The synthesis of cholesterol esters by acyl-CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) is an important component of cellular cholesterol homeostasis. Cholesterol ester formation also is hypothesized to be important in several physiologic processes, including intestinal cholesterol absorption, hepatic lipoprotein production, and macrophage foam cell formation in atherosclerotic lesions. Mouse tissue expression studies and the disruption of the mouse ACAT gene (Acact) have indicated that more than one ACAT exists in mammals and specifically that another enzyme is important in mouse liver and intestine. We now describe a second mammalian ACAT enzyme, designated ACAT-2, that is 44% identical to the first cloned mouse ACAT (henceforth designated ACAT-1). Infection of H5 insect cells with an ACAT-2 recombinant baculovirus resulted in expression of a ~46-kDa protein in cell membranes that was associated with high levels of cholesterol esterification activity. Both ACAT-1 and ACAT-2 also catalyzed the esterification of the 3β-hydroxy group of a variety of oxysterols. Cholesterol esterification activities for ACAT-1 and ACAT-2 exhibited different IC₅₀ values when assayed in the presence of several ACAT-specific inhibitors, demonstrating that ACAT inhibitors can selectively target specific forms of ACAT. ACAT-2 was expressed primarily in mouse liver and small intestine, supporting the hypothesis that ACAT-2 contributes to cholesterol esterification in these tissues. The mouse ACAT-2 gene (Acact2) maps to chromosome 15 in a region containing a quantitative trait locus influencing plasma cholesterol levels. The identification and cloning of ACAT-2 will facilitate molecular approaches to understanding the role of ACAT enzymes in mammalian biology.

The ability to synthesize sterol esters is fundamental to most eukaryotic cells. Sterol esterification is thought to participate in the maintenance of cell membrane sterols at levels optimal for normal cell function. In mammalian cells, cholesterol is the predominant cellular sterol, and cholesterol esterification is catalyzed by the enzyme acyl-CoA:cholesterol acyltransferase (E.C. 2.3.1.26, ACAT)¹ (reviewed in Refs. 1–3). Oxygenated sterols (oxysterols) and their esters also are found in mammalian tissues (4). Although oxysterol esterification likely is catalyzed by ACAT (5), a systematic analysis of the enzyme (5) and substrates involved has not been done.

ACAT activity has been implicated in a number of physiologic processes (1). In the small intestine, ACAT has been proposed to play a role in cholesterol absorption by maintaining a free cholesterol diffusion gradient across the enterocyte surface through the formation of cholesterol esters intracellularly (6, 7). Cholesterol ester formation by ACAT also has been hypothesized to be important for the assembly and secretion of apolipoprotein B–containing lipoproteins in the intestine and the liver (reviewed in Ref. 8). In the adrenal glands and other steroidogenic tissues, ACAT synthesizes cholesterol esters that accumulate in cytosolic droplets where they can serve as cholesterol substrate stores for steroidogenesis. In macrophages, ACAT generates intracellular cholesterol esters that are stored as cytosolic lipid droplets, a characteristic feature of macrophage foam cells in atherosclerotic lesions (9). Because of ACAT’s apparently prominent role in cholesterol metabolism, a number of ACAT inhibitors have been developed for use as anti-atherosclerosis agents (reviewed in Ref. 10). Some of these act by inhibition of intestinal cholesterol absorption (10) or hepatic lipoprotein synthesis and secretion (11, 12), and others, as specific inhibitors of macrophage foam cell formation (13).

Recent evidence has suggested that more than one ACAT exists in mammals. A human ACAT cDNA was first identified from a macrophage cDNA library by Chang and co-workers (14). The disruption of the mouse homolog of this ACAT gene (Acact) yielded viable, ACAT-deficient (Acact−/−) mice that were characterized by tissue-specific reductions in cholesterol esters (15). Cholesterol ester stores were markedly reduced in adrenal cortices and cultured peritoneal macrophages; however, substantial levels of ACAT activity were present in...
Acat-/- livers, and intestinal cholesterol absorption was normal, indicating that another ACAT was active in these tissues (15). Studies examining the tissue distribution of Acat mRNA expression also supported the hypothesis that more than one ACAT exists (16), as did previous biochemical (17) and ACAT expression studies showing differences between liver and aorta/macrophage ACAT activities. Taken together, these data led us to hypothesize that a second ACAT contributes to cholesterol esterification activity in the liver and small intestine. A precedent for eukaryotic organisms having more than one sterol esterification enzyme was established for precedent for eukaryotic organisms having more than one sterol esterification activity in the liver and small intestine. A precedent for eukaryotic organisms having more than one sterol esterification enzyme was established for Saccharomyces cerevisiae, which have two genes encoding sterol esterification enzymes, ARE1 and ARE2 (19, 20). Although the reason for yeast having two genes is unclear, the disruption of both was necessary to render this organism deficient in sterol esterification (19, 20).

In this study, we report the identification and cloning of a second mammalian ACAT, designated ACAT-2. We have expressed the ACAT-2 cDNA in insect cells and characterized its sterol esterification activity in detail. In addition, the ability to express ACAT-1 and ACAT-2 in the low-background insect cell system enabled us to compare the two enzymes with respect to substrate preferences and response to inhibitors. The characteristics of the mouse ACAT-2 gene product and its tissue distribution support the hypothesis that ACAT-2 may contribute to cholesterol esterification activity in the liver and intestine.

MATERIALS AND METHODS

Cloning of ACAT-2 cDNA—An EST (accession number R10292) was identified by BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/blast-help.html) of the human EST data base with sequences from ACAT-1. Degenerate primers derived from the EST sequences were subsequently used to amplify a partial ACAT-2 cDNA from mouse liver cDNA. Sequences for the 5' and 3' ends of the ACAT-2 cDNA were determined by 5'- and 3'-rapid amplification of cDNA ends methodology using a mouse spleen Marathon ReadyTM cDNA library (CLONTECH, Palo Alto, CA). ACAT-2 sequences have been deposited in GenBank (accession number AF078751).

Generation of Recombinant Baculoviruses—Mouse ACAT-2 and human ACAT-1 baculovirus transfer vectors were constructed by inserting ACAT-2 coding sequences (amplified from mouse liver cDNA) or ACAT-1 coding sequences (amplified from HepG2 cell cDNA) downstream of the polyhedrin promoter of the pVL1392 vector (PharMingen, San Diego, CA). Vectors were co-transfected with viral baculogold DNA into Sf9 insect cells, according to the manufacturer's protocol (PharMingen Kit), to generate recombinant baculoviruses. Viruses were plaque-purified and high titers of recombinant baculoviruses were generated by two rounds of amplification in Sf9 cells. Cells were cultured at 27 °C in Grace's medium (Life Technologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum.

Expression Studies—For protein expression studies, H5 insect cells were cultured in optimized serum-free Express-Five medium (Life Technologies), supplemented with 20 mM l-glutamine. Cells were plated on day 0 at 8.5 x 10⁶ cells per 100-mm dish and infected on day 1 with high-titer baculovirus stocks at an optimal multiplicity of infection that was determined empirically. On day 3 (48 h after infection), cells were collected by low-speed centrifugation and washed twice with phosphate-buffered saline. Cell pellets were resuspended in 0.1 M sucrose, 0.05 M KCl, 0.04 M KH₂PO₄, 0.03 M EDTA (pH 7.2) and homogenized by 10 passages through a 27-gauge needle. Cell homogenates were fractionated by centrifugation at 100,000 g for 1 h at 4 °C. The cell membrane fraction (pellet) was resuspended in the isolation buffer, and aliquots were frozen (−80 °C) for activity assays. Protein concentrations were determined using a DC Protein Assay kit (Bio-Rad).

Metabolic Labeling—For metabolic labeling, H5 insect cells were plated on day 0 at 2.9 x 10⁶ cells per 60-mm dish and infected at day 1 with high-titer baculoviruses at empirically determined multiplicity of infections. On day 3 (48 h after infection), cells were washed and incubated in methionine-free and cysteine-free medium (SF900 II, Life Technologies) for 1 h. The medium was then replaced by 3 ml of methionine-free and cysteine-free medium containing 715 µCi of [35S]methionine and [35S]cysteine (Amersham Pro-Mix, Amersham) and aliquots were frozen (−80 °C) for activity assays. Protein concentrations were determined using a DC Protein Assay kit (Bio-Rad).

ACAT Activity Assays—The rate of incorporation of [14C]oleoyl-CoA into cholesterol esters was determined using a [14C]oleoyl-CoA incorporation assay (18, 26) in which the cell membranes were labeled with [14C]oleoyl-CoA and the rate of incorporation of [14C]oleoyl-CoA into cholesterol esters.
(Amersham) into cholesterol esters was assayed essentially as described by Erickson et al. (21). Reactions were performed at 37 °C for 5 min with 100 μg of cell membrane protein and 25 μM oleoyl-CoA (specific activity = 18 μCi/μmol). Exogenous cholesterol (20 nmol) was added as phosphatidylcholine (PC):cholesterol (4:1 molar ratio) liposomes to measure apparent V_{max} activities (22).

**Determination of Fatty Acyl-CoA Specificity—** ACAT activity was assayed essentially as described (22), except that unlabeled fatty acyl-CoAs and [14C]cholesterol (51.3 mCi/mmol, NEN Life Science Products, Boston, MA) were used. Parallel assays were performed with [14C]oleoyl-CoA (57 mCi/mmol, Amersham) and unlabeled cholesterol provided as PC liposomes (molar ratio = 0.2). The relative competition of fatty acyl-CoAs for cholesterol esterification was assessed using [14C]oleoyl-CoA diluted to ~20,000 dpm/nmol with unlabeled fatty acyl-CoAs (~1.6, mol:mol dilution) and unlabeled cholesterol:PC liposomes (molar ratio = 0.7). The abilities of [14C]oleoyl-CoA and [14C]palmitoyl-CoA (55.0 mCi/mmol, Amersham) to catalyze cholesterol esterification were assessed directly. Fatty acyl-CoAs, egg PC, and cholesterol were from Sigma. Briefly, membrane proteins (25 μg) were incubated in 0.25 M sucrose, 1 mM EDTA, 100 mM Tris-HCl (pH 7.5) containing 400 μg of bovine serum albumin and cholesterol:PC liposomes (final volume 500 μl). The reaction was started by the addition of 5 nmol of fatty acyl-CoA and carried out for 2 min at 37 °C. The reaction was terminated by the addition of CHCl₃:methanol (2:1, v:v), and the products analyzed as described (21). All assays were in the linear range with respect to assay time and protein concentration.

**Determination of Acyl Acceptor Substrate Specificity—** The ACAT as-
say described above was used, except that acceptors other than cholesterol were added to the incubations as acceptor:PC liposomes (molar ratio = ~0.2) or, in the case of ethanol, added directly. The acceptors included ethanol, retinol, tocopherol, β-sitosterol, lanosterol, vitamins D₃ and D₄, diacylglycerol, and a number of oxysterols (25-hydroxy-, 27-hydroxy-, 7α-hydroxy-, 7β-hydroxy, 7-keto-, 24(S)-hydroxy-, 24(R)-hydroxy-, 24(S),25-epoxy-, and 24(R),25-epoxycholesterol). The sterols were assessed at three different concentrations (4, 8, and 16 μg) at a constant sterol:PC molar ratio (~0.2). Acceptors were from Sigma or Steraloids (Wilton, NH). 24(S)-Hydroxy- and 24(R)-hydroxycholesterol (23, 24), 24(S),25-epoxy-, and 24(R),25-epoxycholesterol were prepared as described (24–26). The reaction products were extracted and separated by thin-layer chromatography (21), and the plates exposed to x-ray film for 4 days to visualize the radioactive spots. Fatty acyl ester standards were used to identify the migration positions of the products. The spots were cut out and counted in a liquid scintillation counter (21).

Inhibitor Studies—Cholesterol esterification activity was assayed as described above (using [14C]oleoyl-CoA and cholesterol:PC liposomes [molar ratio = 0.2]) after a 5-min preincubation of the membranes at 37 °C with 1 mM PMSF, 0.1 mM DTT, and 0.1 mM PHMB. Inhibitors were purchased from Sigma.

The specific ACAT inhibitors PD 132301–2 (5′-[2,6-bis(1-methyl-ethyl)-phenyl]-N′-[1-[4-(dimethyl-amino)phenyl]cyclopentyl]methylurea, hydrochloride), CI-976 (2,2-dimethyl-N-(2,4,6-trimethoxyphenyl)dodecanamide), and CI-1011 ((2,4,6-trisopropylphenyl)acetyl)sulfamic acid, 2,6-disisopropyl-phenyl ester) were provided by Parke-Davis Pharmaceuticals (Ann Arbor, MI). Stock solutions (20 mM) of the inhibitors were prepared in dimethyl sulfoxide and stored at –20 °C. Membrane proteins (100 μg for ACAT-1 and 50 μg for ACAT-2) were preincubated with cholesterol:PC liposomes and different inhibitors (final Me2SO concentration = 2.5%) for 30 min on ice. After equilibrating for 5 min at 37 °C, the reaction was started by adding [14C]oleoyl-CoA. Assays were performed otherwise essentially as described above.

ACAT-2 Expression in Mouse and Human Tissues—For RT-PCR analysis, total RNA was prepared from fresh mouse tissues, human hepatoma cells (HepG2), human small intestine, or human fibroblasts using Trizol (Life Technologies), and cDNA was synthesized using random hexamer primers and Superscript reverse transcriptase (Life Technologies). For mouse ACAT-2, a 531-base pair fragment was amplified using sense (5′-ACTGTGCCTGGGATCTTTTGTGTC-3′) and antisense (5′-TCTCGCCGGGTGCCATCAGCGGAT3′) primers. For human ACAT-2, a 742-base pair fragment was amplified using sense (5′-TCTATCCCGTCATGCTG-3′) and antisense (5′-GGTCCACATCAAGCA-3′) primers.
RESULTS

A human EST cDNA (accession R10292) that was highly similar to the C-terminal region of ACAT-1 was identified from BLAST data base searches. By using the EST sequence, a full-length cDNA containing the entire coding sequence was subsequently isolated from mouse liver and designated ACAT-2. The mouse ACAT-2 cDNA is predicted to encode a 525–amino acid protein that is 44% identical to mouse ACAT-1 (31) (Fig. 1A). The ACAT-2 protein has multiple hydrophobic domains, and its hydrophobicity plot is strikingly similar to that for ACAT-1 (Fig. 1B). The analysis of mouse ACAT-2 sequences with a transmembrane prediction program (http://ulrc3.unil.ch/software/TMPRED_form.html) indicated that ACAT-2 may have eight transmembrane-spanning regions (amino acid residues 125–143, 154–181, 192–220, 265–285, 304–326, 351–389, 439–459, and 475–496). Using the same program, mouse ACAT-1 was predicted to have seven or eight transmembrane regions. The alignment of the mouse ACAT-2 protein sequence with those of other ACAT gene family members (Fig. 2) reveals many conserved residues, especially in the C termini of these proteins. Each family member has a potential tyrosine phosphorylation site and a conserved serine residue (amino acid 248 of ACAT-2) that has been implicated in the catalytic activity of hamster ACAT-1 (32).

To determine if ACAT-2 was capable of catalyzing sterol esterification, we expressed its cDNA in H5 insect cells using a baculovirus expression system. Insect cells were chosen for these expression studies because they have very low levels of cellular cholesterol and lack significant cholesterol esterification activity (33). Metabolic labeling of cellular proteins in insect cells 48 h after infection demonstrated the expression of a ∼46-kDa protein (Fig. 3A). The apparent molecular mass of ACAT-2 on SDS-polyacrylamide gels was smaller than that predicted from the amino acid sequence (60.7 kDa), a finding similar to that reported for ACAT-1 (14). For ACAT-1, it has been suggested that the apparent size of the protein is less than predicted due to high amounts of SDS binding (33). Cell membrane preparations from insect cells infected with the ACAT-2 baculovirus were characterized by high levels of cholesterol esterification activity (Fig. 3B). The amount of ACAT activity present in ACAT-2-infected cells was consistently 10-fold higher than that in cells infected with virus containing ACAT-1. However, metabolic labeling studies (Fig. 3A) indicated that considerably more ACAT-2 protein may have been expressed than ACAT-1. Membrane cholesterol esterification activity was dependent upon the amount of ACAT-2 protein expressed, as demonstrated by a time course of ACAT-2 virus infection (Fig. 4).

The ability to express ACAT-1 or ACAT-2 independently in insect cells enabled us to examine several biochemical properties of the enzymes. The esterification of cholesterol by both ACAT-1 and ACAT-2 was dependent on the presence of a fatty acyl-CoA substrate; in its absence, cholesterol esterification activity greater than background was not observed (data not shown). Of the fatty acyl-CoAs tested, ACAT-1 showed a slight preference for oleoyl, while that for ACAT-2 was palmitoyl > linoleoyl > oleyl > arachidonyl (Table I). The ability of unlabelled fatty acyl-CoAs to compete with [14C]oleoyl-CoA for incorporation into cholesterol esters by ACAT-1 or ACAT-2 was also examined. For ACAT-1, palmitoyl, linoleoyl, and to a lesser extent, arachidonyl, competed with oleyl for incorporation into cholesterol esters (Table II). For ACAT-2, both linoleoyl and

### Table I

| Experiment | Relative activities (units) |
|------------|-----------------------------|
|            | Oleyl-CoA | Arachidonyl-CoA | Linoleoyl-CoA | Palmitoyl-CoA |
| ACAT-1     |           |               |               |               |
| 1          | 1.0       | 0.4           | 0.7           | 0.9           |
| 2          | 1.0       | 0.8           | 0.7           | 0.6           |
| ACAT-2     |           |               |               |               |
| 1          | 1.0       | 0.6           | 1.4           | 1.4           |
| 2          | 1.0       | 0.7           | 1.4           | 1.7           |

### Table II

| Experiment | Relative activities (units) |
|------------|-----------------------------|
|            | Oleyl-CoA | Arachidonyl-CoA | Linoleoyl-CoA | Palmitoyl-CoA |
| ACAT-1     |           |               |               |               |
| 1          | 1.0       | 0.9           | 0.8           | 0.7           |
| 2          | 1.0       | 0.8           | 0.8           | 0.8           |
| ACAT-2     |           |               |               |               |
| 1          | 1.0       | 1.3           | 0.7           | 0.8           |
| 2          | 1.0       | 1.3           | 0.8           | 0.8           |

### Table III

| Experiment | [14C]Oleyl-CoA | [14C]Palmitoyl-CoA |
|------------|---------------|-------------------|
| ACAT-1     | 529           | 334               |
| ACAT-2     | 1823          | 1573              |
palmitoyl competed with oleoyl for incorporation into choles-
terol esters, but arachidonyl did not. When cholesterol esteri-
fication was measured using [14C]oleoyl-CoA or [14C]palmitoyl-
CoA directly, ACAT-1 had 58% higher activity with oleoyl-CoA
compared with palmitoyl-CoA, while ACAT-2 showed a negli-
gible difference (16%) (Table III).

Fatty acyl acceptor specificities also were examined for the
expressed proteins. Esterification activity was not detected for
either ACAT-1 or ACAT-2 with ethanol, retinol, tocopherol,
β-sitosterol, lanosterol, vitamins D₃ and D₄, or diacylglycerol as
acceptors (not shown). However, in addition to esterifying cho-
terol, both ACAT-1 and ACAT-2 esterified the 3β-hydroxyl
group of a variety of oxysterols. All oxysterols analyzed exhib-
ted greater esterification capability than cholesterol itself,
with the exception of 7β-hydroxycholesterol for ACAT-1 (Fig.
5A) and 25-hydroxycholesterol for ACAT-2 (Fig. 5B). For
ACAT-1, the highest levels of incorporation into sterol esters
(more than 5-fold greater than for cholesterol) were for 24(R)-
hydroxycholesterol, 7α-hydroxycholesterol, and 27-hydroxy-
cholesterol. For ACAT-2, 24(R)-hydroxycholesterol, 27-hy-
droxycholesterol, and 7β-hydroxycholesterol were esterified at
rates 2–4-fold higher than for cholesterol.

We also examined the effects of added oxysterols on mem-
brane cholesterol esterification, using the small but significant
mass of endogenous membrane cholesterol as a substrate. For
ACAT-1, cholesterol esterification was increased approxi-
mately 2.5-fold by 27-hydroxycholesterol and 24(S),25-epoxy-
cholesterol, but it was decreased substantially by 7α-hydroxy-
cholesterol, 25-hydroxycholesterol, and 24(S)-hydroxy-
cholesterol (Fig. 6A). For ACAT-2, none of the oxysterols
increased cholesterol esterification; instead, they either had
little effect or decreased it by 40–60% (Fig. 6B).

The effects of nonspecific (PMSF, PHMB, and progesterone)
and specific inhibitors of ACAT were examined next. PMSF,
which inhibits enzymes utilizing serine in their active site,
inhibited both ACAT-1 and ACAT-2 by 20–40% (Fig. 7).
PHMB, which acts on enzymes that require sulfhydryl groups
for activity, inhibited ACAT-1 to a greater extent than ACAT-2
(90% and 55%, respectively), and progesterone, a compound
known to inhibit ACAT (34), inhibited both enzymes by
approximately 70% (Fig. 7). Membranes from ACAT-1 and
ACAT-2 virus–infected cells were also assayed in the presence of ACAT-specific inhib-
itors PD 132301–2, CI-976, and CI-1011. PD 132301–2 inhibited both ACAT-1 and ACAT-2 to similar degrees (IC₅₀ < 1 μM) (Fig. 8A). In contrast, CI-976 inhibited some selectivity for the expressed enzymes. ACAT-1 activity was suppressed by CI-976 with an apparent IC₅₀ of ~5 μM, whereas ACAT-2 activity was more sensitive with an apparent IC₅₀ of ~1 μM (Fig. 8B). A significant difference in selectivity was also observed for CI-
1011, which also inhibited ACAT-2 (IC₅₀ ~ 2.5 μM) more than
ACAT-1 (IC$_{50}$ > 10 μM) (Fig. 8C).

ACAT-2 mRNA was detected in a variety of mouse tissues by RT-PCR with the highest levels of expression in the liver, small intestine, and embryo liver (Fig. 9A). Northern analysis of mouse tissues demonstrated a ~2-kb ACAT-2 mRNA in the liver and small intestine (Fig. 9B). To determine if ACAT-2 was also expressed in the liver and intestine in humans, we performed RT-PCR experiments on cDNA from HepG2 cells (a human hepatoma cell line), human intestine, and human fibroblasts. ACAT-2 mRNA was detected in HepG2 cells and small intestine, but not in fibroblasts (Fig. 9C). In a separate experiment, we also detected ACAT-2 mRNA in cDNA from human liver (not shown).

The mouse ACAT-2 gene (Acact2) was localized to mouse chromosome 15 by linkage analysis from an interspecific backcross (Fig. 10). The hybridization of BamHI-digested DNA of the parental strains with the Acact2 cDNA probe resulted in the detection of single fragments in DNA from each (C57BL/6J = 10 kb and Mus spretus = 8.8 kb) and both fragments in DNA from the F1 progeny. The segregation pattern of the M. spretus allele among the backcrossed mice revealed linkage to markers on chromosome 15; linkage was not detected with markers on any other chromosome. No recombination was observed between Acact2 and the microsatellite marker D15Mit16 (0/65 mice). The following gene order was obtained (distance ± S.E., in centiMorgans): centromere-D15Mit31- (3.6 ± 2.5 centiMorgans)-Ppara-(9.2 ± 3.6)-D15Ucla3, Pou6f1- (1.5 ± 1.5)-Acact2, D15Mit16. A human EST for Acact2 (accession R10292) has been mapped to a homologous region on human chromosome 12q13.3-q15 (http://www.ncbi.nlm.nih.gov/XREFdb).

**DISCUSSION**

Prior studies, including the disruption of the mouse ACAT-1 gene (Acact) (15) and tissue expression studies of Acact (16), indicated that more than one ACAT exists in mammals. In the current study, we report the cloning and expression of a second mammalian ACAT, ACAT-2. The expression of the ACAT-2 cDNA in insect cells, which normally lack ACAT activity, resulted in high levels of fatty acyl CoA–dependent cholesterol esterification in isolated cell membranes, thereby establishing that this cDNA encodes a sterol esterification enzyme.

The predicted protein sequence for mouse ACAT-2 shows similarities to that for mouse ACAT-1 and other members of the sterol acyltransferase gene family. This family of proteins are most similar in their C termini, suggesting that this region contains domains important for enzyme catalysis. The motif MKX(H/Y)SF in the mid-region of the proteins is conserved in all family members; the serine residue in this motif is necessary for catalytic activity in the hamster ACAT-1 ortholog (32). A tyrosine residue located within a tyrosine phosphorylation consensus motif also is found in most family members. The hydrophobicity analyses for mouse ACAT-1 and ACAT-2 suggest that the proteins may have seven or eight transmembrane regions, consistent with their being intrinsic membrane proteins.

The expression of ACAT-1 or ACAT-2 independently in insect cell membranes made it possible to characterize the two activities separately with respect to substrate specificities and response to inhibitors. The level of cholesterol esterification specific activity for ACAT-1 was similar to that reported previously in insect cell expression studies (33). ACAT-2-containing membranes catalyzed cholesterol esterification at a consis-
ACAT-2, a Second Acyl-CoA:Cholesterol Acyltransferase

Fig. 9. Tissue expression pattern of ACAT-2 mRNA. A, RT-PCR analysis of mouse tissues. cDNA was prepared from mouse tissues, and a 531-base pair mouse ACAT-2 fragment was amplified as described under "Materials and Methods." The amplified band was confirmed to be specific for ACAT-2 by hybridization with an internal primer. Amplification of glyceraldehyde-3-phosphate dehydrogenase was used as a control for cDNA integrity. B, Northern analysis of mouse tissues. Total RNA was prepared from mouse tissues and samples (10 μg) were analyzed by Northern blotting as described under "Materials and Methods." Blots were probed with a 32P-labeled 1.5-kb cDNA fragment that hybridizes to mouse ACAT-2 sequences. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) served as the internal control. The autoradiogram for mouse ACAT-2 was exposed for 18 h. C, RT-PCR analysis of human tissues. cDNA was prepared from human hepatoma cells (HepG2), and human small intestine and fibroblasts. A 742-base pair mouse ACAT-2 fragment was amplified as described under "Materials and Methods." The amplified band was confirmed to be specific for ACAT-2 by hybridization with an internal primer. Amplification of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) served as the internal control. The amplified bands (including a smaller amplification product for HepG2 cDNA) were confirmed to be specific for ACAT-2 by hybridization with an internal primer (not shown). The significance of the smaller amplification product in the HepG2 cDNA is currently unknown.

Fig. 10. Genetic mapping of Acact2 to mouse chromosome 15 by linkage analysis using an interspecific backcross [(C57BL/6J X Mus spretus)F1 X C57BL/6J]. The chromosome is drawn to scale with the centromere at the top and the distance of the most distal marker from the centromere indicated at the bottom (cum, in centiMorgans). The ratios of the number of recombinants to the total number of informative mice and the recombination frequencies ± standard errors (in centiMorgans), for each pair of loci, are indicated to the left. For pairs of loci that cosegregate, the upper 95% confidence interval is shown in parentheses. The localization of Acact2 is supported by a LOD score of 17. Ucla markers were reported (28) or are unpublished data. References for other linked loci can be obtained from the Mouse Genome Database (http://www.informatics.jax.org). The filled box represents a previously mapped quantitative trait locus for plasma lipoprotein levels; the length of the box indicates an estimated 95% confidence interval for the most likely location of the underlying gene (57).

oxysterols can be toxic to cells (38–40), a potential role for ACAT-1 in macrophages may be to convert free oxysterols to oxysterol esters as part of a detoxification mechanism. The ability of ACAT-2 to esterify both cholesterol and oxysterols would be expected for an intestinal ACAT activity because dietary oxysterols derived from oxidized cholesterol are absorbed in both animals (41–43) and humans (44). Oxysterols also are esterified by liver microsomes (5). Of note, two oxysterols involved in bile acid metabolism, 7α-hydroxycholesterol and 27-hydroxycholesterol, were esterified by both ACAT enzymes, suggesting a potential role for ACAT in regulating bile acid metabolism. Recently, specific oxysterols, in particular 24(S),25-epoxycholesterol and 24(S)-hydroxycholesterol, were shown to be ligands for nuclear transcription factors LXRα and LXRβ (45, 46). LXRα has been implicated in the sterol-mediated regulation of cholesterol-7α-hydroxylase expression (45). The finding that these oxysterols are esterified by ACAT-1 and ACAT-2 raises the possibility that ACAT activity may modulate the effects of oxysterols on LXR-responsive genes.

The effects of oxysterols on cholesterol esterification activity were mixed for ACAT-1 in this expression system. Both 27-hydroxycholesterol and 24(S),25-epoxycholesterol stimulated cholesterol esterification, consistent with the possibility that ACAT-1 may be allosterically regulated by oxysterols (33, 47). In contrast, a number of oxysterols were inhibitory, in partic-
ular 25-hydroxycholesterol. The reason for this result, which differs from findings reported previously (33), is unclear, but could relate to differences in ACAT assay methods. In contrast, none of the added oxysterols stimulated ACAT-2 activity, suggesting that ACAT-2 may not have an allosteric binding site for the up-regulation of cholesterol esterification by specific oxysterols. For both ACAT-1 and ACAT-2, the inhibition of cholesterol esterification by oxysterols suggests that, in some instances, oxysterols may compete with cholesterol for the active site. Of interest in this regard is that oxysterols are reported to inhibit cholesterol absorption (43).

The fatty acyl-CoA specificity for ACAT-1 was broad with some preference for oleoyl-CoA, similar to results reported previously for ACAT-1 expressed in yeast cells deficient insterol esterification (48). ACAT-2 exhibited a preference for linoleoyl-CoA or palmitoyl-CoA over oleoyl-CoA and had the least activity with arachidonoyl-CoA. The lower activity with oleoyl-CoA was unexpected because ACAT-2 is expressed predominantly in the liver and small intestine and oleoyl-CoA was reported to be the preferred substrate for hepatic and intestinal ACAT activities (49, 50).

The finding that ACAT-1 and ACAT-2 activities responded differently to ACAT inhibitors may be of interest with regard to development of drugs designed specifically to target ACAT in selected tissues. Responses of ACAT-1 and ACAT-2 to ACAT inhibitors differed with the biochemical class. PD 132301-2, a urea-based compound whose use is associated with adrenal gland toxicity in animals (51, 52), markedly inhibited both enzymes. The IC50 data for this compound are similar to those reported previously (53). CI-976, a fatty acid amide (13), and CI-1011, an acyl sulfamate (54), have been used successfully without adrenal gland toxicity in animals. Both compounds demonstrated selective inhibition of ACAT-2 compared with ACAT-1. For CI-976, this result was surprising inasmuch as this drug has been used in animal studies to inhibit macrophage ACAT selectively without affecting plasma lipid levels (13). CI-1011, a potent hypocholesterolemic agent (54) currently under investigation in human trials, also exhibited a high degree of selectivity toward ACAT-2. Data from human subjects administered CI-1011 at 500 mg/day indicate that the maximal drug concentration ranges from ~7 to 13 μM, depending on the fat content of the diet (55), suggesting that at these concentrations, both ACAT-1 and ACAT-2 would be inhibited significantly. These studies demonstrate that the ability to express the two enzymes independently may provide a useful screen for designing inhibitors with narrow specificity profiles and low toxicity. It is important to realize, however, that the results in both the inhibitor and substrate preference studies reflect data obtained from enzymes expressed in insect cells. The findings therefore may not necessarily reflect what occurs in mammalian cells, where potential differences in post-translational modifications of the enzymes or differences in the membrane lipid environment may occur.

Previous studies (15, 16) indicated that ACAT-1 likely is not the major cholesterol esterification enzyme in mouse liver or intestine. Although it remains to be determined whether ACAT-2 is responsible for the bulk of the activity in these tissues, several findings make it an attractive candidate. First, ACAT-2 is expressed predominantly in the liver and the small intestine. Its mRNA was detected specifically in mouse liver and small intestine by Northern analysis and in human intestinal samples by RT-PCR analysis. ACAT-2 mRNA also was detected in HepG2 cells, a human hepatoma cell line that expresses high levels of ACAT activity (56). In agreement with

our findings, Rudel and co-workers (2) have detected ACAT-2 mRNA expression in the liver and intestine of primates by Northern blotting. The mouse ACAT-2 gene mapping data also are consistent with a possible role for ACAT-2 in intestinal cholesterol absorption or lipoprotein production by the liver or intestine. A quantitative trait locus influencing plasma low density lipoprotein and very low density lipoprotein cholesterol levels in response to high-fat feeding has been mapped to a region on mouse chromosome 15 from crosses of NZB/B1NJ and SM/J mice (57). The mapping of Acact2 to this region makes it an attractive candidate gene for this locus.

It remains to be determined why mammals or yeast (19, 20) have a need for more than one sterol esterification enzyme. These studies comparing ACAT-1 and ACAT-2 suggest that evolutionary conservation of the two enzymes may relate either to different substrate preferences or different patterns of tissue expression. Another hypothesis is that the two enzymes have fundamentally different functions within cells. For example, it is possible that cholesterol esters generated by ACAT-1 may be preferentially directed toward intracellular cytosolic droplets for storage, whereas cholesterol esters generated by ACAT-2 may be directed to secretion from the cell by entering the lipoprotein assembly process in tissues like the liver and intestine. This hypothesis is supported by the observation that ACAT-1-deficient mice lacked cholesterol ester droplets in adrenocortical cells and macrophages, but had no apparent defects in lipoprotein production or cholesterol absorption (15). To answer these and other questions related to the biology of ACAT enzymes, the identification and characterization of an ACAT-2 cDNA provides a valuable tool.

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Note Added in Proof—Anderson et al. (58) and Oelkers et al. (59) report the identification of ACAT-2 in primates and humans, respectively, in this issue.

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