthesis of a 114-bp Probe by PCR—Two degenerate oligonucleotide sense primers based on the amino acid sequence of the N-terminal end (EVLVDADPF) and one antisense primer for a sequence toward the C-terminal end were designed. Two G residues and a restriction endonuclease site for BglII were incorporated at the 5’ end of the sense primers. Similarly, a restriction endonuclease site for EcoRI was included at the 5’ end of the antisense primer. Fig. 1 shows the primers used for the amplification of the cDNA library. The reaction mixture contained 100 nM of each primer (50 μM), 10 μl of 10 × Taq polymerase buffer, 10 μl of dNTP mixture (2.5 mM each), 1 μl of 5’ rapid amplification of cDNA ends-ready rat brain cDNA (Clontech), 5 μl of MεSO4, and water to make 100 μl. The first cycle of the PCR was carried out as follows: 94°C for 1 min, 50°C for 30 s, raised to 72°C in the next 30 s followed by 90 s at 72°C. The subsequent 40 cycles were all the same except the denaturation (94°C) was done for 30 s, 10 μl of the reaction mixture was subjected to 3% NuSieve agarose gel electrophoresis, and a band of the expected size (114 bp) was seen for both the primer combinations (Broustas, 1995). This PCR product was purified from the remaining reaction mixture by preparative agarose gel electrophoresis (Uhlen, 1993).

Preparation and Sequencing of the Probe—The purified PCR-amplified DNA was ligated to PGM-T vector (Promega) and introduced into E. coli XL1-Blue by electroporation. Digestion of the resulting plasmids by EcoRI and BglII and examination of the product by electrophoresis in 3% NuSieve agarose gel (Uhlner, 1993) showed that the expected 114-bp fragment was present. The PCR amplified DNA was released from the plasmids by digestion with EcoRI and BglII, followed by purification by agarose gel electrophoresis.

Preparation of Probe—A second probe was prepared from a clone (ACT 4.0) that was isolated by screening a rat brain cDNA library with the 114-bp probe (see “Results”). The plasmids isolated from ACT 4.0 were digested with EcoRI and KpnI, and the resulting 250-kb DNA probe (see Fig. 2) was purified by agarose gel electrophoresis.

Synthesis of Radiolabeled Probes—Radiolabeled probes were generated by the random primer extension method (Feinberg and Vogelstein, 1983) using (alpha-32P)dATP, the Klenow fragment of E. coli DNA polymerase I and the denatured probes (Uhlner, 1993). After the reaction, the oligonucleotides were separated from the unincorporated labeled and nonlabeled nucleotides using a 1-mL G-75 gel filtration column. The labeled probes, eluted in the void volume of 0.1 × SET buffer, were used directly for screening (1 × SET = 1% SDS, 10 mM Tris, pH 7.4, 10 mM EDTA).

Screening of Rat Brain λ ZAP I cDNA Library—E. coli cells and λZAP I phage were mixed at two different concentrations, plated on agar plates, and incubated overnight at 37°C. The plaques were lifted on either Nytran nylon membrane (first screening) or nitrocellulose papers (second screening) using standard protocols (Sambrock et al., 1989). The filters were first prehybridized at 37°C in a solution (pH 7.2) containing 50% formamide, 5× SSC, 10 mM NaCl, 20 mM sodium phosphate, 10 mM HEPES, 5 mM EDTA, 100 μg/ml herring sperm DNA, and 0.1% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll. After 3 h, the radioactive DNA probe (~107 dpm) was added to the solution containing the blots, which were further incubated at 37°C overnight with gentle shaking. Blots were washed two times at 60°C and at room temperature once in 0.5% SDS, 10 mM Tris, 1 mM EDTA, pH 7.4, dried at room temperature, and then autoradiographed using Kodak X-Omat film. Four to five sequential screenings resulted in purified recombinant phage clones.

In Vivo Excision to Generate the Plasmids—The purified λZAP I clones were added to the E. coli cells (XL1-Blue) infected with the helper phage R-408 (Short et al., 1988) and incubated at 37°C for 3 h according to the conditions described (Stratagene). After the tubes were heated at 70°C for 20 min to kill the E. coli cells (and the phage) and centrifuged, and the supernatant was used to infect a fresh batch of E. coli cells in the presence of ampicillin. Individual colonies were isolated from these cells by streaking them on LB-ampicillin plates and then propagating them in liquid culture. Plasmids were isolated from the harvested cells by alkaline lysis and purified by ethanol precipitation followed by CsCl gradient equilibrium ultracentrifugation procedure (Sambrock et al., 1989). The DNA inserts in these pBluescript SK- plasmids were analyzed by restriction analysis using EcoRI, XhoI, and KpnI enzymes.

DNA Sequencing—The alkali-denatured double-stranded purified plasmid DNA was sequenced using the dideoxynucleotide termination method (Sanger et al., 1977) using the appropriate primers and the Sequenase 2.0 sequencing kit from U.S. Biochemical Corp. The initial primers used were the oligonucleotides complementary to the T3 (5’ end) and T7 (3’ end) promoter regions present in the vector on either side of the insert. The generated nucleotide sequences were then utilized to synthesize new primers to extend the sequence information. This was done until the complete sequence of both the strands could be deduced. Sequencing was also done at the University of Michigan DNA sequencing Core facility using automated (Applied Biosystem) thermal sequencing, which provided additional sequence data. Alignment of the nucleotide residues and translation to amino acids were done using the Genetics Computer Group sequence analysis programs. The validity of the restriction sites was confirmed. A summary of the strategy used and the results obtained is given in Fig. 2.

Induction of ACT in E. coli—E. coli containing clones ACT 1.1 and 5.1 were grown in LB broth containing ampicillin (0.1 mg/ml) with constant shaking at 37°C to the mid-log phase (A600 ≈ 0.3). IPTG (final concentration, 1 mM) was then added to the experimental flasks, and the cultures were further aerobically incubated at 37°C. Aliquots of the cultures were taken at different time periods from which bacterial cells were harvested by centrifugation at 8,000 × g for 10 min. The bacterial pellets were kept frozen at −20°C before processing. The frozen cells were thawed and then lysed by treatment with lysozyme in the presence of phenylmethanesulfonyl fluoride, followed by deoxycholate treat- ment described by Sambrock et al. (1989). The endotoxin rich solution was reduced by treatment with DNase I, and then the solutions were centrifuged at 100,000 × g for 30 min. The supernatants were collected, and their ACT activity was determined with palmitoyl CoA as the substrate by the assay described by Ui et al. (1979). Protein content of the supernatant was measured by the method of Lowry et al. (1951) using the reagent from Pierce and bovine serum albumin as the standard.

Northern Blotting—Rats were fed powdered food containing 0.05% Wy-14,643 for 4 days. Total RNA was prepared from brain, liver, and tests of these rats (and control rats) by homogenization in 5 mM guani- dinium isothiocyanate, 0.5% β-mercaptoethanol, and 0.5% N-lauroyl sarcosine (Chirgwin et al., 1979). The homogenate was layered over a solution of 5.7 M CsCl and centrifuged at 41,000 rpm for 20 h in a Beckman SW41 rotor. The RNA pellets were rinsed with 0.1 × SET and resuspended in water. The RNA was fractionated on a 1% agarose gel in

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formaldehyde and transferred to Nyttran membrane. A radioactive riboprobe was synthesized by using the linearized plasmid isolated from clone ACT 5.0 as the template. One microgram of the plasmid was incubated at 37 °C with 10 units (1 μl) of EcoRI at pH 8.0 overnight. To the mixture was then added 2 μl of 10 × transcription buffer (400 mM Tris-HCl, pH 8.0, 80 mM MgCl2, 20 mM spermidine, 500 mM NaCl), 1 μl of 200 mM dithiothreitol, 1 μl each of 10 mM ATP, CTP, and GTP, 1 μl of 0.1 mM UTP, 5 μl of [α-32P]UTP (650 Ci/mmol, 100 mCi/ml), 1 μl of T7 RNA polymerase (10 units), and RNase-free water to make the total volume 20 μl. The mixture was incubated at 37 °C for 60 min. 1 μl (2 units) of RNase-free DNase was then added to the reaction mixture, which was further incubated at 37 °C for 15 min to hydrolyze the template DNA. The [32P]RNA generated was used directly for hybridization.

The RNA blot was first incubated in the hybridization buffer (5% SDS, 400 mM sodium phosphate, pH 7.2, 1 mM EDTA, 1 mg/ml bovine serum albumin, 50% formamide) at 60 °C for 4 h. The radioactive probe was then added to the solution, and the blot was further incubated with gentle shaking at 60 °C for 22 h. The blot was washed two times with 0.5 × SET buffer at 60 °C followed by one wash with the same buffer at room temperature. After air drying, the blot was used for autoradiography by exposing x-ray film to it at −70 °C.

RESULTS

Partial Amino Acid Sequence of the Purified Thioesterase—The enzyme was resistant to Edman degradation, indicating that its N terminus may be blocked. The enzyme was, therefore, cleaved with CNBr, and two major peptides (8.0 and 11.0 kDa) were isolated by SDS-polyacrylamide gel electrophoresis. Upon sequencing, both the fragments had the same partial amino acid sequence at the N-terminal end, implying that the 8-kDa fragment is nested within the 11-kDa peptide. From the degradation results, the following sequence (38 amino acids) was reliably established: EIVELVDADIPYPVDNOSKRYRASSAFFTYYVSNLOEGKPL. From this sequence, degenerate sense and antisense primers were synthesized with a BglII site at the 5′ end of the sense primers and an EcoRI site at the 5′ end of the antisense primer as described under “Experimental Procedures.” The primers (Fig. 1) were used to amplify the segment using rat brain cDNA and mouse brain cDNA as templates. From both these templates, one band of the expected size (114 bp) was found to be the main product of the PCR (Broustas, 1995). The band was excised, subcloned into the EcoRI site of the PGEM-T vector, and transfected into E. coli as described above. The plasmid was purified by alkaline lysis miniprep and then sequenced using the T7 promoter region of PGEM-T as a primer.

The nucleotide sequence of the PCR product predicted a protein sequence that matched the partial amino acid sequence of the enzyme except for the terminal amino acid (ATG instead of a codon for L).

Cloning of cDNA Encoding the Acyl-CoA Thioesterase—The PCR-generated 114-bp ACT fragment was used for screening a rat brain λZapII cDNA library. Initial screening yielded 20 positive clones from which six were selected for in vivo excision. The sizes of the cDNA inserts in the plasmids from different clones were determined after digestion with EcoRI and XhoI and were found to be 0.3, 0.6, and 1.3 kb for clones ACT 3.0, ACT 5.0, and ACT 4.0 respectively. The inserts in the plasmids ACT 4.0 and ACT 5.0 were sequenced as described under “Experimental Procedures.” The sequence of ACT 5.0 was found to be wholly contained within the ACT 4.0 sequence (Fig. 2). ACT 4.0 was found to be a 1300-bp-long oligonucleotide containing an open reading frame of 1010 nucleotides (Broustas, 1995). The region corresponding to the 114-bp PCR fragment was found between nucleotides 725 and 838. A putative stop codon was located at position 1013; however, a start codon (ATG) could not be located at the 5′ end. The length of the open reading frame was also shorter than expected for a 40-kDa protein. From these and also from the homology of the sequence with a human brain EST clone (see below), it was concluded that this cDNA did not encode the entire thioesterase and that a segment at the 5′ end containing the start codon was missing (Broustas, 1995).

To isolate clones containing the missing 5′ end, the cDNA library was again screened, this time with a 250-bp probe generated from the 5′ end of ACT 4.0. Among the new clones, ACT 1.1 and ACT 5.1 were found to contain the largest inserts: 1.5 kb and 1.4 kb, respectively. The nucleotide sequences of these two clones were determined using the same strategies described under “Experimental Procedures.” The ACT 1.1 insert was found to be a 1400-bp-long polynucleotide that completely contained the nucleotide sequence of ACT 4.0, as mentioned above. The sequence of ACT 5.1 was similar to 1.1 but

Fig. 1. Primers used for the synthesis of probe by PCR. The sequence of various degenerate primers and the corresponding amino acids for the sense and antisense strands are shown. N in the primer sequence indicates that all four nucleotides were used.

Fig. 2. Sequencing strategies and restriction map for brain ACT cDNA clone. The map of the consensus sequence is shown, the black box shows the open reading frame, and the white boxes represent the untranslated 3′ and 5′ ends. The sites for the restriction endonucleases used to generate probes and confirm the sequence are shown. The start of translation of the lacZ gene within pBluescript SK- is also indicated, along with the T7 and T3 promoter regions. The hatched boxes indicate the positions and sizes of the four clones used for sequencing the cDNA. The arrows indicate the direction of sequencing using either sense (5′x) or antisense (3′x) primers. MCS, multiple cloning site.
was truncated at both the 5’ and 3’ ends (1350 bp). Both these clones contained a putative start methionine codon (ATG) at the 5’ end. A comparison of the clones with respect to their relative lengths and positions and a restriction map is given in Fig. 2. The sequence of ACT 1.1, which contained the full-length sequence of clones 5.1, 4.0, and 5.0, is shown in Fig. 3. This cDNA contained a 1074-bp open reading frame with an untranslated region of 93 bp at the 5’ end, and a consensus polyadenylation signal (AAATAAA) 16 bp upstream from the 3’ end. Translation of this open reading frame yielded a 358-amino acid protein with a calculated molecular mass of 39.7 kDa, which is in close agreement with the molecular mass of 40.9 kDa deduced from SDS-polyacrylamide gel electrophoresis analysis of the enzyme (Broustas and Hajra, 1995). The deduced amino acid sequence for the cDNA clone ACT 1.1 is given in Fig. 3. No nucleotide sequence differences were found for any of the cDNAs described.

Homology of Acyl-CoA Thioesterase cDNA to Other cDNAs—The nucleotide sequence encoding brain acyl-CoA thioesterase did not show any homology to the known sequences of other acyl-CoA thioesterases nor to the thioesterase domain of fatty acid synthase. Neither the nucleotide sequence nor the translated amino acid sequence showed any significant homology to any known protein sequence stored in Genbank or other available data bases. However, significant homologies were seen to a number of human brain EST clone sequences (Adams et al., 1991). Sequences of five different brain EST clones (T 74295, M 62036, F 12498, T 32226, Z 41960) are homologous to the ACT sequence presented here. An analysis of the homologies found with four of these human EST clones, which in combination cover the whole length of the ACT 1.1 clone, is presented in Table I.

Expression of ACT in E. coli—Both clone ACT 5.1 and ACT 1.1 contain the full open reading frame for the thioesterase (Fig. 2). Examination of the sequences showed that in clone 1.1 but not 5.1 the start codon, ATG, of the open reading frame is “in-frame” with the start codon of the split lacZ (β-galactosidase) gene present in the pBluescript-SK+ phagemid. Therefore, it was reasoned that if an inducer for the lacZ gene product (β-galactosidase) was added to these E. coli clones, a fusion protein with an N-terminal fragment of β-galactosidase (plus the multiple cloning site and the normally untranslated 5’ end of ACT) and acyl-CoA thioesterase would be expressed in the bacteria. This was shown to be the case by adding the β-galactosidase inducer IPTG to a growing culture of E. coli containing the ACT 1.1 clone. On addition of IPTG, there was a strong induction of acyl-CoA thioesterase activity, compared with the control culture where no IPTG was present (Fig. 4A). As expected, IPTG did not induce acyl-CoA thioesterase activity in the ACT 5.1-containing E. coli (Fig. 4A). The protein content of the extracts were similar for the bacteria grown with or without IPTG varying from 0.8 mg/ml (0 h sample) to 8–9 mg/ml (24–36 h samples) in both the cultures.

The properties of the induced thioesterase in the E. coli extract were very similar to those of brain cytosolic acyl-CoA thioesterase, as seen by its inhibition by bovine serum albumin, detergents, diethylpyrocarbonate, and p-hydroxymercuribenzoate but not by N-ethylmaleimide or phenylmethanesulfonyl fluoride (data not shown). The Km of the induced E. coli enzyme with palmitoylCoA was 4.5 μM, similar to that of the brain ACT (6.0 μM). Substrate specificity of the recombinant enzyme was broad with respect to the chain length (Fig. 4B), similar to that of the rat brain ACT (Broustas and Hajra, 1995).

**Fig. 3. Nucleotide and deduced amino acid sequence of cDNA encoding brain ACT.** The single line underlined region corresponds to the partial amino acid sequence determined directly from the enzyme, which was used to generate the probe for screening. The polyadenylation signal is shown by the double lines.

**Table I Homology of cDNA encoding rat brain ACT with human EST clones**

| EST clone number | Nucleotide position of ACT | Nucleotide homology | Polynucleotide | Translated (ORF) polypeptide | % |
|-----------------|---------------------------|---------------------|---------------|------------------------------|---|
| T 47295         | 22–396                    | 76.3                | 86.0          |                              |   |
| T 74295         | 314–765                   | 89.4                | 96.7          |                              |   |
| M 62036         | 728–1083                  | 86.8                | 93.2          |                              |   |
| T 35417         | 1006–1236                 | 82.0                | 87.0          |                              |   |

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Esterases have a serine at the active site in a GXXXG motif, but not inhibited by active site serine-specific reagents such as phenylmethylsulfonyl fluoride, indicating that serine is also involved in the catalysis. The brain enzyme is also strongly inhibited by diethylpyrocarbonate, indicating that a histidyl moiety may be involved in catalysis. An active site histidine has been demonstrated in mammary gland thioesterase II, but the mammary gland enzyme is also inhibited by phenylmethylsulfonyl fluoride, indicating that serine is also involved in the catalysis. The brain enzyme is also strongly inhibited by p-hydroxymercuribenzoate, a sulfhydryl agent, indicating that a thiol group may be involved in catalysis.

The deduced amino acid sequence of brain ACT does not seem to have any consensus functional sequence motifs, and the sequence itself does not yield any clues regarding its catalytic function. Therefore, it seems that the ACT sequence presented here is unique and represents a new class of enzyme utilizing long chain acyl-CoAs as substrates. Considering that brain ACT is a cytosolic (soluble) enzyme, there is a comparable distribution of hydrophobic amino acids along the polypeptide backbone, except for the region of amino acid residues 140–156, where no hydrophobic region is present in the deduced amino acid sequence. A hydropathy plot indicates that the hydrophobic amino acids are almost evenly distributed along the polypeptide backbone, except for the region of amino acid residues 140–156, where no hydrophobic amino acids are present.

Though the ACT sequence shows no homology to any known protein sequence, a very strong homology was seen with a number of human brain EST clones as presented in Table I. The translated amino acid sequences of these clones have a higher homology with the deduced polypeptide sequence of ACT than the nucleotide sequences because of species-specific codon selection. The sequences of these clones overlap to cover the entire deduced length of rat brain ACT cDNA (Table I). There is greater than 90% homology between the translated region of the combined human EST clones and rat brain ACT, and in the positions where the amino acids do differ, the replacement amino acid is usually homologous, bringing the sequence similarity close to 100%. Such phylogenetic conserva-
enzyme activity is highest in brain followed by testis (to the known tissue distribution of the enzyme. In rats the expressed only in rat brain and testis, which corresponds well antibody production and also for detailed studies on its struc-

tion of brain ACT in

and Cronan, 1993; Naggert shown not to be deleterious to the growth of the bacteria (Cho

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induction(320)
catalytic activity. It is of interest to note that this high level of

physiological function of this enzyme.

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ACT mRNA was found in normal rat liver, but ACT mRNA is present in the liver of Wy-14,643-fed rats (Fig. 5). The cytosolic ACT activity in liver is also greatly increased in these rats (from 0.01 to 0.2 units/mg protein). Though the Northern blot analysis qualitatively indicates that the ACT mRNA level in testis is also increased in the Wy-14,643-fed rats (Fig. 5B), no change was observed in the activity of ACT in testis of these animals.2 Yamada et al. (1994) also observed no increase in ACT activity in testis of di(2-ethylhexyl)phthalate-fed rats. The induction of ACT mRNA in liver and the similarity of properties between the brain ACT and the induced liver thioesterase reported by other laboratories (Kawashima et al., 1981; Yamada et al., 1994) indicate that either the same isoform or an enzyme that is structurally very similar to the brain ACT is induced in liver.

The physiological function of this cytosolic thioesterase is not clear. The enzyme activity is associated with the lipid metabolic activity of tissues (Svensson et al., 1995b) with highest activity in brain and testis, tissues that have high steroidogenic activity. We have postulated that this enzyme regulates peroxi-

somal fatty acid oxidation, one of the functions of which is to produce acetyl CoAs, the building block for steroids and also for fatty acids (Broustas and Hajra, 1995; Broustas, 1995). The increase in activity of this enzyme in liver after induction correlates with the increase in peroxisomal β-oxidation of fatty acids and the increase in liver weight of the animals, indicating that the high activity of ACT is associated with the high rate of membrane lipid biosynthesis. Our findings suggest that differences exist in the regulation of this enzyme in brain, where it is constitutive, and liver, where it is highly induced by hypolipidemic peroxisome-proliferating agents. The induction of proteins in rodent liver by these agents has been shown to be effected via cytosolic receptors (PPAR), which, after binding to the hypolipidemic agents, are translocated to the nucleus. Within the nucleus, PPARs have been shown to form heterodimers with retinoid X receptors and act as transcription factors (Green and Wahli, 1994). It is possible that in brain these PPARs are constitutively active due to the presence of endogenous ligands such as free fatty acids. The cDNA does described here and the primary structure of the enzyme pre-

scribed here should prove to be very useful in establishing the mechanism of this tissue-specific regulation, as well as the physio-

logical function of this enzyme.

Cytosolic Acyl Coenzyme A Thioester Hydrolase

Northern blot analysis shows that the mRNA for ACT is expressed only in rat brain and testis, which corresponds well to the known tissue distribution of the enzyme. In rats the enzyme activity is highest in brain followed by testis (~50% of brain activity on a per gram basis) (Kurooka et al., 1972; Yamada et al., 1994). All other organs have very low enzyme activity, which may in part be due to the presence of other isoforms of the enzyme. The surprising finding is that although the specific activity of ACT is lower in testis, the ACT mRNA level is much higher in this tissue than in brain (Fig. 5). No 2 L. K. Larkins and A. K. Hajra, unpublished data.
Cytosolic Acyl Coenzyme A Thioester Hydrolase

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Molecular Cloning and Expression of cDNA Encoding Rat Brain Cytosolic Acyl-Coenzyme A Thioester Hydrolase

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