Tissue specific changes in acyl-CoA:cholesterol acyltransferase (ACAT) mRNA levels in rabbits

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Abstract A human cDNA clone (K1) was recently isolated that encodes functional acyl-CoA:cholesterol acyltransferase (ACAT) protein (Chang et al. *J. Biol. Chem.* 1993. **268:** 20747-20755). We used the K1 clone to screen a rabbit liver cDNA library and isolated a 919 base pair partial rabbit cDNA ($ACAT_{14b}$) that was greater than 90% homologous with the human nucleotide sequence. Northern blotting using the rabbit ACAT $cDNA_{14b}$ revealed the existence of at least six related mRNA species (ranging from 6.2 to 1.7 kb) in various rabbit tissues. Using an RNAse protection assay, ACAT $mRNA_{14b}$ was detected in twelve separate rabbit organs. Adrenal gland contained the highest concentrations of ACAT $mRNA_{14b}$ (per μg of total RNA) being 20-, 30-, and 50-fold higher than small intestine, aorta, and liver, respectively. Additional studies with isolated liver cell populations revealed that rabbit hepatic nonparenchymal cells contained 30-fold more ACAT $mRNA_{14b}$ (per μg of total RNA) than parenchymal cells. To determine whether ACAT $mRNA_{14b}$ levels are regulated in vivo, rabbits were fed for 4 weeks a high fat/high cholesterol diet (HFHC; 0.5% cholesterol, 3% coconut oil, 3% peanut oil) at which point they were either kept for an additional 4 weeks on the HFHC-diet or switched to the HFHC-diet plus CI-976 (50 mg/kg), a potent and specific ACAT inhibitor; another group of rabbits was fed a chow diet for the entire 8 weeks. The HFHC-diet caused a 2- and 3-fold increase in hepatic and aortic ACAT $mRNA_{14b}$ levels, respectively, in comparison to chow-fed animals; there was no change in adrenal or small intestine levels. CI-976 treatment lowered ACAT $mRNA_{14b}$ levels by 60% and 40% in liver and aorta, respectively, in comparison to the HFHC controls; again there was no change in adrenal or small intestine levels. These data indicate that ACAT $mRNA_{14b}$ levels increase in a tissue specific manner in response to dietary fat and cholesterol.—**Pape, M. E., P. A. Schultz, T. J. Rea, R. B. DeMattos, K. Kieft, C. L. Bisgaier, R. S. Newton, and B. R. Krause.** Tissue specific changes in acyl CoA:cholesterol acyltransferase (ACAT) mRNA levels in rabbits. *J. Lipid Res.* 1995. **36:** 823-838.

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In eukaryotes, acyl-CoA:cholesterol acyltransferase (ACAT) catalyzes cholesteryl ester formation from fatty acyl-CoA and cholesterol. The cholesteryl esters are either packaged and secreted as lipoproteins or stored as intracellular lipid droplets to provide a steroid reserve for membrane, hormone, or bile acid synthesis. In vivo these processes are cell-type dependent. For instance, intestinal mucosal ACAT re-esterifies absorbed cholesterol providing the chylomicron cholesteryl ester component (1, 2). Hepatocytes also secrete cholesteryl esters in the form of apolipoprotein B (apoB)-containing lipoproteins; indeed, high hepatic cholesteryl ester content is associated with increased apoB production (3). Although storage of cholesteryl esters is a normal cellular process such as in steroid hormone secreting organs, excess storage in non-lipoprotein secreting tissues can lead to certain pathologies. A prevalent disorder is the accumulation of cholesteryl esters in arterial macrophages to form foam cells that are a hallmark of atherosclerosis. Overall, ACAT plays a role in metabolism by regulating dietary cholesterol absorption, affecting hepatic secretion of apoB-containing lipoproteins, providing reserves for steroid hormone production, and stimulating formation of macrophage foam cells. Thus, the selective pharmacological inhibition of ACAT activity in intestinal, hepatic, and arterial tissue has been considered a viable approach for limiting progression or promoting regression of atherosclerosis (4).

There are multiple mechanisms regulating ACAT activity. They are generally divided into four categories: substrate (cholesterol) availability, activation/inactivation, viscotropic effects, and altered enzyme mass (5-8). First, regulation of ACAT activity by cholesterol availability has been demonstrated both in vitro and in vivo. Exogenous

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; apoA-I, apolipoprotein A-I; apoB, apolipoprotein B; apoE, apolipoprotein E; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HFHC diet, high fat/high cholesterol diet; PCR, polymerase chain reaction; CETP, cholesteryl ester transfer protein.

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cholesterol addition to microsomal membranes results in a marked elevation in oleate incorporation into cholesteryl esters compared to microsomes without exogenous cholesterol added ((8) and references therein). Thus, ACAT does not appear to be a rate-limiting enzyme in the classic sense, but, rather an enzyme regulated by simple substrate availability. Mechanisms for how the ACAT substrate pool can be regulated have been proposed. For example, recent work by Okwu et al. (9) suggests sphingomyelin may alter intracellular cholesterol trafficking by modifying the ACAT substrate pool. A second control mechanism involves activation/inactivation which may include covalent modifications or allosteric effects. Modulation of ACAT activity by a phosphorylation/dephosphorylation mechanism has been proposed (10); however, recent functional (11) and structural evidence (i.e., the lack of classic phosphorylation sites in the primary ACAT protein K1 sequence (12)) has called the relevance of these data into question. Allosteric modulation of ACAT activity is suggested from studies in macrophages and CHO cells that imply the existence of a cycloheximide-sensitive inhibitor of ACAT, although this factor may function by modulating the ACAT substrate pool (13-15). The importance of the ACAT substrate pool is borne out further in recent work by Cheng and coworkers (16, 17) who demonstrated that ACAT protein K1 expressed in insect Sf9 cells can be activated by cholesterol and oxysterol through allosteric mechanisms. Pharmacological inhibition of ACAT activity may also operate through allosteric mechanisms that are at present poorly defined (18). A third control mechanism for regulating ACAT activity involves viscotropic effects. Physiological or cellular treatments can alter the fatty acid content of the membranous environment where ACAT resides (19, 20). Diets that alter microsomal composition and membrane fluidity affect ACAT activity and are the primary data in support of this control mechanism (21, 22). Finally, indirect evidence exists suggesting changes in arterial ACAT enzyme mass with diet (6, 23, 24), but similar data for liver are lacking. Direct evidence supporting these changes in enzyme mass requires tools such as specific antibodies. Although ACAT-specific antibodies have not yet been generated, the recent cloning of a human cDNA clone ($ACAT_{K1}$) that encodes a polypeptide essential for ACAT catalysis (12, 16, 17) will accelerate synthesis of these important reagents. We have used the ACAT $cDNA_{K1}$ to isolate a portion of the rabbit homolog ($ACAT_{14b}$) to begin investigating molecular mechanisms controlling ACAT activity. In this report we characterize ACAT $mRNA_{14b}$ distribution in rabbit tissues, the sizes of ACAT $mRNA_{14b}$ synthesized, and the regulation of ACAT $mRNA_{14b}$ levels by a high fat/high cholesterol diet in the absence and presence of a potent and specific ACAT inhibitor, CI-976.

MATERIALS AND METHODS

Isolation of rabbit ACAT $cDNA_{14b}$

Nine different polymerase chain reaction (PCR, (25)) primers varying in lengths of 21-27 bases were designed and synthesized based on the coding sequence of the human ACAT $cDNA_{K1}$. Four of the nine primers would presumably prime first strand cDNA synthesis from the rabbit liver RNA template (near the 3'-end of mRNA) while five primers would hybridize to the cDNA strand (near the 5'-end of the cDNA) to allow amplification. First strand synthesis was also primed with oligo dT and random hexamers before using various primer combinations in the PCR. To increase the probability of amplifying rabbit ACAT cDNA we performed the PCR under three different cycling conditions. Two hundred forty PCR conditions were tested. First strand priming and subsequent PCR were performed using the Perkin-Elmer RNA PCR Kit (Perkin-Elmer, Norwalk, CT). Amplification products were analyzed by agarose gel electrophoresis which revealed that 60 reactions produced products of predicted size. Those reactions were further characterized by Southern blot analysis (26) using a random primed HincII-HincII fragment of the human ACAT $cDNA_{K1}$ (12). Ten PCR reactions showed hybridization of the labeled human ACAT $cDNA_{K1}$ to bands of predicted size. Amplification products from the "positive" PCRs were ligated into pCRII using the TA Cloning Kit (Invitrogen, San Diego, CA) as described by the manufacturer. Individual bacterial colonies were screened by colony hybridization (27) with a random primed HincII-HincII fragment of the human ACAT $cDNA_{K1}$ (12). Two positive colonies were identified. The plasmid DNA was subsequently isolated and both strands of each cDNA insert were sequenced using the chain termination method of Sanger, Nicklen, and Coulson (28). Both colonies contained an identical 919 bp cDNA insert that was 90% homologous with the human ACAT $cDNA_{K1}$ (12); the plasmid DNA for these clones was named pRQV-ACAT/Rb_{14b}.

The first strand cDNA synthesis that eventually produced the rabbit ACAT $cDNA_{14b}$ sequences contained 1 μg of rabbit liver total RNA that was primed with random hexamers. The PCR was performed with the following primers: 5'-ATCGAATTCAGCAGAGGCAGAGG AATTGA-3' (corresponding to 1561-1581 of the human sequence (12)) and 5'-ACTGATATCGCACACCTGGCA AGATGGAG-3' (corresponding to 2490-2510 of the human sequence (12)); the cycling conditions in the GeneAmp 9600 System consisted of a 95°C denaturation step for 1 min, followed by 35 cycles of 55°C for 30 sec, 72°C for 60 sec, and 95°C for 30 sec, with a final elongation step of 72°C for 3 min.

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RNA isolation, Northern blots, and internal standard/RNAse protection assay

Total RNA was isolated from tissue and cells using the single-step method of Chomczynski and Sacchi (29); RNAzol reagent (Biotecx Laboratories Inc., Houston, TX) was used for these isolations. Poly A+ RNA was isolated from total RNA using the mRNA Separator Kit (Clonetech Laboratories Inc., Palo Alto, CA). RNA concentrations were determined by absorbance at 260 nm; the 260/280 nm ratio of all samples was 1.9–2.0. Human poly A+ RNA was purchased from Clonetech.

Poly A+ RNA was separated by electrophoresis in a formaldehyde-containing gel essentially as described (27). Without prior treatment of the gel, RNAs were transferred to supported nitrocellulose (Schleicher and Scheull, Keene, NH) by capillary transfer with 20 × SSC (20 × is 3 M NaCl, 0.3 M sodium acetate, pH 7.0 (27)). After transfer, RNA molecular size standards on the nitrocellulose were marked and the filter was subsequently heated under vacuum at 80°C for 1 h. The membrane was prehybridized in 50% formamide, 5 × SSPE (20 × is 3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA, pH 7.4), 5 × Denhardt's solution (50 × is 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 0.2% SDS, 200 μg/ml salmon testes DNA, and 200 μg/ml Torula RNA for 1 h at 60°C. After prehybridization, 0.2 volumes of 50% dextran sulfate and 5 × 10^6 cpm/ml of radiolabeled riboprobe were added to the prehybridization bag. Hybridization was performed overnight at 60°C after which the membrane was washed at 70°C in 0.1% SSC, 0.1% SDS (3 × 30 min per wash). The membranes were analyzed using a Molecular Dynamics 400E PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RNA probes for Northern analysis were derived from human or rabbit ACAT cDNA depending on the experiment. For the human probe, the human ACAT $cDNA_{K1}$ in pBluescript II SK(+) was digested with HincII to yield a 721bp DNA fragment (1557–2278 in the human sequence (12)) that was subsequently isolated and subcloned into the SmaI site of pGEM-4Z (termed pRQV-ACAT/Hu). pRQV-ACAT/Hu was linearized with EcoRI (located in the multiple cloning site of the vector) to synthesize radiolabeled antisense probe with T7 RNA polymerase to a specific activity of 3 × 10^8 cpm/μg using the Riboprobe Gemini II system (Promega Corp., Madison, WI). For rabbit, radiolabeled antisense probe for Northern blots was synthesized with SP6 RNA polymerase and pRQV-ACAT/Rb_{14b} that had been linearized with Not I (located in the multiple cloning site of the vector) to a specific activity of 3 × 10^8 cpm/μg.

A rabbit internal standard ACAT RNA was synthesized using T7 RNA polymerase and pRQV-ACAT/Rb_{14b} that had been linearized with HindII (a site internal to the ACAT $cDNA_{14b}$), and a radiolabeled antisense probe was synthesized with SP6 RNA polymerase and pRQV-ACAT/Rb_{14b} that had been linearized with Not I. These RNAs and tissue total RNA was used to quantitate ACAT $mRNA_{14b}$ levels by an internal standard/RNAse protection assay as described in detail elsewhere (30, 31). Radiolabeled antisense probes and internal standard RNAs used for HMG-CoA reductase and LDL-R mRNA quantitation have also been described (31). Typically 30–50 μg of total RNA and 20–30 pg of internal standard were used in each hybridization reaction.

In one experiment RNAse One (Promega Corp., Madison, WI) was used in the RNAse protections assay instead of the RNAse A/RNAse T1 combination (30).

Isolation of rabbit hepatic cell types

Rabbit liver parenchymal and nonparenchymal cell populations were isolated as previously described (31).

Animals and diets

Male New Zealand White rabbits weighing approximately 1.5 kg on arrival were randomly assigned to chow (n = 8) or 0.5% cholesterol, 3% coconut oil, 3% peanut oil (HFHC) diet groups (n = 14). Animals were initially meal-fed 40 g of food per day and this was gradually increased to 70 g by the end of 4 weeks. After 4 weeks on the HFHC diet the mean plasma cholesterol level was 1582 ± 48 mg/dl (n = 14). Animals with pre-established hypercholesterolemia were randomly divided into two groups: one group remained on the HFHC diet (n = 7) while the other received the HFHC diet plus 50 mg/kg CI-976 by diet admix (n = 7); this feeding scheme continued for an additional 4 weeks. It has been previously shown that under these conditions peak plasma levels of CI-976 exceed by 10-fold the in vitro ACAT IC_{50} value using liver microsomes (32). Chow-fed animals (n = 8) were fed the diet for the entire 8 weeks. Animals were killed approximately 20 h after their last meal by carbon dioxide inhalation and blood was taken by heart puncture and placed into tubes containing EDTA. Various tissues were also removed for lipid and mRNA quantitation experiments.

Measurements of lipids, lipoproteins, and apolipoproteins

Plasma cholesterol (33) and triglycerides (34) were measured enzymatically using an Abbott VP Series II Bichromatic Analyzer with Boehringer Mannheim reagents. Cholesterol distribution among lipoproteins was determined by high-performance gel chromatography as described (35) except that the Beckman System GOLD was used (36). Liver total and free cholesterol concentrations were determined in isopropanol extracts as described (37). Whole-plasma apolipoprotein B, apolipoprotein A-I, and apolipoprotein E were determined by rocket immunoelectrophoresis (38) as described (39).

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Assay for microsomal ACAT activity

ACAT activity in hepatic microsomes was measured using the methods of Burrier et al. (40) with modifications described here. Liver homogenates were prepared in 0.25 M sucrose, 1 mM EDTA, 0.01 M Tris (pH 7.4) using a Polytron PT3000 (Brinkmann). Microsomes were isolated from homogenates by clarification of samples (15 min, 4°C, 6300 *g*) followed by pelleting from the supernatants (1 h, 14°C, 105,000 *g*). Microsomal pellets were resuspended in 0.25 M sucrose, 0.01 M Tris (pH 7.4). ACAT reactions were carried out in 50-μl volumes with 10 μg of microsomal protein using buffer conditions identical to those described by Burrier et al. (40). Exogenous cholesterol, when included, was added as cholesterol:phosphatidylcholine (0.5 molar ratio) vesicles without deoxycholate reconstitution of microsomes (41). After a 15-min preincubation, [^{14}C]oleoyl CoA (New England Nuclear) was added to a final concentration of 10 μM (specific activity = 5.8 mCi/mmol). Fifteen min after addition of oleoyl-CoA, reactions were terminated by direct application to silica gel thin-layer chromatography plates (Whatman LK6D). Dried plates were developed in hexanes–diethyl ether–acetic acid 85:15:1 (by volume). Incorporation of radioactivity into cholesteryl esters was measured using a PhosphorImager (Molecular Dynamics).

Statistical analysis

Statistical comparison among diet groups was determined by one-way analysis of variance (ANOVA) using InStat software (GraphPad Software, San Diego, CA).

RESULTS

Cloning of rabbit ACAT $cDNA_{14b}$

We prepared an "ACAT-enriched" plasmid cDNA library from rabbit liver. To do this we designed PCR primers based on the human ACAT $cDNA_{K1}$ sequence and then performed reverse transcriptase-based PCR under numerous conditions. Our aim was to find a primer set with sufficient homology to a putative rabbit ACAT K1 homolog that a sequence corresponding to the cDNA could be amplified. A primer set which amplified a DNA of predicted size was identified (i.e., based on the human ACAT $cDNA_{K1}$ sequence). The amplification products were cloned and the resultant "enriched" library was screened with the human ACAT $cDNA_{K1}$. Two positive colonies were identified; sequence analysis revealed they were identical and shared greater than 90% homology with the 1581 to 2499 region of the human ACAT $cDNA_{K1}$ sequence (**Fig. 1** and **Fig. 2**). The partial rabbit cDNA (919bp) was termed ACAT $cDNA_{14b}$ and is the homolog of the human ACAT $cDNA_{K1}$. If the mRNA coding regions for this protein are of similar size in rabbits and humans then ACAT $cDNA_{14b}$ (919bp) spans greater than 50% of the coding region.

Size and tissue distribution of $ACAT_{14b}$ RNAs

To determine the number of ACAT $mRNA_{14b}$ species in rabbits, we used an RNA probe derived from the partial ACAT $cDNA_{14b}$ to perform Northern blot analysis on adrenal, small intestine, liver, and aorta poly A+ RNA

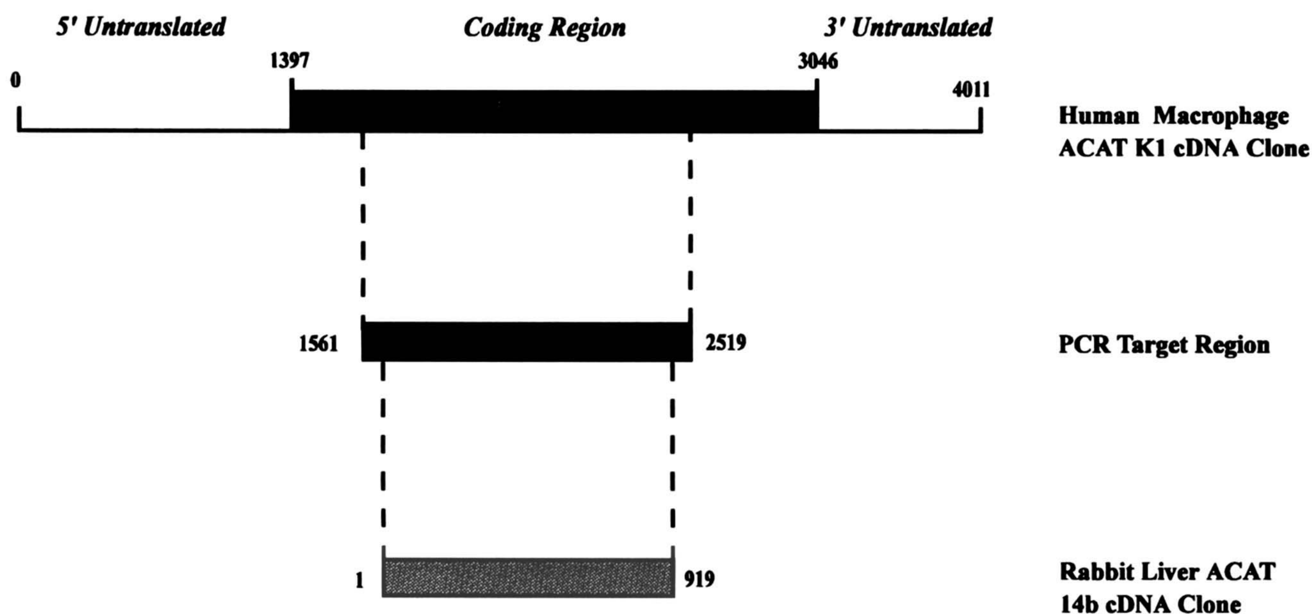

Fig. 1. Cloning of a partial rabbit ACAT $cDNA_{14b}$. The human ACAT $cDNA_{K1}$ (12) is depicted at the top of this schematic. The rabbit sequence targeted for amplification corresponded to the 1561–2519 region of the K1 clone (second line). The third line shows the isolated partial rabbit ACAT $cDNA_{14b}$ and its relationship to the human sequence. This sequence was cloned into a transcription vector and the resultant plasmid provided the template for synthesis of RNA probes used in Northern blot and RNAse protection assays.

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Rabbit ACAT 14b  AGCCACTTTTTTGAAGGAAGTTGGCAGTCACTTTGATGATTTTGTGACCAATCTGATTGAAAAATCGGCATCATTAGAC    80
Human ACAT K1         T     A                                           C        G  A                1660

Rabbit ACAT 14b  AATGGTGGATGTGCTCTCACAACTTTTTCCATTCTTAAAGAAATGAAAAACAATCACAGAGCTAAAGACCTGAGAGCACC   160
Human ACAT K1            G  C           C     TG     G   G GA         C  T     G  G  TT              1740

Rabbit ACAT 14b  TCCAGAGCAGGGAAAGATTTTCGTTGCAAGGCGATCTCTCTTGGATGAGCTGTTTGAAGTGGACCACATCAGGACAATAT   240
Human ACAT K1          A  A           TA          C        A     A   C                   A           1820

Rabbit ACAT 14b  ATCACATGTTCATCGCCCTCCTCATTCTCTTTATCCTCAGCACTCTTGTGGTAGATTACATCGATGAAGGAAGGCTGGTG   320
Human ACAT K1                T  T                             A     A           T                      1900

Rabbit ACAT 14b  CTTGAGTTCAATCTCCTGTCTTACGCTTTTGGCAAACTTCCTACTGTTGTTTGGACCTGGTGGACCATGTTCCTGTCTAC   400
Human ACAT K1                GC           T            T       C                   T                   1980

Rabbit ACAT 14b  ACTTTCAATTCCCTATTTCCTGTTTCAACATTGGGCCAATGGCTACAGCAAGAGCTCTCATCCACTGATGTATTCTCTCT   480
Human ACAT K1     T     G          T               CG  C      T        T        G     CCG            2060

Rabbit ACAT 14b  TCCATGGCTTACTTTTTATGGTCTTCCAGCTTGGAATTCTAGGTTTTGGGCCAACGTATATTGTATTAGCATATACACTG   560
Human ACAT K1              T     C   A        A     G             A     A   G    G                   2140

Rabbit ACAT 14b  CCACCAGCTTCCCGTTTCATTGTTATACTCGAGCAGATTCGTTTGATAATGAAGGCCCATTCATTTGTCAGAGAGAACGT   640
Human ACAT K1                  G     CA     T                TG             C                        2220

Rabbit ACAT 14b  GCCTCGGGTACTAAATTCAGCTAAGGAGAAATCAAGCACTGTTCCAATACCCACAGTCAACCAGTACTTGTACTTCTTGT   720
Human ACAT K1                                                       T              T           A     2300

Rabbit ACAT 14b  TTGCTCCTACCCTGATCTACCGGGACAGCTATCCCAGGACTCCCACTGTAAGATGGGGTTATGTTGCTATGCAGTTTGCA   800
Human ACAT K1                 T        T                A                        C      A            2380

Rabbit ACAT 14b  CAGGTCTTTGGCTGCCTTTTTTATGTGTACTATATCTTTGAGCGGCTCTGTGCCCCGTTGTTTCGGAATATCAAACAGGA   880
Human ACAT K1               T   T    C           C        AA    T        C                           2460

Rabbit ACAT 14b  GCCCTTCAGCGCTCGTGTCCTCGTCCTGTGTATATTTAA                                             919
Human ACAT K1                      T  G     A   G                                                    2499
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Fig. 2. Nucleotide sequence of rabbit ACAT $cDNA_{14b}$ and comparison to the corresponding region of human ACAT $cDNA_{K1}$. Numbering for the human clone is based on the published sequence (12).

(**Fig. 3A**). The probe recognized at least four RNA species in the adrenal and aorta and at least six in the small intestine and liver. The sizes ranged from 6.2 kb to 1.7 kb (Fig. 3A).

To determine whether similar sized ACAT $mRNA_{K1}$ and $mRNA_{14b}$ species were present in human and rabbit tissues, poly A+ RNA from the same four human tissues (liver, adrenal, small intestine, and aorta) were analyzed by Northern blot using a riboprobe derived from human ACAT $cDNA_{K1}$ (Fig. 3B). The probe hybridized to several bands in the human samples; under these assay conditions and with these human samples, the smaller 1.9 to 1.7 kb species present in rabbit liver and intestine were not evident in the human samples. This may be related to the sensitivity of the assay or the fact that some of these RNA preparations are from individuals who died from causes that affected the species present. Alternatively, the smaller species may not be made in human liver and intestine. Nonetheless, it is apparent that the ACAT $mRNA_{14b}$ species in rabbit are about 500 bp shorter than the corresponding mRNA species in humans.

To more accurately quantify ACAT $mRNA_{14b}$ levels in rabbit tissues, an RNAse protection assay was developed. In this assay, the plasmid containing the 919 bp partial rabbit ACAT $cDNA_{14b}$ was used to synthesize a radiolabeled RNA probe and a non-radioactive internal standard RNA. The internal standard corrects for variations in sample handling and hybridization efficiencies which should be relatively constant in all hybridization reactions (30). Using this assay, ACAT $mRNA_{14b}$ was detected in all tissues analyzed (**Fig. 4**). Adrenal gland contained the most ACAT $mRNA_{14b}$ (per μg of total RNA) with omental fat, brain, and small intestine having appreciable levels. After correcting the counts in the band corresponding to probe protected by authentic rabbit ACAT $mRNA_{14b}$ ("A" in Fig. 4) by the counts in the band corresponding to probe protected by the internal standard ("IS" in Fig. 4), the abundance of the ACAT $mRNA_{14b}$ in the tissues analyzed was as follows: adrenal > omental fat > small intestine > brain > pancreas, spleen > lung > heart > aorta > liver > kidney > skeletal muscle. Adrenal contained 20-, 30-, 50-fold more ACAT $mRNA_{14b}$ (per μg of total RNA) than small intestine, aorta, and liver, respectively. From these analyses, it was apparent that an additional band migrated slightly faster than the predicted size of probe that would be protected by the authentic ACAT $mRNA_{14b}$ (Fig. 4, most pronounced in the adrenal lane). This band could have

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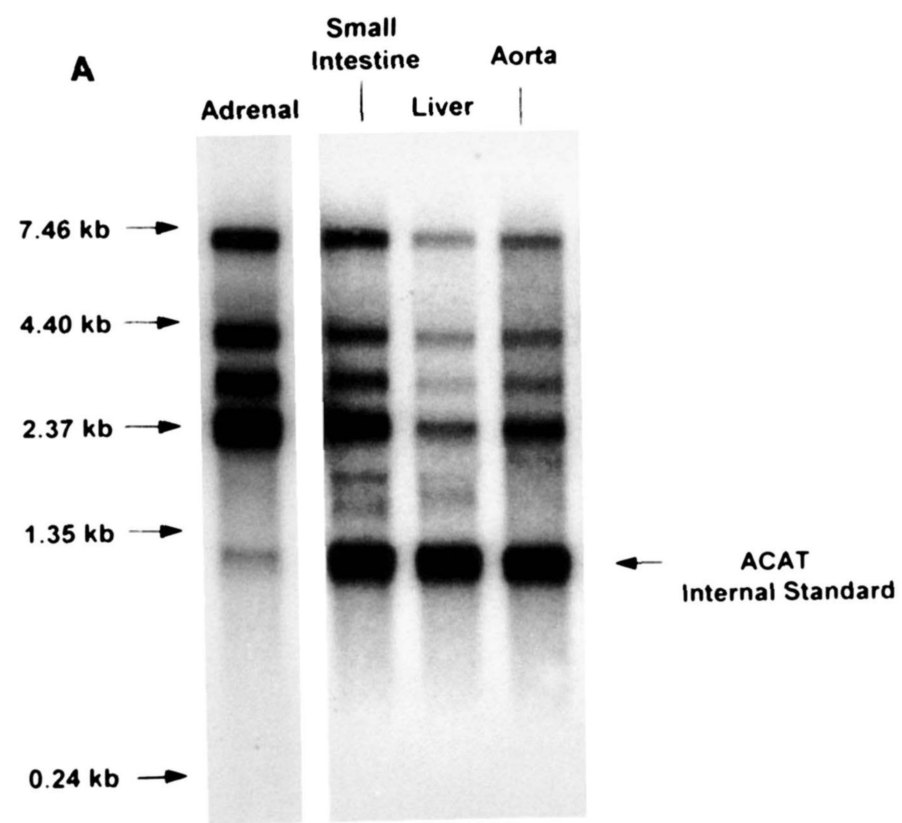

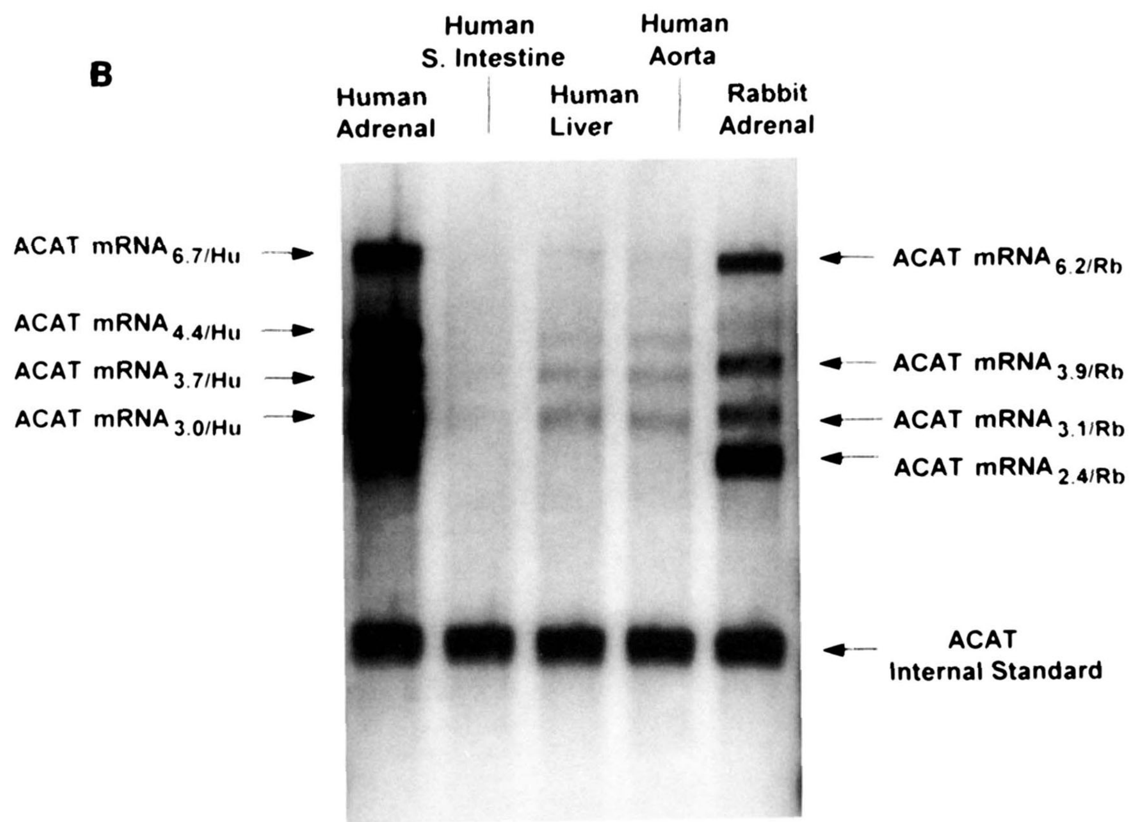

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Fig. 3. Northern blot analysis of rabbit and human ACAT RNAs. (A) Poly A+ RNA (5 μg) from the indicated rabbit tissues was isolated, separated on a formaldehyde-containing gel, and transferred to nitrocellulose. ACAT $mRNA_{14b}$ species on the membrane were detected with a radiolabeled RNA probe containing sequences from the entire 919 bp ACAT $cDNA_{14b}$ (see Fig. 1). As a positive control, a non-radioactive internal standard RNA (ACAT RNA_{14b} sense strand) was synthesized from the same plasmid used to generate the antisense RNA probe. Fifteen picograms of internal standard RNA was added to each poly A+ sample before separation on the gel; it migrates at approximately 950 bases. The signal intensity from the internal standard RNA is directly related to the exposure time of each lane to the PhosphorImager screen. To illustrate, adrenal ACAT $mRNA_{14b}$ species are in greater abundance than those of the other tissues; a shorter exposure was used. In all cases, the variable size ACAT $mRNA_{14b}$ species can be related to the internal standard independent of PhosphorImager exposure time. (B) Poly A+ RNA (2.5 μg) from the indicated human tissues was analyzed as described above except that a riboprobe derived from a 721 bp fragment of the human ACAT $cDNA_{K1}$ was used to detect ACAT $mRNA_{14b}$ species. Rabbit adrenal poly A+ was included in this analysis for comparison of ACAT mRNA sizes; thus, the human probe can cross-hybridize to rabbit ACAT $mRNA_{14b}$ sequences.

been due to the presence of another form of ACAT $mRNA_{14b}$ which differed in the coding region, i.e., containing a deletion in the region where the probe hybridizes. Investigating this possibility was imperative as Kinnunen, DeMichele, and Lange (42) have presented evidence that different forms of ACAT protein exist in rabbit. To test whether the smaller band contained sequences highly homologous to riboprobe derived from ACAT $cDNA_{14b}$, we used another RNAse, RNAse One, to digest single-stranded regions of hybrids formed during the hybridization reaction (Fig. 4B). RNAse One, unlike RNAse A or RNAse TI, can cleave the phosphodiester bond at all four bases (43); thus, it is more adept at digesting slightly mismatched regions within an RNA:RNA hybrid. The data indicated that the faster migrating band disappeared with RNAse One treatment while the slower migrating band was not affected by RNAse One. These data suggested that the smaller RNA species and probe are probably not identical in sequence. Whether the smaller RNA species was derived from an RNA that con-

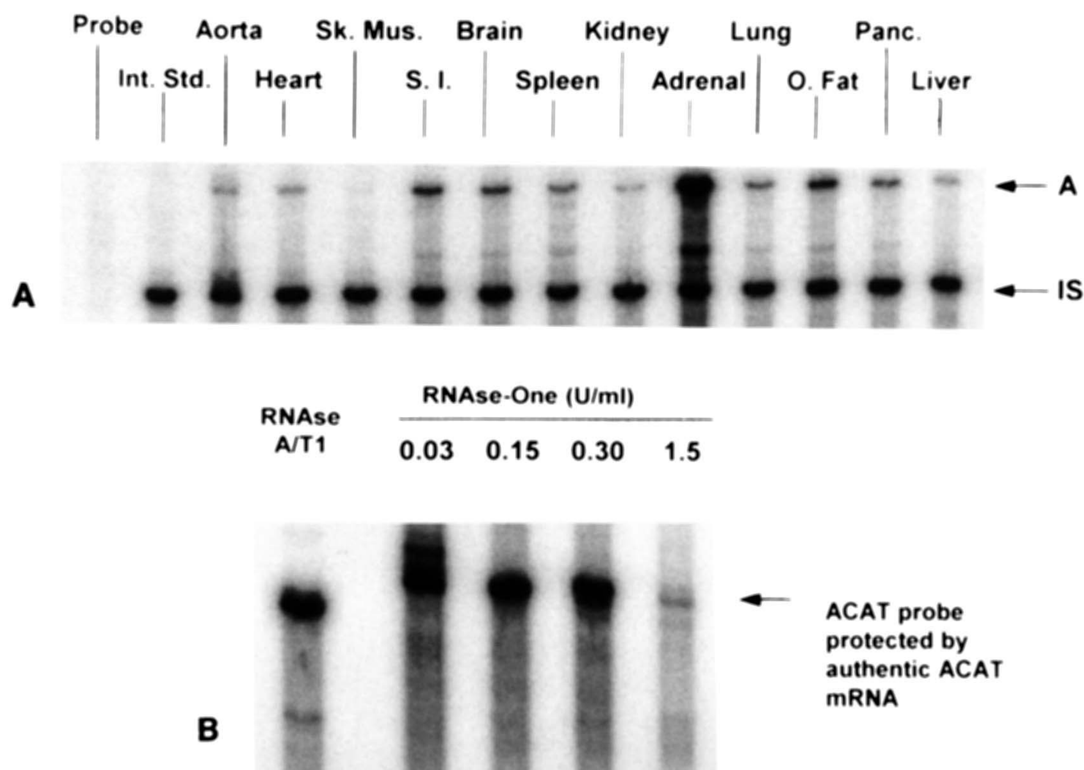

Fig. 4. Tissue distribution of ACAT $mRNA_{14b}$ in rabbit tissues. (A) Total RNA was isolated from each tissue (Sk. mus., skeletal muscle, S.I., small intestine, O. fat, omental fat, panc., pancreas). Equal amounts of tissue total RNA from two rabbits were pooled and ACAT $mRNA_{14b}$ content was determined by the internal standard/RNAse protection assay. "Probe" indicates control hybridization reaction that included only the radiolabeled antisense riboprobe. "Int. Std." indicates a control hybridization reaction including only internal standard RNA and radiolabeled probe. "IS" and "A" mark the electrophoretic migration of probe protected by the internal standard and authentic ACAT $mRNA_{14b}$, respectively. Each hybridization reaction contained 40 μg of total RNA, 20 pg of internal standard RNA, and excess radiolabeled probe. (B) Probe was hybridized with 40 μg of total RNA from rabbit adrenal under the standard RNAse protection conditions (no internal standard was included in this hybridization). After the overnight hybridization at 45°C, single-stranded RNA was digested with either the standard RNAse A/RNAse T1 combination (RNAse A/T1 lane) or with different concentrations of RNAse One (0.03, 0.15, 0.30, 1.5 U/ml). Note that at the low concentration (0.03 U/ml) digestion is not complete, while at the high concentration (1.5 U/ml) there is excessive digestion of hybrids by this enzyme.

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tained some sequences homologous to the riboprobe derived from ACAT $cDNA_{14b}$ and thus is related is unclear at present.

In light of the RNAse One-based protection assay, it is not possible to unequivocally state that the probe protected (band "A" in the gel) in the RNAse protection assay represents all ACAT $mRNA_{14b}$ species present in the Northern analysis. In fact, the lower stringency of Northern analysis (although very stringent by Northern standards) compared to the RNAse One-based protection assay, and the presence of multiple bands in the Northern may mean that some are ACAT $mRNA_{14b}$-related. Nonetheless, it should be noted that: *1)* the riboprobe derived from ACAT $cDNA_{14b}$ was the same used in the RNAse protection assays and Northern blot analysis (Fig. 3); *2)* no additional bands (even further down the gel lanes (Fig. 4B), data not shown) except those protected by the authentic mRNA were present in any of the samples treated with RNAse One; and *3)* both the RNAse protection assay and Northern blot analysis yield similar quantitative changes for ACAT $mRNA_{14b}$ levels between tissues (compare Fig. 3A and Fig. 4). These data indicate that the signal from band "A" in the protection assay is concordant with the overall signal from the various ACAT $mRNA_{14b}$ species by Northern blot analysis. In any event, band "A" in the protection assay represents sequences probably identical to the 919 base probe while the Northern analysis could be showing not only those sequences identical to the 919 base probe but also some minor ACAT $mRNA_{14b}$-related species. Unequivocal statements concerning these multiple mRNA species, such as whether they are ACAT $mRNA_{14b}$-related or represent ACAT $mRNA_{14b}$ species at various stages of processing, will require mapping, cloning, and sequencing the various ACAT $mRNA_{14b}$ species.

Hepatic cell distribution of ACAT $mRNA_{14b}$

As the liver plays such a central role in lipid metabolism, we isolated hepatic parenchymal and nonparenchymal cells to determine their relative contribution to hepatic ACAT $mRNA_{14b}$ levels (**Fig. 5**). Rabbit hepatic nonparenchymal cells contained about 30-fold more ACAT $mRNA_{14b}$ than parenchymal cells. We also analyzed CETP and apoE mRNA levels in these cell preparations. CETP mRNA, like ACAT $mRNA_{14b}$, predominated in the nonparenchymal cells while apolipoprotein E mRNA was primarily in the parenchymal cells, consistent with our previous findings (31).

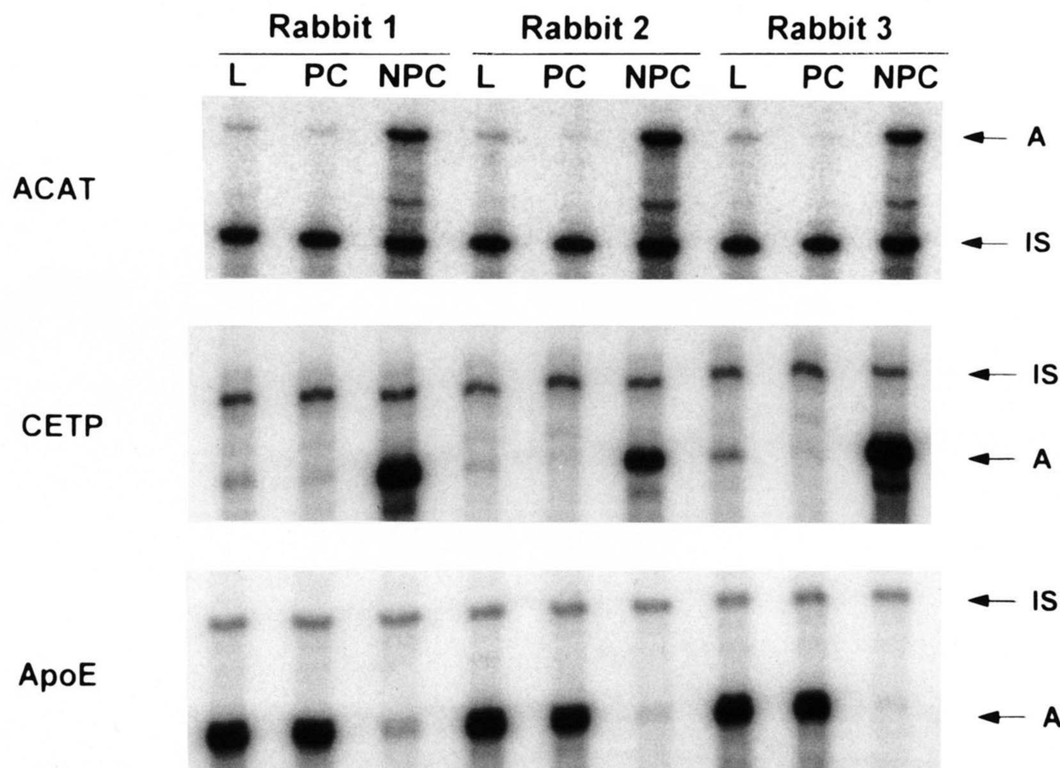

Fig. 5. Expression of ACAT $mRNA_{14b}$, CETP mRNA, and apoE mRNA in rabbit liver cell populations. Total RNA was isolated from intact liver (L) or isolated parenchymal cells (PC), and nonparenchymal cells (NPC). Abundance of the indicated mRNA was measured by the internal standard/RNAse protection assay. The amount of total RNA and internal standard was as follows: ACAT, 40 μg total RNA/15 pg internal standard; CETP, 30 μg/30 pg; and apoE, 5 μg/40 pg. These analyses were performed for three different chow-fed rabbits. "IS" and "A" mark the electrophoretic migration of probe protected by the internal standard and authentic ACAT $mRNA_{14b}$, respectively.

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TABLE 1. Effect of a high fat/high cholesterol (HFHC) diet and CI-976 on plasma lipids and apolipoproteins

	Plasma Lipids (mg/dl)					Plasma Levels (arbitrary units)		
Treatment	Total-C	VLDL-C	LDL-C	HDL-C	TG	ApoB	ApoE	ApoA-I
Chow (n = 8)	104 ± 9	4 ± 1	62 ± 9	38 ± 2	63 ± 5	100 ± 12	100 ± 4	100 ± 16
HFHC (n = 7)	2749 ± 230[a]	1968 ± 183[a]	669 ± 45[a]	112 ± 8[a]	149 ± 15[a]	286 ± 23[a]	804 ± 36[a]	62 ± 8
HFHC + CI-976 (n = 7)	671 ± 68[b]	216 ± 41[b]	419 ± 30[b]	37 ± 3[b]	111 ± 10	312 ± 33	351 ± 31[b]	74 ± 8

Numbers are expressed as mean ± SEM.
[a] $P < 0.001$ compared to chow-fed controls.
[b] $P < 0.001$ compared to HFHC-fed controls.

In vivo regulation of ACAT $mRNA_{14b}$ levels

To investigate ACAT $mRNA_{14b}$ regulation in vivo, we fed rabbits three different diets and measured plasma lipid parameters, liver cholesterol content, and ACAT $mRNA_{14b}$ levels in liver, small intestine, adrenal, and aorta. The dietary treatments included a group fed a chow diet for 8 weeks, another fed the HFHC diet for 8 weeks, and a third group fed the HFHC diet for 8 weeks in which the diet contained 50 mg/kg CI-976 for the last 4 weeks.

Table 1 shows the diet-induced changes in plasma lipids and apolipoproteins. The HFHC diet elevated plasma total cholesterol more than 25-fold relative to the chow-fed animals; this included formation of VLDL and elevation of LDL and HDL cholesterol. Plasma triglycerides increased about 2.5-fold with the HFHC diet. Animals with a pre-established hypercholesterolemia (1500 mg/dl after 4 weeks on the HFHC diet) when treated with CI-976, showed about a 75% decrease in total plasma cholesterol compared to the HFHC-fed controls, mostly due to a reduction in VLDL (89%) although the absolute concentrations of LDL (37%) and HDL (67%) cholesterol were also lowered. There was a nonsignificant 26% decrease in plasma triglycerides with CI-976 treatment. Unlike chow-fed animals in which most cholesterol resided in LDL (58%) and HDL (39%), in the HFHC-fed animals plasma cholesterol appeared mainly in the VLDL (71%) and LDL (25%). Treatment of HFHC-fed animals with CI-976 reduced the percentage of cholesterol in VLDL to 30% of the total. Thus, the relative percent of total cholesterol in LDL was similar in chow-fed and HFHC + CI-976 groups (i.e., approximately 60% of total). In the HFHC-fed animals, apoB and apoA-I plasma levels were not altered by drug treatment while apoE levels were reduced 56%. These data suggest that CI-976 ACAT inhibition markedly reduces cholesterol concentrations in apoB-containing lipoproteins largely by reduction in particle size and composition rather than number.

Diet and drug treatment also had a marked effect on hepatic lipids (**Table 2**). Changes in total hepatic cholesterol content in each group nearly paralleled the changes in total plasma cholesterol. Compared to HFHC-fed controls, CI-976 treated animals showed markedly reduced levels of both free (65%) and esterified (80%) cholesterol and of the percent cholesterol esterified.

We measured ACAT $mRNA_{14b}$ levels in various organs of animals treated with the different diets (**Fig. 6A**). Hepatic ACAT $mRNA_{14b}$ levels were elevated 2.2-fold in the HFHC-fed animals while CI-976 treatment lowered ACAT $mRNA_{14b}$ levels to those near the chow-fed level. As expected (44), hepatic LDL-receptor and HMG-CoA reductase mRNA levels were reduced 60% and 20%, respectively, in the HFHC-fed animals compared to chow-fed controls (Fig. 6B and 6C). Hepatic HMG-CoA reductase mRNA levels were reduced 43% in CI-976-treated animals compared to HFHC-fed controls while LDL-receptor mRNA was unaffected.

To further characterize expression in this model, the levels of ACAT $mRNA_{14b}$ in other tissues were analyzed (Fig. 6). ACAT $mRNA_{14b}$ levels in adrenal and small intestine were not affected by either the HFHC-diet or drug treatment. However, there was a marked elevation of ACAT $mRNA_{14b}$ levels in the aorta of HFHC-fed animals (3-fold) and CI-976 limited this elevation to 60% of control although the data did not reach statistical significance ($P = 0.07$); aortic ACAT $mRNA_{14b}$ levels in chow-fed and CI-976-treated animals were not statistically different. In contrast to ACAT $mRNA_{14b}$, HMG-

TABLE 2. Effect of the HFHC diet and CI-976 on hepatic cholesterol levels

	Liver Cholesterol (mg/g)		
Treatment	Total	Free	Esterified
Chow (n = 8)	2.14 ± 0.1	2.04 ± 0.1	0.1 ± 0.1
HFHC (n = 7)	22.7 ± 1.9[a]	6.34 ± 0.2[a]	16.4 ± 1.2[a]
HFHC + CI-976 (n = 7)	7.71 ± 0.7[b]	3.03 ± 0.1[b]	4.69 ± 0.5[b]

Values are expressed as mean ± SEM.
[a] $P < 0.001$ compared to chow-fed controls.
[b] $P < 0.001$ compared to HFHC-fed controls.

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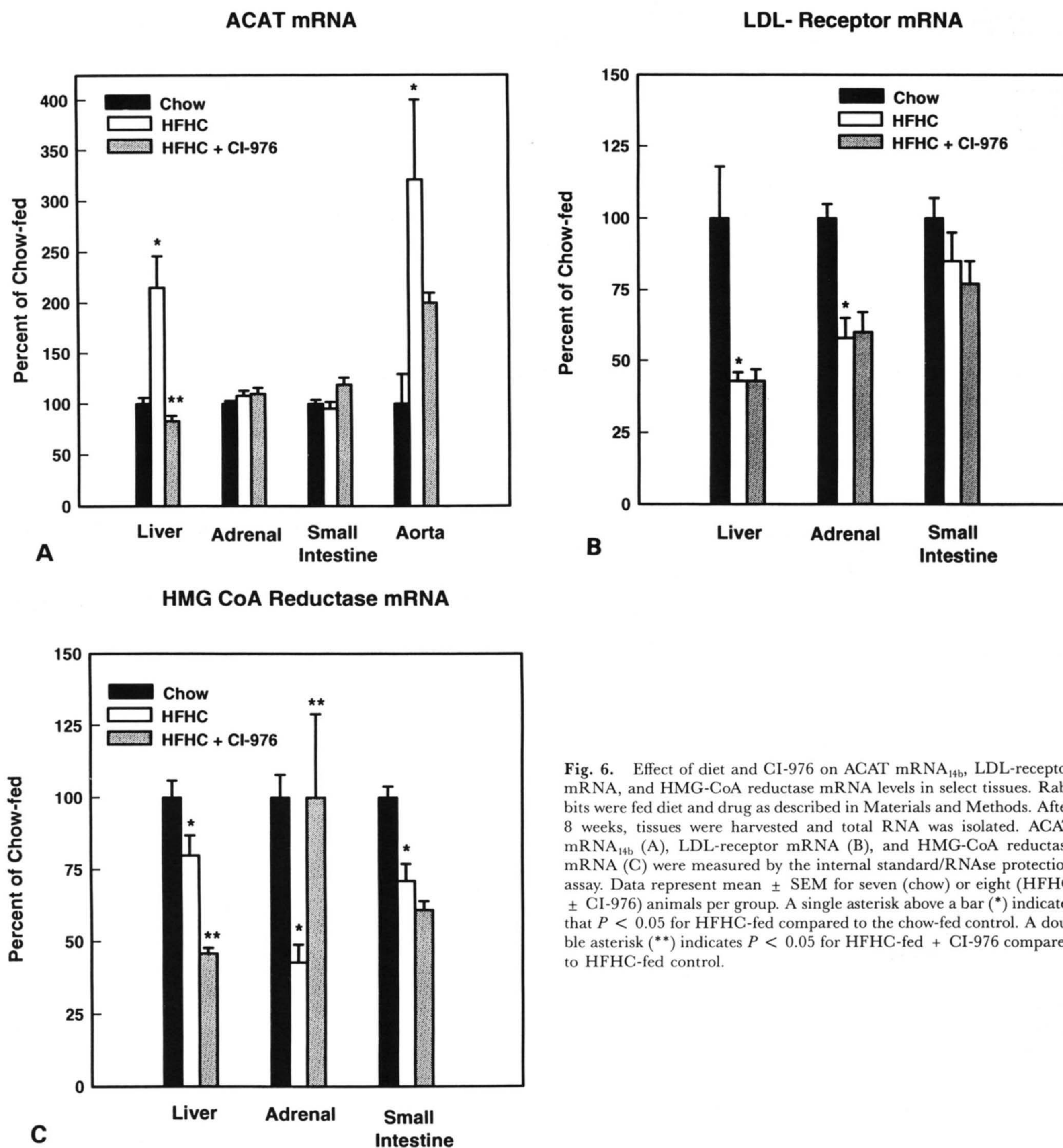

Fig. 6. Effect of diet and CI-976 on ACAT $mRNA_{14b}$, LDL-receptor mRNA, and HMG-CoA reductase mRNA levels in select tissues. Rabbits were fed diet and drug as described in Materials and Methods. After 8 weeks, tissues were harvested and total RNA was isolated. ACAT $mRNA_{14b}$ (A), LDL-receptor mRNA (B), and HMG-CoA reductase mRNA (C) were measured by the internal standard/RNAse protection assay. Data represent mean ± SEM for seven (chow) or eight (HFHC ± CI-976) animals per group. A single asterisk above a bar (*) indicates that $P < 0.05$ for HFHC-fed compared to the chow-fed control. A double asterisk (**) indicates $P < 0.05$ for HFHC-fed + CI-976 compared to HFHC-fed control.

CoA reductase mRNA levels in adrenal fell 57% in the HFHC-fed animals while LDL-receptor mRNA levels were reduced by 42% compared to chow-fed controls. CI-976 treatment prevented the fall in HMG-CoA reductase mRNA levels in adrenal while the drug did not affect LDL-receptor mRNA levels. There were only minor changes in small intestine for HMG-CoA reductase and LDL-receptor mRNAs under the various conditions. These data indicated that ACAT $mRNA_{14b}$ levels (in addition to HMG-CoA reductase and LDL-receptor) can be regulated in a tissue-specific manner depending on the dietary and pharmacological state.

As multiple forms of ACAT $mRNA_{14b}$ exist, we further characterized the expression of these RNAs under the various physiological conditions. Equal amounts of total RNA from each animal in the treatment group were

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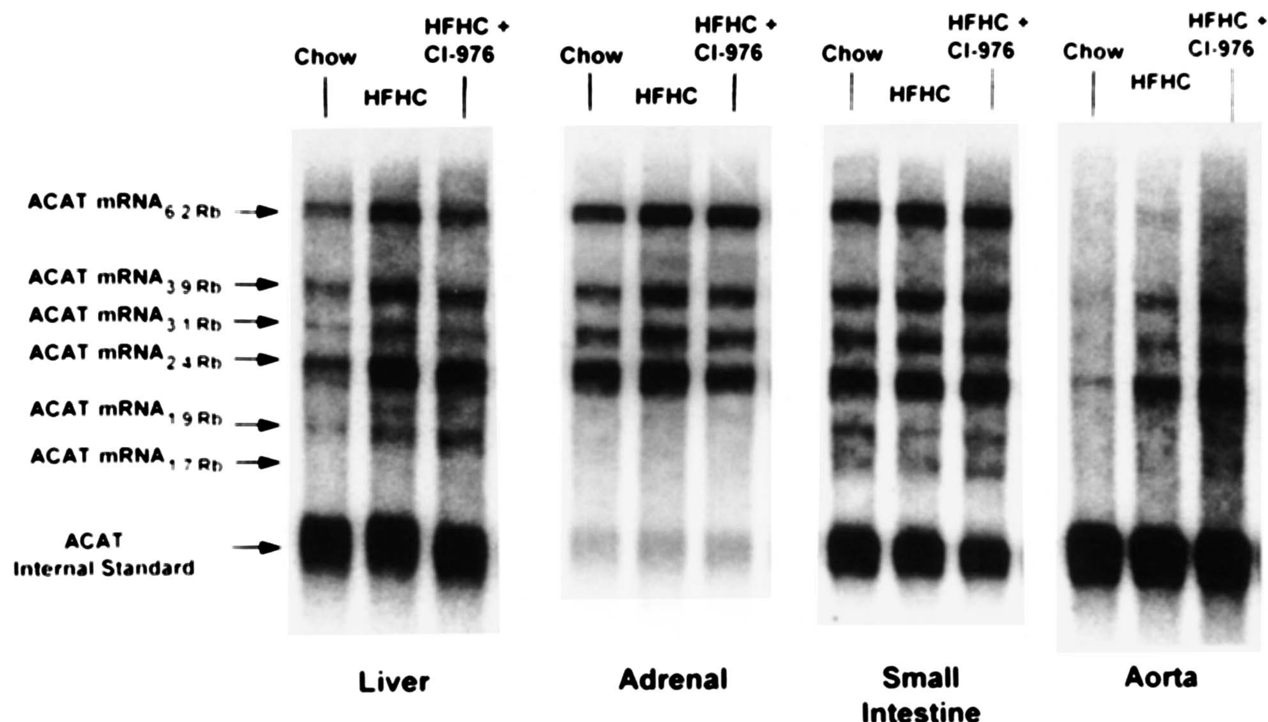

Fig. 7. Effect of diet and CI-976 on ACAT $mRNA_{14b}$ distribution by Northern blot analysis. Poly A+ RNA was isolated from equal amounts of pooled total RNA isolated from tissues of the same rabbits used to generate data in Fig. 6. Poly A+ RNA (approximately 2 μg) was separated on 1% formaldehyde-containing agarose gels and transferred to nitrocellulose. The nitrocellulose was incubated with radiolabeled riboprobe derived from the entire 919 bp ACAT $cDNA_{14b}$ (see Fig. 1). As a positive control, a non-radioactive internal standard RNA was synthesized from the same plasmid used to generate the antisense RNA probe. Fifteen picograms of internal standard RNA was added to each poly A+ sample before separation on the gel. It migrates at approximately 950 bases. The signal intensity from the internal standard RNA is directly related to the exposure time of each lane to the PhosphorImager screen. Thus, for lane to lane comparisons the relative ratio of internal standard RNA to authentic ACAT $mRNA_{14b}$ (1.7-6.2) can be used.

pooled and poly A+ RNA was isolated and analyzed by Northern analysis to determine whether there was altered expression of specific ACAT $mRNA_{14b}$ species (**Fig. 7**). No marked changes in the various ACAT $mRNA_{14b}$ species were observed when analyzed under these conditions.

To determine whether hepatic microsomal ACAT activity was altered in this rabbit model, a separate experiment was performed. We confirmed the hepatic changes in ACAT $mRNA_{14b}$ levels in response to diet and drug (**Table 3**). Furthermore, the hepatic ACAT $mRNA_{14b}$ increase in response to the HFHC diet was associated with an increase in hepatic microsomal ACAT activity (Table 3). Without exogenous cholesterol added to the assay, microsomal ACAT activity was 17-fold higher in animals fed the HFHC diet compared to chow-fed controls. When exogenous cholesterol was added to the assay, the difference in activity was maintained; however, only a 4-fold difference was observed between the HFHC-fed group

TABLE 3. Effect of the HFHC diet and CI-976 on hepatic ACAT $mRNA_{14b}$ and microsomal ACAT activity

Treatment	ACAT $mRNA_{14b}$, percent of chow	ACAT Activity: Without Exogenous Cholesterol	ACAT Activity: With Exogenous Cholesterol
		pmol/min/mg	*pmol/min/mg*
Chow (n = 6)	100 ± 6	40 ± 22	273 ± 81
HFHC (n = 6)	228 ± 17[a]	705 ± 84[a]	1109 ± 314
HFHC + CI-976 (n = 6)	75 ± 18[b]	482 ± 70	852 ± 204

Values are expressed as mean ± SEM.
[a] $P < 0.05$ compared to chow-fed controls.
[b] $P < 0.05$ compared to HFHC-fed controls.

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and the chow-fed group. Microsomal ACAT activity from animals treated with or without CI-976 was not statistically different, whether the assay was performed in the presence or absence of exogenous cholesterol; this could be due to drug washout during isolation of the microsomes.

DISCUSSION

Cholesteryl ester synthesis is catalyzed by ACAT, a microsomal protein or protein complex with a molecular mass of about 200 kDa as determined by radiation inactivation techniques (45, 46). The hydrophobic nature of the protein has made purification and characterization difficult. However, two reports have been published recently claiming the isolation of a cDNA encoding a protein with ACAT activity (12, 47). The primary amino acid sequence of the cholesterol-esterifying protein described by Chang et al. (human ACAT protein K1 (12)) bears little overall homology with any known protein sequence while the protein identified by Becker et al. (47) is identical to human carboxylesterase. This discrepancy bears on the interpretation of the present work as we have analyzed the expression of the rabbit homolog of the human ACAT protein K1. Thus, a limited critique of the identity of the clones in question is essential for evaluating the relevance of the ACAT $mRNA_{14b}$ presented in this report.

It is clear that Chang and coworkers have isolated a cDNA that encodes a polypeptide essential for ACAT catalysis (12, 16, 17, 48). The most compelling data is that insect Sf9 cells infected with baculovirus containing the ACAT $cDNA_{K1}$ displayed specific activities at least 10-fold higher than various human cell lines when assayed in vitro; there was no detectable ACAT activity in Sf9 cells infected with baculovirus lacking the ACAT $cDNA_{K1}$ (17). Thus, transfection of the human ACAT $cDNA_{K1}$ into either a mutant CHO cell line that lacks ACAT activity (12) or a non-mammalian cell line (17) results in a marked increase in ACAT activity. These results do not prove that ACAT protein K1 is the ACAT holoenzyme or, for that matter, the only protein capable of esterifying cholesterol. Furthermore, the possibility exists that ACAT protein K1 is part of a larger hetero- or homo-multimeric protein complex that catalyzes the overall ACAT reaction (48). Unequivocal statements regarding these issues must await purification of the ACAT protein K1. Nonetheless, this protein is a critical component in the esterification of cholesterol.

The cholesterol-esterifying protein isolated by Becker et al. (47) is identical to carboxylesterase. The purified protein showed ACAT activity in a liposome-based system. In addition, overexpression of the carboxylesterase protein in CHO cells resulted in a 3-fold increase in ACAT activity in cell homogenates and about a 20-fold increase in intracellular cholesteryl ester. The specific role carboxylesterase may play in cholesteryl ester metabolism is unclear at present. The carboxylesterases are a family of serine esterases and are thought to be active in modifying xenobiotics (49). With respect to these properties and the question of ACAT, others have shown that the irreversible serine esterase inhibitor, PMSF, does not affect ACAT activity in rabbit or rat liver microsomes (42, 46) which are a rich source of carboxylesterase (50). Also, Ozols (50) has provided evidence that carboxylesterase is localized in the lumen of the endoplasmic reticulum (ER); as activated fatty acids cannot freely traverse the ER membrane, it is not clear what role carboxylesterase may play physiologically. Finally, overexpression of 7α-hydroxylase, which presumably perturbs intracellular cholesterol and lipid metabolism, has been shown to elevate cholesteryl ester mass in CHO cells (51); it is possible that overexpression of carboxylesterase may also indirectly affect cholesteryl ester synthesis by altering substrate availability. In light of these data, it appears that the physiological role for carboxylesterase as related to cholesteryl ester metabolism remains to be determined. Transgenic animals lacking carboxylesterase or ACAT protein K1, if viable, will permit investigations into the relative physiological importance of each.

The present report focuses on characterizing mRNA expression and regulation of the rabbit homolog of human ACAT protein K1. As this protein is an essential component in ACAT catalysis (17), we hypothesized that altered ACAT activities induced by an atherogenic diet (23, 52–56) can be partly due to control mechanisms involving changes in the level of its mRNA. A critical tool required for this study was the isolation of a rabbit cDNA homologous to the human ACAT $cDNA_{K1}$. This clone was obtained by screening a rabbit liver cDNA library with the human ACAT $cDNA_{K1}$; the 919 base pair rabbit cDNA was termed ACAT $cDNA_{14b}$. That the partial cDNA isolated is the rabbit homolog of human ACAT $cDNA_{K1}$ is evident from the high degree of sequence homology (90%). Isolation of the rabbit ACAT $cDNA_{14b}$ permitted the development of assays for quantitative (internal standard/RNAse protection assay) and qualitative (Northern blot analysis) assessment of ACAT $mRNA_{14b}$. These assays allowed characterization of ACAT $mRNA_{14b}$ expression in rabbits and assessment of its molecular regulation.

The abundance of rabbit ACAT $mRNA_{14b}$ in various tissues differs somewhat from the microsomal ACAT activities in rabbit tissues as reported by Kinnunen et al. (42). In a chow-fed rabbit (n = 1), high ACAT activity was found in adrenal, intermediate activity in liver, kidney, lung, fat, spleen, and low activity in small intestine, pancreas, heart, brain, aorta. Abundance of ACAT $mRNA_{14b}$ in chow-fed rabbits (pooled average of two chow-fed rabbits) shows high levels in adrenal, intermediate levels in fat, small intestine, brain, pancreas, spleen,

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lung, and low levels in liver, heart, aorta, kidney; ACAT $mRNA_{14b}$ was barely detectable in skeletal muscle. These differences may reflect the limitations of the microsomal ACAT activity assay (see ref. 57) and in general, the complex nature of ACAT regulation (8). Tissue differences in microsomal composition and the presence or absence of other microsomal proteins that affect esterification may explain why ACAT activity does not correlate directly with ACAT $mRNA_{14b}$ levels. Furthermore, as the present report shows, the massive increase in hepatic cholesteryl ester content caused by feeding rabbits the HFHC diet was accompanied by a modest increase in hepatic ACAT $mRNA_{14b}$ levels. Clearly, other mechanisms play a major role in cholesteryl ester metabolism.

A novel finding from this study was that ACAT $mRNA_{14b}$ was present in hepatic nonparenchymal cells at a level approximately 30-times that of parenchymal cells (on a per microgram of total RNA basis). From our data it is not possible to determine whether the increase in ACAT $mRNA_{14b}$ levels in the HFHC-fed state occurred in one or both cell populations. Nonetheless, although nonparenchymal cells contain 30-fold more ACAT $mRNA_{14b}$, adjustment for the fact that parenchymal cells contain about 20- to 25-fold more total RNA per cell and make up 65% of liver cell number, the parenchymal and nonparenchymal cells each contribute about an equal amount of ACAT $mRNA_{14b}$ per gram of tissue (31). This distribution of ACAT $mRNA_{14b}$ is nearly the same as that for CETP in rabbits (31). It is interesting to note that hepatic CETP mRNA levels also increase significantly in both rabbits (58) and monkeys (59) in response to a HFHC diet suggesting that a common regulatory pathway may exist for these two presumably sterol-responsive genes. Indeed, it is of interest to identify those factors that modulate $ACAT_{14b}$ gene expression.

In vivo experiments demonstrated that hepatic and aortic ACAT $mRNA_{14b}$ levels were elevated by high fat/high cholesterol feeding; whether this reflects a change in ACAT protein 14b mass is not clear. There are only limited data available, all indirect, suggesting that ACAT activity is regulated by control mechanisms involving changes in protein mass. The reasons for this are 3-fold. First, there are inherent difficulties in negating the effect of cellular lipids on the microsomal ACAT activity assay. Second, ACAT specific antibodies are not yet available for mass determination. Third, most studies investigating ACAT activity or esterification rates in cell culture have been performed in an acute fashion. With respect to the last point, Drevon, Weinstein, and Steinberg (60) showed that esterification and ACAT activity increased within minutes after exposing rat parenchymal cells to 25-hydroxycholesterol. Similar effects on ACAT activity and esterification rates have been observed when cholesterol or a precursor of its synthesis is delivered to macrophages, intestinal cell lines, hepatoma cells, CHO cells, and fibroblasts (61–64). In each case esterification rates and ACAT activity were measured after only a brief exposure to cholesterol or its precursor (typically less than 24 h and usually 4 h or less). In some cell culture systems differences in the rate of cholesteryl esterification were observed as well as differences in microsomal ACAT activity in the absence of exogenous cholesterol (41, 64, 65). However, when exogenous cholesterol was added to the microsomes from these cells, the differences in ACAT activity were abolished, suggesting that substrate availability rather than protein mass changes were responsible (41, 64, 65). This is consistent with work by Cheng et al. (17) who demonstrated that cholesterol and oxysterol are allosteric activators of ACAT protein K1 catalytic activity.

In more chronic experiments, ACAT activity has been measured in microsomes from animals on various diets. These studies revealed differences in ACAT activity even in substrate excess (55, 56, 66); in most cases the changes were 2-fold or less. In this report, we demonstrate that liver microsomes from HFHC-fed rabbits have 4-fold higher ACAT activity compared to microsomes from chow-fed rabbits even when the assay is performed in substrate excess. Although we cannot conclude that ACAT protein mass was altered in these microsomes due to the possible existence of allosteric or viscotropic effects on the enzyme, it hints at the possibility that this type of control exists for ACAT. Indeed, although we were not able to directly measure ACAT protein 14b mass due to the lack of a specific antibody, ACAT $mRNA_{14b}$ levels increased 2-fold after rabbits were fed the HFHC diet for 8 weeks. Definitive statements relating ACAT protein 14b mass to ACAT $mRNA_{14b}$ mRNA levels and rates of gene transcription, mRNA decay, and translation cannot be ascertained until a specific antibody for ACAT protein 14b is available; this reagent is currently being made. However, transcriptional control of ACAT $mRNA_{14b}$ does not appear to be the primary mechanism for altering cholesteryl ester metabolism in liver, small intestine, and adrenal for the following reasons. *1)* A massive increase in hepatic cholesteryl ester content (at least 40-fold) in response to the HFHC diet is accompanied by a modest 2-fold increase in ACAT $mRNA_{14b}$ levels. *2)* There were no changes in intestinal or adrenal ACAT $mRNA_{14b}$ levels even though these tissues in rabbits have been shown to increase microsomal ACAT activities in response to cholesterol feeding (42). In these tissues other mechanisms may be operative including substrate (cholesterol) availability, translational control, specific activities of other proteins involved in cholesteryl ester metabolism, or even cell population dynamics. The last point is especially important when interpreting ACAT activity or mRNA changes in aorta.

We observed that the HFHC diet caused a 3-fold increase in aortic ACAT $mRNA_{14b}$ levels. The increase in ACAT $mRNA_{14b}$ levels in aorta needs to be considered in

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light of the cellular diversity in a lesioned versus nonlesioned aorta. Although there is not extensive lesion development in rabbit aortas after 8 weeks on an atherogenic diet, there can be a significant influx of lipid-laden macrophages into the vessel wall after 4 weeks (67). Thus the increase in ACAT $mRNA_{14b}$ levels may not be due to the specific increase in ACAT $mRNA_{14b}$ molecules per cell but rather reflect the number of macrophages in the vessel. Indeed, in these same aorta samples we found that apoE mRNA levels were increased about 4-fold in the HFHC-fed animals in comparison to chow-fed animals (M. E. Pape, unpublished observation); the increase in apoE mRNA in lesioned vessels from rabbits has been partly attributed to an accumulation of macrophages in the subintima of the arterial wall (68). Whether a similar mechanism involving macrophage infiltration or replication is responsible for the hepatic increase in ACAT $mRNA_{14b}$ cannot be answered at present. However, liver and aorta from cholesterol-fed animals contain numerous macrophages while adrenals and small intestine, two organs that did not display elevated ACAT $mRNA_{14b}$ levels in response to the HFHC diet, contain relatively few macrophages. The high abundance of ACAT $mRNA_{14b}$ in hepatic nonparenchymal cells, a population that includes Kupffer cells, further bears on this point. It is conceivable that exposure of the liver to the HFHC diet for 8 weeks may have induced some type of liver disease which resulted in an influx of macrophages. Isolation of individual cell types from the various organs and investigating ACAT $mRNA_{14b}$ regulation in response to cholesteryl ester loading will shed light on this issue.

Not only did the nutritional state alter ACAT $mRNA_{14b}$ levels but treatment of rabbits with an ACAT inhibitor, CI-976, also resulted in tissue specific changes in ACAT $mRNA_{14b}$ levels. In rabbits with pre-established hypercholesterolemia, CI-976 maintained hepatic ACAT $mRNA_{14b}$ levels to those of chow-fed animals. As in the HFHC-fed state, ACAT $mRNA_{14b}$ levels were not changed in adrenal and small intestine in response to the drug. The decreased abundance of aortic ACAT $mRNA_{14b}$ upon drug treatment may reflect a reduction in the number of macrophages in the vessel as CI-976 has been shown to markedly reduce lesion development in this model independent of changes in plasma cholesterol (37). The mechanism by which CI-976 mediates these changes in ACAT $mRNA_{14b}$ does not appear to be direct. In the pre-established hypercholesterolemia rabbit model, lowering plasma cholesterol to the same levels with CI-976 or β-sitosterol, a non-absorbable cholesterol absorption inhibitor, resulted in a similar reduction in hepatic ACAT $mRNA_{14b}$ levels (M. E. Pape, unpublished observation). It appears that the effect of CI-976 on ACAT $mRNA_{14b}$ is through its ability to inhibit cholesterol absorption in the gut (32).

In summary, ACAT $mRNA_{14b}$ metabolism is a complex process involving multiple forms of the RNA expressed in various tissues and is regulated by both nutritional and pharmacological factors. The relatively small changes in ACAT $mRNA_{14b}$ levels in contrast to the marked hypercholesterolemia suggest that substrate availability may be a primary mechanism for determining cellular cholesteryl ester stores. Nonetheless, investigations into transcriptional and posttranscriptional mechanisms controlling ACAT $mRNA_{14b}$ metabolism will provide further insight into the physiological and cellular mechanisms of cholesterol esterification and homeostasis. ■

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