Identification of a Form of Acyl-CoA:Cholesterol Acyltransferase Specific to Liver and Intestine in Nonhuman Primates

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Richard A. Anderson‡‡§, Charles Joyce§§, Matthew Davis§, Jerry W. Reagan, Michelle Clark§, Gregory S. Shelness**§, and Lawrence L. Rudel§§§§

From the Arteriosclerosis Research Program, Departments of ‡‡Biochemistry, §§Comparative Medicine, **Internal Medicine, and ¶¶Pathology, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157

The present study demonstrates that two different forms of the intracellular cholesterol esterification enzyme acyl-CoA:cholesterol acyltransferase (ACAT) are present in the nonhuman primate hepatocyte; one is similar to that originally cloned from human genomic DNA, here termed ACAT1, while a second gene product, termed ACAT2, is reported here. The primate ACAT2 gene product was cloned from an African green monkey liver cDNA library. Sequence analysis of an isolated, full-length clone of ACAT2 cDNA identified an open reading frame encoding a 526-amino acid protein with essentially no sequence similarity to the ACAT1 cDNA over the N-terminal 101 amino acids but with 57% identity predicted over the remaining 425 amino acids. Transfection of the cloned ACAT2 cDNA into two different mammalian cell types resulted in the production of abundant ACAT activity which was sensitive to ACAT inhibitors. Northern blot analysis showed that the ACAT2 mRNA was expressed primarily in liver and intestine in monkeys. In contrast, ACAT1 mRNA was expressed in almost all tissues examined. Topologic predictions from the amino acid sequence of ACAT2 indicate that it has seven trans-membrane domains in a configuration that places the putative active site of the enzyme in the lumen of the endoplasmic reticulum. This orientation of ACAT2 in the endoplasmic reticulum membrane, in addition to its expression only in liver and intestine, suggests that this enzyme may have as its primary function, the secretion of cholesterol esters into apoB-containing lipoproteins.

The intracellular formation of cholesterol esters catalyzed by the action of the enzyme acyl-CoA:cholesterol acyltransferase (ACAT; EC 2.3.1.26) appears to be nearly ubiquitous in mammalian cells (1). Elucidation of the details of the structure and catalytic mechanism of ACAT and of the regulation of its activity have been stymied by the difficulty in isolating and purifying an active form of this membrane-associated enzyme. It has taken the isolation of a cDNA for ACAT from human genomic DNA, accomplished through functional complementation of mutant Chinese hamster ovary cells lacking ACAT activity, to initiate progress in understanding the biochemistry of ACAT function (2). The mRNA for this ACAT is expressed in most human tissues and cDNAs with nearly identical ACAT sequences have likewise been found in a variety of tissues from mouse, hamster, and rabbit (3–5).

Several functions can be attributed to cholesterol esterification by ACAT. The enzyme appears to modulate the potentially toxic effects of cholesterol in cell membranes. By attaching a fatty acid to the free hydroxyl group of cholesterol, physical properties of the cholesterol molecule are changed and the solubility of esterified cholesterol in the lipids of the cell membrane is limited. Cholesteryl esters accumulate in lipid droplets in the cytoplasm, and maintenance of a balance between the free and esterified forms of cholesterol in a cell is believed to be a component of regulation of cholesterol signaling pathways (6).

Evidence is also accumulating that ACAT activity is important for cholesterol transport in lipoproteins by the liver and intestine. The majority of cholesterol absorbed from the intestinal lumen by the mucosal cell is esterified by ACAT (7) and incorporated into chylomicron particles (8). Furthermore, the secretion of apoB-containing lipoproteins by the liver appears to depend on an extent on ACAT-catalyzed cholesterol esterification (9–11). Formation of cholesteryl esters in the primate liver is promoted by oleic acid-enriched diets (12) and is catalyzed by ACAT (13). Recent work from our laboratory has shown that hepatic secretion of cholesteryl esters in apoB-containing lipoproteins, as monitored during isolated liver perfusion, was strongly associated with the extent of coronary artery atherosclerosis in nonhuman primates fed atherogenic diets, (12), and was sensitive to inhibition by ACAT inhibitors (11). Interestingly, the extent of diet-induced cholesteryl ester accumulation in the liver, while correlated to the rate of hepatic cholesteryl ester secretion, was not as highly correlated to the extent of coronary artery atherosclerosis as was the secretion rate (12). One possible explanation was that the hepatic ACAT enzyme that catalyzed the formation of the cholesteryl esters of lipoproteins was different from the enzyme that catalyzed the formation of cholesteryl esters of intracellular lipid droplets. In any case, an important role for hepatic ACAT in the development of coronary artery atherosclerosis was indicated.

Conceptually, the presence of various forms of ACAT differentially distributed in tissues and conceivably present in separate cellular compartments is consistent with the increasing evidence for multiple roles of sterol esterification in cholesterol...
metabolism. The possibility that more than one ACAT enzyme is present in at least some tissues of mammalian species was highlighted by the mouse knockout experiment showing that disruption of the ACAT gene analog to the human ACAT gene cloned by Chang et al. (2), did not alter the pattern of intestinal cholesterol absorption or hepatic cholesteryl ester accumulation when cholesterol was fed (14). Furthermore, the work of Sturley and colleagues (15) and Yu et al. (16) demonstrated two different ACAT analogs in yeast, suggesting that two or more ACAT enzymes in mammalian species would not be unprecedented. Additional work in the Sturley (17) and Farese laboratories (18) identifying at least two ACAT-related gene products (ARGP) in the EST data bases for the human and mouse genomes supports the possibility that more than one ACAT enzyme exists.

In the work reported here, the complete nucleotide sequence has been determined for the cDNA of a second ACAT (ACAT2) isofrom in primates which varies substantially in structure from the initially described human ACAT (2). ACAT2 is present in an organ-specific distribution (only liver and intestine) that differs from the nearly ubiquitous expression pattern seen for the original human ACAT, here termed ACAT1. The specific tissue location and topology prediction of this new ACAT2 isoform appears to adapt it to the particular function of providing cholesteryl esters for lipoprotein secretion from hepatocytes and intestinal mucosa. In this way ACAT2 may complement the ACAT1 analog, the latter being found widely distributed among tissues in the monkey apparently serving more general cell functions.

EXPERIMENTAL PROCEDURES

Materials—For earlier experimental protocols (19), organs from adult male African green monkeys (Cercopithecus aethiops) were collected at the time of necrospy, cut into 1-cm cubes, and snap frozen in liquid nitrogen. Samples were stored at −80 °C until use. Tissues from two adult male cynomolgus monkeys (Macaca fascicularis) fed monkey chow were also collected at necrospy. All experiments with monkeys had been subjected to prior approval by our institutional animal care and use committee. AC29 cells, a Chinese hamster ovary cell line (Invitrogen, San Diego) using RNA purified in our laboratory. 9.5 μg of recombinant plasmid DNA was linearized with ApaI, EcoRI, and BamHI and was transfected into E. coli.
and vena cava were then cannulated. The liver was then continuously flushed with a calcium, magnesium-free Krebs-Henseleit original Ringer bicarbonate solution containing 0.5 mM EGTA, pH 7.4, for approximately 10 min at a rate of 1 ml/min/g liver while the liver was excised from the abdominal cavity. The caudate lobe of the liver was then tied off, removed, and snap frozen. The liver was then suspended on a nylon screen in a closed chamber. Hank's balanced salt solution containing 26 mM Tricine buffer, pH 7.4, 1 mM CaCl$_2$, and 0.05% collagenase (Boehringer Mannheim) was then recirculated for 15–20 min (1 ml/min/g liver). All procedures were done under sterile conditions.

Throughout perfusion, solutions were kept at 37 °C and oxygenated with 100% oxygen. After perfusion, the liver was transferred to a sterile container, Williams E medium containing 0.5% bovine serum albumin was added, and the tissue was teased apart. The resulting hepatocyte suspension was filtered through a 500-μm nylon mesh and washed twice by gentle resuspension in fresh Williams E medium with 0.5% collagenase (Boehringer Mannheim) and then recirculated for 15–20 min (1 ml/min/g liver). All procedures were done under sterile conditions.

From the remaining cell suspension, Kupffer cells were collected by centrifugation at 550 g for 5 min, at 4 °C. Hepatocyte yield was typically in excess of 1.5 × 10$^8$ cells/liver. The degree of contamination with hepatocytes, checked microscopically, was estimated to be <10%.

Structure Prediction—Topology and secondary structure predictions from the deduced ACAT amino acid sequences were performed using the Predict Protein service maintained by the Protein Design Group, European Molecular Biology Laboratory, Heidelberg, Germany (30, 31).

RESULTS

Cloning and Sequence Analysis—As the initial step in the cDNA cloning strategy, RT-PCR using monkey liver mRNA as template was performed with primer sequences whose design was based on a partial human ACAT-related gene sequence termed ARGP2 by Sturley et al. (17). These primers were used to generate a monkey ACAT-like cDNA fragment used subsequently to screen the monkey liver cDNA library. Aligning the nucleotide sequence similarities in ARGP2 and human ACAT1, a 355-nucleotide segment was chosen that corresponded to the presumed COOH-terminal portion of the coding sequence and the adjacent 3′-noncoding region. The resulting PCR product displayed 94% identity in nucleotide sequence to the region in human ARGP2 but only about 50% identity to the cognate patch in human ACAT1.

Screening of 7.5 × 10$^4$ colonies from the green monkey liver plasmid cDNA library with this probe yielded 13 positive signals that appeared on duplicate filters. Five of these were purified to homogeneity. The nucleotide sequence of the longest insert, present in plasmid pACAT2.23, is shown in Fig. 1. A 5′-rapid amplification of cDNA ends determination involving primer-mediated extension of total RNA from African green monkey liver was performed in triplicate and confirmed that the sequence shown represents the full extent of the 5′-end of this message (data not shown).

Examination of this full-length cDNA sequence indicates that the structure of the mRNA encoding this new African green monkey ACAT protein is clearly distinct from that of the human ACAT. Assuming that the initiation of translation is at the initial ATG codon at nucleotide 80 (Fig. 1), the 5′-untranslated region for the monkey ACAT-like message is only 79 nucleotides long in contrast to the 1396 nucleotides for human ACAT1 mRNA (2). The 3′-end untranslated region of the pACAT2.23 cDNA insert consists of 421 nucleotides that contain the conventional AATAAA polyadenylation signal 24 residues upstream from the run of A residues that terminates the cDNA. The position of this motif is 45 nucleotides downstream of the first of 2 polyadenylation sequences that appear in the 965 nucleotide 3′-untranslated region of the human macrophage ACAT message (2).

The amino acid sequence deduced from the cDNA insert in pACAT2.23 is shown in Fig. 1. Translation beginning at the first methionine codon gives rise to an open reading frame of 526 amino acids residues. This compares to 551 amino acids for human and monkey ACAT1. Consistent with the sequence of human ACAT1, the NH$_2$-terminal amino acid sequence of the new enzyme does not appear to encode a signal peptide sequence (33).

The deduced AA sequence for the African green monkey ACAT-related protein appears completely unrelated to that of the published sequence of human ACAT for the initial 101 amino acids, but from that point to the end of the protein, the sequences are 57% identical. In Fig. 1 the identical residues are indicated by shading. In particular, the amino acid pattern in the region centered around the DWWN residues near position 384 are highly conserved in all of the reported ACAT and ACAT-like genes (15, 34). The primary structure of the region surrounding the serine residue at position 249 in the monkey ACAT2 sequence, which represents the cognate of the essential serine at position 269 in the hamster ACAT (4), also closely resembles the corresponding region in human ACAT1 (34). Other primary structural elements in the monkey sequence include a leucine heptad motif between amino acids 342 and 363 and 2 potential N-glycosylation motifs at N329 and N387, only the second of which is present in human ACAT.

Prediction of the topology of the African green monkey ACAT2 protein from the deduced amino acid sequence indicates the existence of 7 potential transmembrane domains, including amino acid residues 124–141, 162–179, 205–222, 306–323, 344–366, 418–435, and 440–461, with the amino-terminal 123 amino acids as the only long span predicted to be intracytoplasmic (Fig. 2). In this model, 26% of the amino acids in the protein are predicted to interact directly with the ER membrane as transmembrane helices. Most of the remainder of the peptide, including the essential serine at position 249, is predicted to reside within the ER lumen.

Comparison of African Green Monkey ACAT1 and ACAT2—To further characterize the structure of the newly discovered ACAT2 and compare its properties to ACAT1, cell-free translations were performed. A cDNA representing the African green monkey form of human ACAT1 was produced by RT-PCR (see “Experimental Procedures”). The amino acid sequence deduced from this cDNA differs from the human sequence at only 11 positions, most of which are conservative. The mRNAs for ACAT1 and ACAT2 were transcribed in vitro and used to program rabbit reticulocyte lysate translation extracts. Translation products were resolved by SDS-PAGE and detected by autoradiography. As observed in Fig. 3, lanes 1 and 2, translation of ACAT1 (A1) and ACAT2 (A2) mRNA gave products with apparent molecular masses of ~50 and 47 kDa, respectively.

To confirm that the newly discovered ACAT2 is an integral membrane protein and to determine if any of the potential sites for N-linked glycosylation are utilized, translations were repeated in the absence (−) or presence (+) of nuclease-treated rough microsomes (RM). Following translation, samples were adjusted to 0.1 M Na$_2$CO$_3$, pH 11.5, and separated into pellet (P)
and supernatant (S) fractions by centrifugation. Most of the protein was recovered in the carbonate supernatant when ACAT1 and ACAT2 were translated in the absence of RM (compare lanes 7 and 8; 11 and 12). In contrast, when translations were performed in the presence of RM, a significant proportion of the protein was recovered in the carbonate pellet, indicating successful integration into the microsomal membranes. In neither case, however, was a mobility shift detected upon translation in the presence of RM suggesting that neither ACAT is modified by N-linked glycosylation (compare lanes 8 and 9; 12 and 13). To confirm that the microsomal membranes were glycosylation competent, mRNA for the ileal bile acid transporter protein was translated. The primary translation product of ileal bile acid transporter is 33 kDa (Fig. 3, asterisk) and is recovered in the carbonate supernatant. Upon addition of RM, most of the protein underwent glycosylation at two sites resulting in a mobility shift corresponding to an apparent increase in size of 5 kDa (2). The glycosylated product was recovered primarily in the carbonate pellet (lane 5).

Expression—AC29 cells have been shown to lack ACAT activity, protein, and mRNA (20), and we did not find significant activity in microsomes made from AC29 cells that had not been transfected (Table 1). Transfection of cultured AC29 cells with monkey ACAT2 cDNA in either the pcDNA3 vector or pCMV5 vector gave levels of ACAT activity near 2,000 pmol/min/mg microsomal protein. The levels of activity achieved were essentially the same for the two vectors and were comparable to the activity found in the rat liver microsomes run with each assay as a control. Microsomes from transfected AC29 cells were incubated with two different ACAT inhibitors, one from Parke-Davis, PD138412 at 3.0 μM, and one from Pfizer, CP113818 at 0.2 μM, both of which had been shown to be effective against ACAT activity in monkey liver microsomes (11). Both inhibitors decreased activity by 80 to 90%.

ACAT activity was also measured in COS-1 cells, which were transfected with the pcDNA3 bearing the monkey ACAT2 cDNA using the DEAE-dextran procedure. COS cells have endogenous ACAT activity, but after transfection, ACAT activity was over 10-fold higher than background indicating that the monkey ACAT2 cDNA conferred ACAT activity to these cells as well (Table 1). As an additional control, AC29 cells were transfected with monkey ACAT1 cDNA. These transfections gave ACAT activities 100-fold over background and similar to those...
found with the African green monkey ACAT2 cDNA. The cell
transfection studies indicate that the monkey ACAT2 clone
encodes an enzyme that confers ACAT activity to mammalian
cells. It is therefore appropriate to name this enzyme ACAT2 to
distinguish it from the human ACAT originally cloned by
Chang and colleagues (2), which we refer to as ACAT1.

**Tissue Distribution**—The tissue distribution of expression of
ACAT1 and ACAT2 was determined by analysis of poly(A)
mRNA isolated from 18 different monkey tissues using North-
ern blots (Fig. 4). These data are typical of studies done on
selected tissues from 5 different African green monkeys and 2
different cynomolgus monkeys. The presence of ACAT2 was
detected with a riboprobe sequence representing the 3′-noncod-
ing portion of the ACAT2 message. A single band consistent
with a 2,200-nucleotide long ACAT2 mRNA appears promi-
nently in the lanes for liver and intestine (Fig. 4B). With the
exception of a less pronounced, single band of 2,600 bases that
appears in the kidney lane, none of the other tissues tested
shows an ACAT2 signal. When liver and intestine from cyno-
molgus monkeys were examined, the additional band at 2,600
bases was typically seen. Probing this Northern blot with a
riboprobe representing sequence in the unique amino-terminal
portion of ACAT2 showed the same pattern of expression ex-
cept that the larger band in the kidney lane was not present
(data not shown), suggesting that hybridization to this larger
band does not represent ACAT2 mRNA.

The smaller, 850-nucleotide band represents the signal aris-
ing from a labeled riboprobe for cyclophilin which was to serve
as a load control. However, the lighter cyclophilin signals that
are evident in the lanes for skeletal muscle, heart, pancreas,
thyroid, and testes are apparently due to the fact that this
particular message is less abundant in these tissues (22) and
thus the cyclophilin signal does not quantitatively correlate
with the amount of mRNA loaded in all lanes; the same low
signals for these tissues were seen on 3 separate Northern
analyses done for this study. Nevertheless, longer exposure of
the autoradiographs still failed to show any ACAT2 signal in

**Table I**

| Enzyme   | Vector        | ACAT Inhibitor | Cell type | n | ACAT activity (pmol/min/mg/protein) |
|----------|---------------|----------------|-----------|---|------------------------------------|
| ACAT2    | pcDNA3+       |                | AC29      | 4 | 12 ± 2                             |
| ACAT2    | pcCMV5        |                | AC29      | 7 | 2,209 ± 123                        |
| ACAT2    | pcCMV5 PD138412 |               | AC29      | 3 | 1,927 ± 209                        |
| ACAT2    | pcCMV5 CP113,818 |             | AC29      | 1 | 548                                |
| ACAT2    | pcDNA3+       |                | COS       | 7 | 3,823 ± 950                        |
| ACAT2    | pcDNA3+       |                | COS       | 5 | 1,359 ± 165                        |
| ACAT1    | pcDNA3+       |                | AC29      | 2 | 1,185 ± 167                        |
| Rat liver| pcDNA3+       |                | AC29      | 1 | 1,868 ± 203                        |
| Microsomes|              |                |           | 5 |                                    |

**Fig. 2.** Representation of the predicted transmembrane topology of monkey ACAT2. Amino acid sequence
extends from the initial methionine (upper area) on the cytoplasmic side to cross the lipid bilayer 7 times in helical con-
formations before ending in the lumen of the ER (lower area).

**Fig. 3.** Cell free translation. mRNAs transcribed by T7 RNA pol-
ymerase were translated in rabbit reticulocyte lysates in the presence (+) and absence (−) of 1 equivalent of nuclease-treated canine pancreas
RM. After translation, samples were placed on ice and 1 equivalent of
RM was added to the minus RM samples. After treatment with sodium
carbonate, pH 11.5, pellet and supernatant fractions were analyzed by
SDS-PAGE and autoradiography. In lanes 1 and 2, ACAT1 (A1) and
ACAT2 (A2) translations were analyzed directly without prior carbon-
ate treatment and centrifugal fractionation. The asterisk beside lane 4
(∗) indicates the position of the ileal bile acid transporter (IBAT) pri-
mary translation product.
any of these lanes. While skeletal muscle mRNA failed to show any appreciable signal with the cyclophilin load control, a prominent band was seen in this lane when a glycerol-3-phosphate dehydrogenase probe was substituted as the load control (data not shown).

The ACAT1 probe, in contrast, hybridized to a characteristic, multi-band array in almost all tissues examined (Fig. 4A). While the highest signal appeared in mRNA from adrenal tissue (note that 5-fold less mRNA was loaded in the adrenal lane), prominent ACAT1 message bands were also visible in RNA from trachea, heart, liver, kidney, adipose tissue, and pancreas. Relative to the high signal in adrenal, the amount of ACAT1 message in liver and particularly in intestine seem disproportionately low in view of the high level of ACAT activity seen in microsomes made from these tissues. Skin mRNA showed an ACAT1 signal almost identical to that in the adjacent lane for aorta in other Northern blots (data not shown).

The sizes of the ACAT1 bands are consistent in all of the monkey tissues examined: 2.1, 2.5, 3.2, and 3.6 kilobase pairs. Except for the largest size, these are close to the values reported for rabbit adrenal tissue (5) but are significantly smaller than the reported sizes of the 4 major bands reported for human adrenal ACAT message.

In an effort to determine whether different ACAT enzymes are expressed in different cell types in monkey liver, a Northern blot comparing the specific ACAT messages in mRNA extracted from isolated cynomolgus monkey hepatocytes, Kupffer cells, and whole liver before cell isolation, is shown in Fig. 5. The banding pattern for ACAT1 is similar in hepatocytes and Kupffer cells, while the signal intensity is approximately double for Kupffer cells compared with hepatocytes. In contrast, the signal for ACAT2 in hepatocytes is significantly greater (~6 times) than in Kupffer cells. Thus, the data suggest that both ACAT1 and ACAT2 are present in hepatocytes, while Kupffer cells contain primarily, if not solely, ACAT1, depending on the degree of hepatocyte contamination. The probes used for ACAT1 and ACAT2 were specific to sequences in each enzyme and the binding efficiency of either probe may be different. Therefore, it is not possible from these data to be able to compare the actual amounts of mRNA for these two enzymes.

When comparing ACAT2 RNA in either cell type to that extracted from a sample of the same liver from which the cells were isolated, the ACAT2 bands in either isolated cell type were lighter in intensity than for fresh whole liver. This may indicate that some loss of ACAT2 mRNA occurred during cell isolation procedures. This is not an unreasonable possibility since the cells remain at 37 °C for some 30 min during collagenase digestion, and then at room temperature for another 60–90 min during cell isolation and washing. The data suggest that ACAT2 mRNA may be more labile than ACAT1 in isolated hepatocytes.

The upper band seen for ACAT2 is the same size as the larger band seen for kidney in Fig. 4. This band was not seen in liver from African green monkeys, but is apparent in cynomolgus monkey liver. The blot of Fig. 5 was reprobed with the 5'-end probe for ACAT2 and this band was not seen (data not shown).

**DISCUSSION**

Evidence in nonhuman primate models has been obtained over the past two decades indicating that the accumulation of cholesteryl esters in low density lipoprotein, with the associated low density lipoprotein particle enlargement, is highly correlated with the extent of coronary artery atherosclerosis (35–38). Most recently, we have shown that the secretion of cholesteryl esters from the liver, as monitored during isolated liver perfusion, is correlated with the enrichment of the low density lipoprotein particles with cholesteryl esters and is also predictive of the extent of coronary artery atherosclerosis (12). The formation of cholesteryl esters in primate liver is catalyzed by ACAT (13), and the secretion of cholesteryl esters and apoB by the perfused primate liver is decreased by ACAT inhibitors (11). When diets rich in monounsaturated fat are fed to African green monkeys, the livers accumulated excess cholesteryl ester in the form of cholesteryl oleate, the secretion of cholesteryl oleate was enhanced, and the extent of coronary artery atherosclerosis occurred out of proportion to the low density lipoprotein and high density lipoprotein cholesterol concentrations (12, 39). All of this evidence points to a key role for hepatic cholesterol esterification by ACAT in promoting diet-induced coronary artery atherosclerosis in primates. Therefore, it was important for us to identify the ACAT enzymes in primate liver as a prelude to study of their regulation.

The evidence supporting the conclusion that the African green monkey cDNA clone we have characterized in this publication is an authentic ACAT enzyme includes, first and foremost, that transfection of mammalian cells with this clone results in high levels of ACAT activity (Table I). The activity in the cells was shown to be associated with the microsomal fraction. The levels of activity were as high as that found in rat liver microsomes and as high or higher as that seen when monkey ACAT1 cDNA was transfected into the cells. Furthermore, the activity was inhibitable by at least two ACAT inhibitors. The AC29 cells from Chang and colleagues (2, 20) used for most of these transfection studies have no ACAT activity, protein, or mRNA, providing a rigorous system in which to characterize ACAT gene expression. A second mammalian cell line, COS-1, was also successfully transfected with ACAT2 to make
One-µg samples of poly(A)+ RNA that was purified from isolated cell populations of hepatocytes (H) and Kupffer cells (K) and from a whole liver biopsy (L) were used to prepare and analyze Northern blots as described in the legend to Fig. 3. The riboprobes used were as described in the legend to Fig. 4 for ACAT1 (top panel), ACAT2 (middle panel), and cyclophilin (bottom panel). Sizes of marker RNAs appear at the right.

Fig. 5. Autoradiograph of Northern blots of mRNAs from liver cell types and whole liver. One-µg samples of poly(A)+ RNA that was purified from isolated cell populations of hepatocytes (H) and Kupffer cells (K) and from a whole liver biopsy (L) were used to prepare and analyze Northern blots as described in the legend to Fig. 3. The riboprobes used were as described in the legend to Fig. 4 for ACAT1 (top panel), ACAT2 (middle panel), and cyclophilin (bottom panel). Sizes of marker RNAs appear at the right.

sure that the outcome was not peculiar to AC29 cells.

The predicted primary structure of the monkey ACAT2 clone is highly analogous to that described for ACAT1. The fact that the first approximately 100 amino acids of each enzyme are of a completely different sequence suggests that this portion of the enzyme may confer separate functions for the two enzymes. However, over the remaining 425 amino acids, a 57% sequence identity occurs for human ACAT1 and monkey ACAT2 suggesting similar functions for this portion of the protein. Easily recognizable aspects of the ACAT2 sequence that may contribute to its function as an ACAT enzyme are: 1) the analog to serine residue 269, which when converted to a leucine residue in SRD-4 cells resulted in an inactive ACAT (4), 2) leucine heptad motifs that may facilitate protein-protein interactions important in the presumed oligomerization that may occur for active ACAT (34), and 3) multiple transmembrane helical domains that may serve to anchor the protein in the ER membrane.

Other evidence that the primary structure of the protein coded for by the monkey ACAT2 clone is as indicated in Fig. 1 was obtained during in vitro translation studies. The primary translation product for the African green monkey ACAT2 cDNA ran at about 47,000 Mr, compared with the primary product for monkey ACAT1, which ran at about 50,000 Mr, during SDS-polyacrylamide gel electrophoresis (Fig. 3). These apparent differences in size are consistent with the presumed differences in the size of the two proteins based on the deduced amino acid sequences. The fact that both enzymes appear to run at molecular weights that are smaller than the predicted sizes based on primary structure has been observed by others (18, 20).

The distribution of ACAT mRNA among 18 different primate tissues is the most detailed comparison yet made available (Fig. 4) and was facilitated by the collection of multiple tissues from monkeys being necropsied for atherosclerosis evaluations in other experiments. The fact that ACAT1 mRNA is present in most tissues, with the possible exception of skeletal muscle, is compatible with the prevalent notion that most cells can esterify excess cholesterol when the need arises. The remarkably high level of ACAT1 mRNA in adrenal, compared with that in other tissues, is likely responsible for the significant amount of cholesteryl ester accumulation that normally occurs in this organ. High adrenal cholesteryl ester levels were dramatically reduced by the disruption of the ACAT1 gene in mice (14).

The presence of multiple bands for ACAT1 mRNA in various tissues has been noted before but the reason is unexplained (2, 5). These data show that the banding pattern was quite similar for most tissues with the 5′-coding region probe used in these studies. We have also probed the same Northern blots with a probe specific to the 3′-end of the ACAT1 coding sequence (data not shown). This probe appeared to show some suggestive differences in band intensity among tissues that were not seen with the 5′-end riboprobe, but the same size range of bands was seen with both probes. Both the 5′- and 3′-end untranslated nucleotide sequences for ACAT1 mRNA are relatively long, especially compared with ACAT2, but any metabolic role for alternate splicing remains uncertain.

With both the 3′- and 5′-end riboprobes to ACAT2 mRNA that were made to sequences dissimilar to any found in ACAT1, the lack of reactivity in tissues other than liver and jejunum was striking, especially considering the near ubiquitous expression of ACAT1. The presence of a larger band in the kidney is believed to represent cross-hybridization with the message for an unidentified protein since the use of a riboprobe specific to the sequences in the coding region near the 5′-end did not identify the larger band in the kidney. Clearly these data suggest that ACAT2 has a specific function related to this specific tissue distribution. While we cannot be sure what this function is, the secretion of apoB-containing lipoproteins is carried out almost exclusively by liver and intestine, and it is possible that ACAT2 is responsible for formation of the cholesteryl esters of newly secreted lipoprotein particles. We have provided suggestive evidence that some ACAT activity is required for apoB particle secretion (11).

In the context of tissue distribution and function, the modeling of the secondary structure of the monkey ACAT1 and ACAT2 enzymes that was done with the Predict Protein technology (30–32) is of interest. The prediction for ACAT2, as shown in Fig. 2, is that there are seven transmembrane domains with the longer peptide loops between these helical transmembrane regions being situated mostly on the lumenal side of the ER membrane. In contrast, for the topology prediction for monkey ACAT1, the model shows eight transmembrane domains. Basically, the same seven domains as found in ACAT2 are also predicted for ACAT1, but one additional transmembrane helix is found at amino acid residues 247–264. This has the effect of moving the putative active site serine at amino acid 269 as well as the remainder of the peptide loops that likely contain other active site residues to the cytoplasmic side of the membrane. The NH2-terminal 100+ amino acids for both enzymes are predicted to be on the cytoplasmic side of the ER membrane, but since there is almost no sequence similarity in this region between ACAT1 and ACAT2, this portion of the protein probably does not contain active site residues.

Thus, the model predictions would be consistent with the active site of the ACAT1 enzyme being on the cytoplasmic side of the membrane where it could possibly facilitate cholesteryl ester incorporation into cytoplasmic lipid droplets, as occurs extensively in the adrenal. In contrast, ACAT2 is predicted to have its active site on the luminal side of the ER membrane (Fig. 2). This orientation may facilitate cholesteryl ester incorporation into apoB-containing lipoproteins, as lipoprotein particle assembly and secretion is known to occur in the ER lumen.
Perhaps it was the inhibition of ACAT2 by ACAT inhibitors that resulted in the decreased rate of appearance in perfusate of apoB and cholesteryl esters in the monkey liver perfusion studies (11). The data in Table I show that ACAT2 is sensitive to two of these ACAT inhibitors. Further work is needed to test these hypotheses.

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