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Human Acyl-Coenzyme A:Cholesterol Acyltransferase 1 (*acat1*) Sequences Located in Two Different Chromosomes (7 and 1) Are Required to Produce a Novel ACAT1 Isoenzyme with Additional Sequence at the N Terminus*

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A rare form of human ACAT1 mRNA, containing the optional long 5′-untranslated region, is produced as a 4.3-kelonucleotide chimeric mRNA through a novel interchromosomal *trans*-splicing of two discontinuous RNAs transcribed from chromosomes 1 and 7 (Li, B. L., Li, X. L., Duan, Z. J., Lee, O., Lin, S., Ma, Z. M., Chang, C. C., Yang, X. Y., Park, J. P., Mohandas, T. K., Noll, W., Chan, L., and Chang, T. Y. (1999) *J. Biol. Chem.* 274, 11060–11071). To investigate its function, we express the chimeric ACAT1 mRNA in Chinese hamster ovary cells and show that it can produce a larger ACAT1 protein, with an apparent molecular mass of 56 kDa on SDS-PAGE, in addition to the normal, 50-kDa ACAT1 protein, which is produced from the ACAT1 mRNAs without the optional long 5′-untranslated repeat. To produce the 56-kDa ACAT1, *acat1* sequences located at both chromosomes 7 and 1 are required. The 56-kDa ACAT1 can be recognized by specific antibodies prepared against the predicted additional amino acid sequence located upstream of the N-terminal of the $ACAT1_{ORF}$. The translation initiation codon for the 56-kDa protein is GGC, which encodes for glycine, as deduced by mutation analysis and mass spectrometry. Similar to the 50-kDa protein, when expressed alone, the 56-kDa ACAT1 is located in the endoplasmic reticulum and is enzymatically active. The 56-kDa ACAT1 is present in native human cells, including human monocyte-derived macrophages. Our current results show that the function of the chimeric ACAT1 mRNA is to increase the ACAT enzyme diversity by producing a novel isoenzyme. To our knowledge, our result provides the first mammalian example that a trans-spliced mRNA produces a functional protein.

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Acyl-coenzyme A:cholesterol acyltransferase (ACAT)[1] is an intracellular enzyme that plays important roles in lipid metabolism. It catalyzes the formation of cholesteryl esters, using long-chain fatty acyl coenzyme A and cholesterol as the two substrates. In mammals, two ACAT genes have been identified (reviewed in Refs. 1–4). The first ACAT gene, *acat1*, was identified by isolating a human cDNA (ACAT cDNA K1) that functionally complements a Chinese hamster ovary cell mutant (clone AC29) lacking endogenous ACAT activity (5). The second ACAT gene, *acat2*, was identified by homology cloning, based on the nucleotide sequence of ACAT1 cDNA. The ACAT1 and ACAT2 proteins share extensive sequence homology at their C-terminal halves but not at their N-terminals. Both enzymes are integral membrane proteins. Human ACAT1 (hACAT1) contains seven transmembranes (6), whereas hACAT2 contains only two detectable transmembranes (7). A conserved histidine (His-460 in hACAT1 and His-432 in hACAT2), located within a long stretch of hydrophobic residues, may serve as an active site for ACAT catalysis (7, 8). Human ACAT1 message and protein are present in many tissues and various cell types examined, including adrenal, kidney, hepatocytes, Kupffer cells, intestinal enterocytes, fibroblasts, macrophages, and neurons in the brain (5, 9–12). In contrast, abundant ACAT2 message, protein, and activity have only been found in intestinal enterocytes (9, 11, 13); weak ACAT2 signals are also detectable in hepatocytes and in macrophages (11, 14). The functional significance of finding both ACAT enzymes in the same cell types (*i.e.* hepatocytes, intestinal enterocytes, and macrophages) is not clear and is currently under investigation. Together, these two isoenzymes participate in various biological processes relevant to cholesterol homeostasis, including intracellular cholesterol storage, lipoprotein synthesis and secretion, steroid hormone synthesis, dietary cholesterol absorption, and macrophage foam cell formation during atherogenesis.

Human ACAT1 gene contains 18 exons (exons Xa, Xb, and 1–16) (15). Unlike almost all other known human genes, the human ACAT1 gene is located in two different chromosomes (1

[1] The abbreviations used are: ACAT, acyl-coenzyme A:cholesterol acyltransferase; h, human; aa, amino acid(s); ER, endoplasmic reticulum; FBS, fetal bovine serum; nt, nucleotide(s); knt, kelonucleotide(s); ORF, open reading frame; PBS, phosphate-buffered saline; UTR, untranslated repeat; HA, hemagglutinin; PMA, phorbol 12-myristate 13-acetate; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; NTP, N-terminal peptide.

and 7), with each chromosome containing a distinct promoter (P1 and P7). The P1 promoter and exons 1–16, which constitute the predicted open reading frame (ORF) are located in chromosome 1. On the other hand, the P7 promoter, contiguous with the optional long exon Xa (1279 bps), is located in chromosome 7 (15). The location of the short exon (Xb; 10 bp) is unknown at present. Northern analyses have revealed the presence of four ACAT1 mRNAs (7.0, 4.3, 3.6, and 2.8-knt), present in almost all of the human tissues and cells examined. These messages share the same coding sequence. The 2.8- and 3.6-knt messages, comprising more than 70–80% of the total ACAT1 mRNAs, are produced from the P1 promoter (15). Specific anti-ACAT1 antibodies (DM10) against the antigenic sites located within the first 131 aa of $ACAT1_{ORF}$ have been produced (10). When AC29 cells were transfected with the plasmid that encompasses the predicted ACAT1 mRNA ORF region (1.7 kb; 550 aa), Western analysis using DM10 antibodies revealed a single ACAT1 protein band with an apparent molecular mass of 50 kDa (10). The same 50-kDa protein is also present in various human cells and tissues examined (10, 11). The 4.3-knt message containing the optional long 5′-UTR that is composed of exons Xa and Xb. Thus, this mRNA is produced from two different chromosomes by a novel RNA recombination event that presumably involves *trans*-splicing (15). This study represents the first example of producing a chimeric mRNA as a result of interchromosomal *trans*-splicing in the human genome (discussed in Ref. 17). The 4.3-knt chimeric ACAT1 mRNA is present in various human tissues and cells examined. However, its functional significance remains unknown. To address this question, in the current study we created various site-specific mutant ACAT1 cDNA plasmids and performed expression studies. The results demonstrate the existence of a novel ACAT1 isoenzyme in transfected cells, as well as in human macrophage cells.

EXPERIMENTAL PROCEDURES

Materials

Cell culture reagents and T4 DNA ligase were from Invitrogen. Anti-rabbit IgG conjugated with horseradish peroxidase was from Pierce. Goat polyclonal antibodies N-20 (against the 78-kDa glucose-regulated protein GRP78, an endoplasmic reticulum marker), rhodamine-conjugated donkey anti-goat IgG, fluorescein-conjugated goat anti-rabbit IgG, and ECL detection reagent were from Santa Cruz Biotechnology (Santa Cruz, CA). The restriction enzymes and agarose were from Promega (Madison, WI). Protease inhibitor mixture, anti-HA antibodies, V8 protease, ANTI-FLAG® M2 affinity gel, and phorbol 12-myristate-13-acetate (PMA) were from Sigma. Precision Plus Protein™ standards (prestained, all blue) were from Bio-Rad. The *Taq*DNA polymerase and dNTPs were from Sino-American Biotech (Shanghai, China). All the oligonucleotides were synthesized with an automated DNA synthesizer at the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Methods

Cell Culture and Treatments—All cell lines were maintained in a basal medium as indicated, supplemented with 10% fetal bovine serum (FBS) and antibiotics, in a 37 °C incubator with humid atmosphere, 5% CO_2, and 95% air. Chinese hamster ovary cell line AC29 was grown in Ham's F-12 medium. Dr. Roger Newton, formerly of Parke-Davis Pharmaceuticals, Ann-Arbor, MI, kindly provided the THP-1 cell line. The THP-1 cells were maintained in RPMI 1640 medium. To trigger differentiation into macrophage-like cells (18, 19), THP-1 cells seeded at a density of 2×10^6 cells per 60-mm dish in 5 ml of 1640 medium containing 10% FBS were grown for 2 days in medium containing PMA (at 0.1 μM), freshly prepared from a 10^3-fold concentrated stock solution in Me_2SO. Human monocytes were isolated according to a published procedure (20). The cells were cultured for up to 16 days in RPMI 1640 medium supplemented with 7% human type AB serum, with a medium change once every other day.

Expression Plasmids—Full-length human ACAT1 cDNA K1 (nt 1–4011) was obtained by digesting the plasmid pBSK-K1 (5) with KpnI and XbaI and inserting the released full-length ACAT1 fragment into the KpnI and XbaI sites of pcDNA3 vector (Invitrogen) to generate the expression plasmid pcDNA3-K1. Partial human ACAT1 cDNA K1 nt 1104–4011 was obtained by digesting pBSK-K1 with XhoI and XbaI, and inserting the partial ACAT1 fragment into the XhoI and XbaI sites of pcDNA3 vector to generate the expression plasmid pcDNA3-K1D1. Three other ACAT1 cDNA K1 fragments (nt 1243–1786, nt 1282–1786, and nt 1304–1786) were generated by PCR, using the following individual forward oligonucleotides (L1D2BF, 5′-ACCG*CTCGAG*TAGTTAAATAG-3′; D13F, 5′-AAG*CTCGAG*GAATTCGGGAGAGCTTC-3′; LDSF, 5′-AGGG*CTCGAG*GTCGACCTTCCTGCTG-3′) with XhoI sites (in italics) and a common reverse oligonucleotide (HAHR, 5′-AAGCGAC*TCTAGA*GATCTAAGAGAGAG-3′) that contains XbaI site (in italics). The amplified fragments were individually purified, digested with XhoI and XbaI, and inserted into the XhoI and XbaI sites of pcDNA3 vector, respectively, to generate the expression plasmids pcAND2, pcAND3, and pcAND4, all of them in-frame with the downstream stop codon TAG present in pcDNA3. Partial ACAT1 cDNA K1 nt 1454–4011 was obtained by digesting pBSK-K1 with Bsu36I and XbaI; the released ACAT1 fragment was inserted into the Bsu36I and XbaI sites of pcAND2, pcAND3, or pcAND4 to generate pcDNA3-K1D2 (containing ACAT1 fragment nt 1243–4011), pcDNA3-K1D3 (containing ACAT1 fragment nt 1282–4011), or pcDNA3-K1D4 (containing ACAT1 fragment nt 1304–4011). Partial ACAT1 cDNA K1 nt 1–1281 was obtained by digesting pBSK-K1 with KpnI and EcoRI and inserting the ACAT1 fragment into the KpnI and EcoRI sites of pcAND3 to generate pcAND containing partial cDNA K1 sequence from nt 1 to 1786. Partial ACAT1 cDNA K1 containing nt 1104–1281 was obtained by digesting pBSK-K1 with XhoI and EcoRI; the released ACAT1 fragment was inserted into the XhoI and EcoRI sites of pcAND3 to generate pcAND1 containing partial cDNA K1 nt 1104–1786. Two ACAT1 cDNA K1 fragments from nt 1243–1786, containing relevant in-frame codon mutations to initiation $ATG_{1397-1399}$ codon of $ACAT1_{ORF}$, were generated by PCR, using the following individual forward oligonucleotides (M41F, 5′-AAG*CTCGAG*TAGTTAAATAGCTATATTTATATATTAGCAGGGCACCCCGAATTC-3′; M12F, 5′-AAG*CTCGAG*TAGTTAAATAGCTATATTTATATATATCTAGGGCACCCCGAATTC-3′), with XhoI sites (in italics) plus codon mutations (underlined), and common reverse HAHR oligonucleotide with XbaI site. The amplified fragments were individually purified, digested with XhoI and XbaI, and inserted into the XhoI and XbaI sites of pcDNA3 vector, respectively, to generate the expression plasmids pcAND2m41 and pcAND2m12, both of them are in-frame with the downstream stop codon TAG present within the pcDNA3 vector sequence. Human ACAT1 cDNA K1 nt 1454–4011 was obtained by digestion with Bsu36I and XbaI of pBSK-K1. The released ACAT1 fragment was inserted into the Bsu36I and XbaI sites of pcAND2m41 and pcAND2m12, to generate pcDNA3-K1D2m41 and pcDNA3-K1D2m12, respectively. The internal codon mutations of ACAT1 cDNA in pcAND2 were created by using a modified two-step PCR method originally described by Higuchi *et al.* (21). Briefly, the first reaction was performed by using the L1D2BF forward oligonucleotides that hybridized to 5′-region of the partial ACAT1 cDNA K1 nt 1243–1786 present in pcAND2, and an internal reverse oligonucleotide (5′-GTTTCTTAGAGAGGCCTTCTCTTCACCCACGGCTGTATTGTCTGAG-3′) for the desired mutated bases that mismatch with the first two ATGs in the $ACAT1_{ORF}$. The second reaction was performed by using an internal forward oligonucleotide (5′-CTCAGACAATACAGCCGTGGGTGAAGAGAAGGCCTCTCTAAGAAAC-3′) for the same desired mutated bases and the above HAHR reverse oligonucleotide that hybridized to the 3′-region of the partial ACAT1 cDNA K1 nt 1243–1786 in pcAND2. The two overlapping DNA fragments amplified by the two different PCRs were then "fused" by denaturing and annealing in a subsequent extension reaction. Finally, the fused and extended product was amplified by PCR using the oligonucleotides L1D2BF and HAHR. The final PCR product was digested with XhoI and XbaI and inserted into the XhoI and XbaI sites of pcDNA3 vector to obtain expression plasmid pcAND2m. The ACAT1 cDNA K1 fragment (nt 1243–1786) with the first three ORF-ATG codons displaced with GCC was achieved from pcAND2m by performing the two-step PCR described above, using the internal forward (5′-TTGAAGCCATTTTTTGCCAAGGAAGTTGGCAGTCACTTTG-3′) and reverse (5′-ACTGCCAACTTCCTTGGCAAAAAATGGCTTCAATTCCTC-3′) oligonucleotides (the desired mutations were underlined). The final PCR product was digested with XhoI and XbaI and inserted into the XhoI and XbaI sites of pcDNA3 vector to obtain the expression plasmid pcAND2m58, upstream of the stop codon TAG from pcDNA3. Human ACAT1 cDNA K1 nt 1454–4011 was obtained by digestion with Bsu36I and XbaI of pBSK-K1. The released ACAT1 fragment was inserted into the Bsu36I and XbaI sites of pcAND2m and pcAND2m58, to generate pcDNA3-K1D2m and

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pcDNA3-K1D2m58, respectively. The ACAT1 cDNA K1 fragment (nt 1304–1786) with the first three ORF-ATG codons displaced with GCC, plus an additional ATG codon inserted upstream to the first mutant GCC codon, was achieved from pcAND2m58 by performing the two-step PCR described above, using the internal oligonucleotide set (M51F, 5′-CTCA-GACAATACAATGGCCGTGGGTGAAGAGAAG-3′/M51R, 5′-CTTCTCT-TCACCCACGGCCATTGTATTGTCTGAG-3′) and common LDSF/HAHR oligonucleotides. After digestion with XhoI and XbaI, the PCR products were individually inserted into the XhoI and XbaI sites of pcDNA3 vector to obtain the expression plasmids pcAND4m62, upstream of the stop codon TAG from pcDNA3. Human ACAT1 cDNA K1 nt 1454–4011 was obtained by digestion with Bsu36I and XbaI of pBSK-K1. The released ACAT1 fragment was inserted into the Bsu36I and XbaI sites of pcAND4m62 to generate pcDNA3-K1D4m62. Partial ACAT1 cDNA K1 fragments containing additional nucleotide sequences for expressing the hemagglutinin epitope tag (HA tag, 9 amino acids Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) at C terminus of human ACAT1 proteins were generated by PCR, using the forward oligonucleotide (5′-CAA*CATATG*TTGT-GTTAGCATATAC-3′) with NdeI sites (italics) and reverse oligonucleotide (5′-AGA*AAGCTT*CTAGAGGGA**GGCGTAGTCCGGGACGTCATATG-GGTA**AAACACGTAACGACAAGTC-3′) with an HindIII site (italics) and additional nucleotide sequences (bold) coding for the HA tag sequence. The amplified fragments were individually purified, digested with NdeI and HindIII, and inserted into the NdeI and HindIII sites of pcDNA3-K1D2, pcDNA3-K1D2m, pcDNA3-K1D2m58, pcDNA3-K1D4, and pcDNA3-K1D4m62 to generate the expression plasmids pcDNA3-K1D2HA, pcDNA3-K1D2mHA, pcDNA3-K1D2m58HA, pcDNA3-K1D4HA, and pcDNA3-K1D4m62HA, as shown in Fig. 6*A*. Inserting the HA tag at the C terminus of ACAT1 protein has little effect on ACAT activity (6). By performing the two-step PCR described above, with the relevant sets of internal oligonucleotides (M6F, 5′-ATATATATCCAGTA-GACCCCGAATTCG-3′/M6R, 5′-CGGGGTCTACTGGATATATATAAAT-A-3′; M2F, 5′-TATATCCAGGGCTAGCCGAATTCGGG-3′/M2R, 5′-CCC-GAATTCGGCTAGCCCTGGATATA-3′; M37F, 5′-GGAGAGCTTCCCTA-GGTCGACCTTCCTGCTG-3′/M37R, 5′-AGGAAGGTCGACCTAGGGAA-GCTCTCCCGAATTC-3′; M38F, 5′-CTTCCTGCTGGCTAGTCTGTGAC-CGCTTC-3′/M38R, 5′-AGCGGTCACAGACTAGCCAGCAGGAAGGTC-3′) with the desired codon mutations (underlined), four final PCR products were obtained from pcAND2. After digestion with XhoI and XbaI, the PCR products were individually inserted into the XhoI and XbaI sites of pcDNA3 vector to obtain the expression plasmids pcAND2m6, pcAND2m2, pcAND2m37, and pcAND2m38, upstream of the stop codon TAG from pcDNA3. Human ACAT1 cDNA K1 nt 1454–4011 was obtained by digestion with Bsu36I and XbaI of pBSK-K1. The released ACAT1 fragment was inserted into the Bsu36I and XbaI sites of pcAND2m6, pcAND2m2, pcAND2m37, and pcAND2m38, to generate pcDNA3-K1D2m6, pcDNA3-K1D2m2, pcDNA3-K1D2m37, and pcDNA3-K1D2m38, respectively. Partial ACAT1 cDNA K1 fragment (nt 1243–1786) containing additional nucleotide sequences for expressing the FLAG epitope tag (FLAG octapeptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) at the C terminus of human ACAT1-NTP was generated by PCR, using the forward oligonucleotide (L1D2BF, 5′-ACCG*CTCGAG*TAGTTA-AATAG-3′) with XhoI sites (italics) and reverse oligonucleotide (5′-AAC-A*TCTAGA*GTCC**CTTGTCGTCGTCGTCCTTGTAGTC**GCCATCTAA-GAGAGAG-3′) with XbaI site (*italics*) and nucleotide sequences (bold and underlined) coding for FLAG octapeptide. The amplified fragments were individually purified, digested with XhoI and XbaI, and respectively inserted into the XhoI and XbaI sites of pcDNA3 to generate the expression plasmid pcAND2-Flag. All plasmids were confirmed by restriction enzyme digestion and by DNA sequencing.

Transfection—Unless stated otherwise, the expression plasmids were individually transfected into various mammalian cells using the method of calcium phosphate coprecipitation described by Liu *et al.* (22) with slight modifications. Briefly, cells were seeded at density of 5×10^5 cells per 60-mm dish in 5 ml of culture medium containing 10% FBS for 36 h. Cells were then incubated sequentially with 5 ml of fresh medium containing 10% FBS for 2 h, then with 5 ml of medium without FBS for 30 min before transfection. 1.2 ml of transfection reagent was first prepared by gently mixing equal volumes of DNA and calcium ion (15 μg of DNA per 60-mm dish, and 125 mM calcium ion at final concentration) in HeBS buffer (final concentration: 21 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM glucose). The transfection reagent was then added to the 5 ml of medium present in each dish. The mixture was incubated with cells at 37 °C for 9 h. Afterward, cells were washed once with PBS, replaced with 5 ml of fresh medium containing 10% FBS, and cultured for another 48 h. To determine the ACAT activities of various constructs expressed in AC29 cells, FuGENE 6 transfection reagent, which gives slightly higher transfection efficiency than the calcium phosphate method in Chinese hamster ovary cells, was used for transfection (see below).

Preparation of the Anti-ACAT1 Antibodies DM58—A GST-$ACAT1_{exonX}$ fusion protein plasmid was created, which contains the first predicted 40 aa, extending from the N-terminal of $ACAT1_{ORF}$, fused in-frame at the C-terminal of the bacterial protein GST. The method used to produce the fusion protein plasmid was the same as the one described earlier (10) to produce the GST-$ACAT1_{1–131}$ fusion protein. The GST-$ACAT1_{exonX}$ plasmid was used to express the fusion protein in *Escherichia coli*. The expressed protein was gel-purified and used to produce antibodies in rabbits. The resultant antisera (designated as DM58) were affinity-purified by using the GST-$ACAT1_{exonX}$ fusion protein as the affinity ligand. The affinity-purified antibodies were stored in 0.1 M Tris-glycine buffer, pH 7.0, under sterile conditions at −80 °C.

Preparation of Protein Samples and Western Blot Analysis—Cells were harvested and lysed with 10% SDS in 50 mM Tris, 1 mM EDTA (pH 7.5), 50 mM dithiothreitol, plus protease inhibitor mixture (Sigma), incubated at 37 °C at various time periods as indicated, and sheared with a syringe fitted with an 18-gauge needle until apparent homogeneity was reached. Protein concentrations of the cell lysates were determined by a modified Lowry method (23). The protein samples were then subjected to 12% SDS-PAGE Western analysis according the a method described previously (11), using 0.5 μg/ml affinity-purified antibody DM10 or 2.5 μg/ml antibody DM58 as the primary antibodies.

MALDI-TOF Mass Spectrometry Analysis of ACAT1-NTP-FLAG—The ACAT1-NTP-FLAG fusion protein (ACAT1-NTP fused with FLAG tag at its C terminus), which contains the N-terminal portion of the 56-kDa human ACAT1, was expressed in AC29 cells by transient transfection and purified using the ANTI-FLAG M2 affinity gel. Briefly, the AC29 cells transiently transfected with the ACAT1-NTP-FLAG construct were grown in F-12 medium with 10% FBS in twenty 100-mm dishes to 70–90% confluency. The cells were washed and detached by scraping in PBS. The cell pellets were collected by centrifugation and dissolved in 3 ml of lysis buffer that contains 1% Triton X-100. The lysates were incubated with ANTI-FLAG M2 affinity gel with gentle mixing at 4 °C for 10 h. After washing the gels with lysis buffer four times, ACAT1-NTP-FLAG was eluted off by using 150 μl of 0.1 M glycine HCl at pH 3.5, then immediately neutralized by adding 15 μl of 0.5 M Tris HCl, pH 7.4, 1.5 M NaCl. The enriched ACAT1-NTP-FLAG fraction was further purified by SDS-PAGE and appeared as a single, 25-kDa protein band. The band was in-gel-digested with trypsin and treated with iodoacetamide. The resultant peptide mixture was desalted and analyzed by MALDI-TOF MS using the instrument Bruker REFLEX™ III MALDI-TOF (Germany), located at the Research Center for Proteome Analysis, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

ACAT Activity Assay—AC29 cells were plated at 1×10^6 cells per 6-well dishes in 2 ml of Medium A (F-12 plus Dulbecco's modified Eagle's medium (1:1) plus 10% fetal bovine serum). Cells were grown for 16 h before transfection. Two micrograms of each plasmid DNA were transfected into each well using the FuGENE 6 transfection reagent according to the manufacturer's instruction. The transfection time was 10 h. Thirty hours later, cells were trypsinized and equally distributed into three wells containing 2 ml/well Medium A and 200 μg/ml G418. Fresh Medium A with G418 was replaced each day. ^{3}H-Oleate pulse in intact cells and Western blotting were performed on the 5th day after the transfection. Treating cells with G418 for several days enriches transfected cells, thus increasing the signal to background ratio for the ACAT activity assay. Oleate pulse was performed in duplicated wells, and Western blot was performed from one well, with cell extracts split into two identical gels for SDS-PAGE and Western blot analysis. The sizes and the levels of the expressed ACAT1 proteins were determined by Western blotting using the HA antibody. The ^{3}H-Oleate pulse assay, measuring [^{3}H]cholesteryl ester formation in intact cells, was performed essentially as described previously (5, 24).

Double Immunofluorescence Staining—Using AC29 cells transiently transfected with various plasmids, or using THP-1 cells treated with PMA for 2 days as indicated, double immunofluorescence staining was performed according to methods described previously (6, 10), pairing either the rabbit antibody DM10 (at 0.5 μg/ml) or DM58 (at 2.5 μg/ml) as indicated, with the goat antibody N-20 (at 2 μg/ml). Rhodamine-conjugated donkey anti-goat IgG diluted at 1:500 and fluorescein-conjugated goat anti-rabbit IgG diluted at 1:500 were used as the secondary antibodies. After carefully washing out the non-reacted secondary antibodies, the specimens were mounted using Dako fluorescent mounting medium (Dako, Carpentaria, CA) and viewed under a laser scanning confocal microscope at 0.36 μm/optical section (Eclipse TE300,

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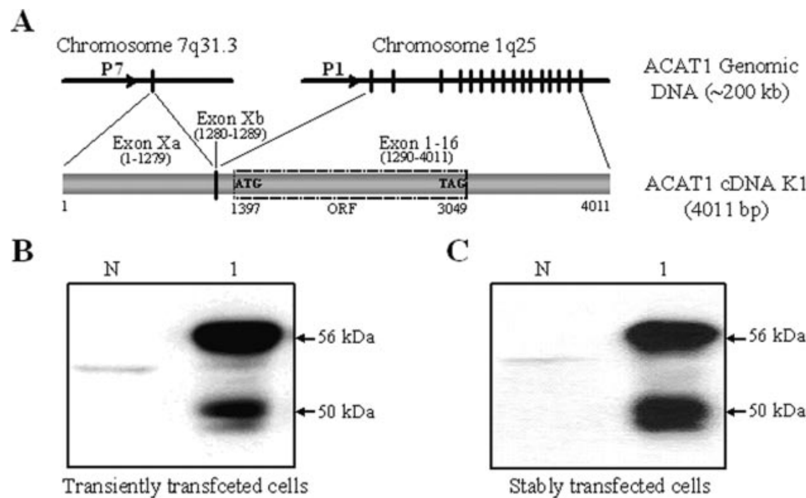

FIG. 1. **The full-length human ACAT1 cDNA K1 produces two ACAT1 proteins with different molecular mass (50 and 56 kDa) in transfected AC29 cells.** *A*, schematic diagram illustrating the relationship between human ACAT1 cDNA K1 and ACAT1 genomic DNAs. The predicted open reading panel (ORF, nt 1397–3049) of ACAT1 cDNA K1 (4011 bp) is *boxed in* with a *dotted line*. The *individual bars* indicate the exons of ACAT1 genomic DNA. The locations of the P7 and P1 promoters are indicated by the *two arrowheads*. *B* and *C*, Western analyses of ACAT1 proteins present in the transiently (*B*, *lane 1*) or stably (*C*, *lane 1*) transfected AC29 cells, using the expression plasmid (1, pcDNA3-K1) that contains the full-length ACAT1 cDNA K1 shown in *A*. Extracts of cells transfected with the expression vector pcDNA3 only were used as a negative control (*lane N*). The methods for transfection and for Western analysis are described under "Experimental Procedures." The transfection and Western blot experiments were repeated three times with similar results.

Nikon, Tokyo, Japan; with a Bio-Rad motorized focus unit). Rhodamine signals were viewed with the red laser beam; fluorescein signals were viewed with the green laser beam.

Immunoelectron Microscopy—Immunoelectron microscopy was performed according to a method described previously (12). Briefly, various transfected AC29 cells were fixed with 4% paraformaldehyde in PBS, pH 7.2, for 30 min at 4 °C. After washing with PBS, the fixed cells were treated with low concentration of saponin and then stained by using the immunoperoxidase method, using DM10 (at 0.5 μg/ml) as the primary antibodies. After visualization with diaminobenzidine for 10 s, the cells were quickly washed and post-fixed with 1% osmium tetroxide at 4 °C for 30 min. After thorough rinsing, cells were dehydrated with a graded series of ethanol and embedded. Thin sections were prepared by using an ultramicrotome and viewed under an electron microscope (Siemens, Germany).

Limited Proteolysis of Recombinant and Endogenous 56-kDa ACAT1—Procedures described previously (25), with minor modifications as described in the Fig. 11 legend, were used to perform limited proteolysis of the 56-kDa ACAT1 protein by V8 protease.

Other Methods—Standard molecular biology techniques were performed according to the methods described by Sambrook *et al.* (26).

RESULTS

Two Human ACAT1 Proteins of Different Molecular Mass Can Be Produced from the Full-length ACAT1 cDNA K1—In our previous work, we had only examined the translation product of the 1.7-kb ACAT1 cDNA, which is composed of exons 1–16. The full-length (4011 bp) human ACAT1 cDNA K1 is composed of the optional exons Xa, Xb, and 1–16. To examine its translation product(s), we constructed the expression plasmid (1, pcDNA3-K1), which contains the full-length ACAT1 cDNA K1, and transfected it into AC29 cells. After transfection, we performed Western analysis using the specific anti-ACAT1 antibody DM10 that recognizes antigenic sites present within the first 131 aa of the $ACAT1_{ORF}$. The results (Fig. 1, *B* and *C*) indicate that in both transiently and stably transfected AC29 cells, a 56-kDa ACAT1 protein band can be detected (Fig. 1, *B* and *C*, *lane 1*), in addition to the 50-kDa band that was produced when the 1.7-kb ACAT1 cDNA was expressed by transfection (10, 27). The control experiment showed that when AC29 cells were transfected with the expression vector pcDNA3 only, no ACAT1-specific signal(s) was detected (Fig. 1, *B* and *C*, *lane N*).

Exonal Sequences of Human acat1 Located at Chromosomes 7 and 1 Are Required to Produce the 56-kDa ACAT1 Protein—To identify the sequences that are essential for producing the 56-kDa protein, we created various expression plasmids that contain different 5′ deletions of the full-length ACAT1 cDNA K1, depicted in Fig. 2*A*, *numbers 2–5*. These plasmids were individually introduced into AC29 cells by transfection. Afterward, extracts were prepared for Western analysis with DM10. The results show that plasmids 1–3 produced both the 50- and the 56-kDa ACAT1 proteins (Fig. 2*B*, *lanes 1-3*), whereas plasmids 4 and 5 produced only the 50-kDa ACAT1 protein (Fig. 2*B*, *lanes 4* and *5*).

The apparent molecular mass of ACAT1 expressed by the 1.7-kb ACAT1 cDNA on SDS-PAGE is 50 kDa, which deviates significantly from the estimated molecular mass (550 aa, 63.8 kDa) deduced from the predicated ORF of ACAT1 cDNA K1. The 50-kDa ACAT1 protein is a hydrophobic and basic protein with an isoelectric point of 9.78. These features may cause the ACAT1 protein to bind more SDS and to migrate faster on SDS-PAGE than a typical protein would. To avoid the uncertainty in molecular mass determination by using the SDS-PAGE method, we produced various expression plasmids that contain the same 5′ deletions as those plasmids described in Fig. 2*A*, but truncated at nt 1786. The resultant expression plasmids, designated as ACAT1-NTP (NTP stands for N-terminal peptide), and diagramed in Fig. 3*A*, all contain the partial $ACAT1_{ORF}$ encoding the first 130 aa of ACAT1 at their C termini. Thus, the translational products should all be recognized by the antibody DM10. These truncated expression plasmids were individually transfected into AC29 cells. The expressed proteins were analyzed by Western analysis with DM10. The results show that plasmids 1–3 produced both a 25-kDa ACAT1 fragment and a 17-kDa ACAT1 fragment (Fig. 3*B*, *lanes 1-3*), whereas plasmids 4 and 5 produced only the 17-kDa ACAT1 fragment (Fig. 3*B*, *lanes 4* and *5*). The difference (8 kDa) in size seen between the 25-kDa fragment and the 17-kDa fragment is similar to the difference (6 kDa) in size seen between the 56-kDa ACAT1 and the 50-kDa ACAT1. Plasmids 2 and 3 described in Fig. 2 and plasmids 7 and 8 in

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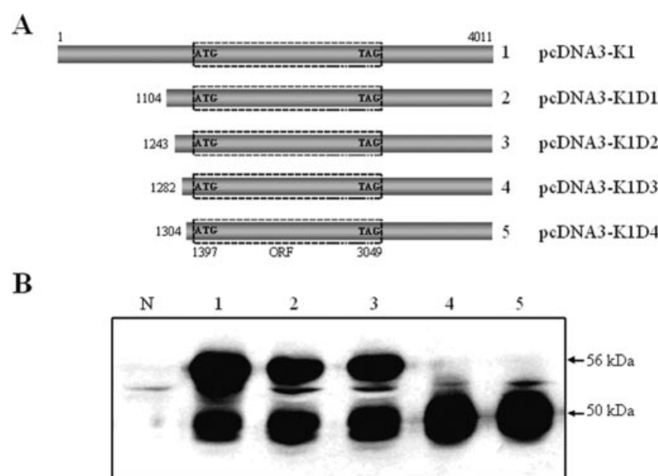

FIG. 2. **The ACAT1 cDNA K1 sequences located in two separate chromosomes are required to produce the 56-kDa ACAT1 protein.** *A*, schematic diagram illustrating the positions of the 5′-end nucleotide of the full-length ACAT1 cDNA and the various partial ACAT1 cDNA K1s present in the expression plasmids 1–5. The predicted ORF (nt 1397–3049) of human ACAT1 cDNA K1 is *boxed in* with a *dotted line*. *Plasmid 1*, full-length cDNA K1. *Plasmids 2* and *3*, partial cDNA K1s containing nt 1104 or 1243–1279; these nucleotides are part of exon Xa located in chromosome 7. *Plasmids 4* and *5*, partial cDNA K1s that do not contain any exon Xa sequence. *B*, Western analysis of human ACAT1s present in AC29 cells (*lanes 1–5*), after the cells were transiently transfected with one of the expression plasmids 1 to 5 (*1*, pcDNA3-K1; *2*, pcDNA3-K1D1; *3*, pcDNA3-K1D2; *4*, pcDNA3-K1D3; *5*, pcDNA3-K1D4) as indicated in *A*, or with the expression vector pcDNA3 only (*lane N*; to serve as a negative control). The transfection and Western blot experiments were repeated three times with similar results.

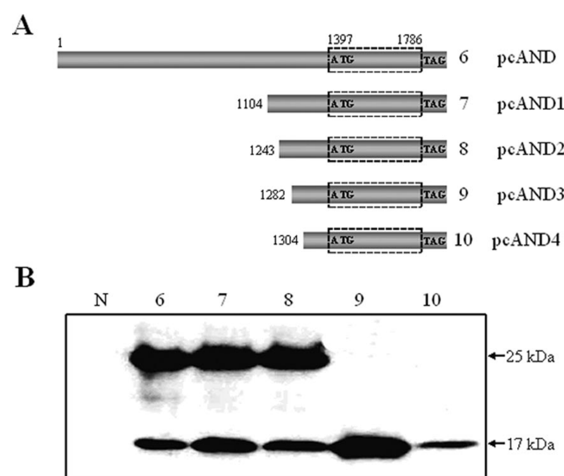

FIG. 3. **The expression patterns of various ACAT1 plasmids truncated at nt 1786.** *A*, schematic diagram illustrating the positions of the 5′-end nucleotide of various ACAT1 cDNA fragments present in expression plasmids 6–10. The partial $ACAT1_{ORF}$ (nt 1397–1786) of human ACAT1 cDNA K1 is *boxed in* with a *dotted line*. The stop codon TAG as indicated in each plasmid is from the expression vector. These fragments are all truncated at nt 1786. *Plasmid 6*, fragment containing nt 1–1279 present in exon Xa. *Plasmids 7* and *8*, fragments containing nt 1104 or 1243–1279 present in *E*xon Xa. *Plasmids 9* and *10*, fragments that do not contain any exon Xa sequence. *B*, Western analysis of various human ACAT1 N-terminal fragments (ACAT-NTPs) present in AC29 cells (*lanes 6–10*), after the cells were transiently transfected with one of the expression plasmids 6 to 10 (*6*, pcAND; *2*, pcAND1; *3*, pcAND2; *4*, pcAND3; *5*, pcAND4) as indicated in *A*, or with the expression vector pcDNA3 only (*lane N*; to serve as a negative control). The transfection and Western blot experiments were repeated three times with similar results.

Fig. 3 contain the 3′ sequences (a total of 176 or 37 nt) of exon Xa upstream of the exon 1 sequences. In contrast, plasmids 4 and 5 described in Fig. 2 and plasmids 9 and 10 in Fig. 3 contain only the exon 1 sequences and the additional downstream exonal sequences. It is known that all of the exon Xa sequence is located in chromosome 7. Thus, the results shown in Figs. 2 and 3 indicate that the 3′ region of the ACAT1 exon Xa sequence located at chromosome 7, the exon Xb sequence, as well as the $ACAT1_{ORF}$ sequence located in chromosome 1, are all required to produce the 56-kDa ACAT1 protein.

The 56-kDa ACAT1 Protein Can Be Recognized by Antibodies Prepared against the Additional Amino Acid Sequence Upstream of the N-terminal of the $ACAT1_{ORF}$—The results described above imply that alternative upstream initiation codon(s), located within the 5′-untranslated region (5′-UTR, nt 1–1396), may be employed to produce the 56-kDa ACAT1 protein, with an additional amino acid sequence extended from the N-terminal of the 50-kDa $ACAT1_{ORF}$. The size of these additional amino acids is between 6 and 8 kDa. Sequence analysis of the full-length ACAT1 cDNA K1 shows that a total of 147 additional nucleotides are located between the initiation codon $AUG_{1397–1399}$ for $ACAT1_{ORF}$ and the in-frame upstream stop codons (UAA, located at nucleotide positions 1247–1249). Starting from the 3′-end, the first 107 of these nucleotides are located in exon 1, the next 10 nucleotides are located in exon Xb, and the last 30 nucleotides are located in exon Xa. Some or all of these 147 nucleotides may serve as an additional coding sequence, to encode up to 49 additional amino acids. Analysis shows that the sequence of the first predicted 40 amino acids is reasonably antigenic. We thus created a GST-$ACAT1_{exonX}$ fusion protein plasmid containing the first predicted 40 aa, extending from the N-terminal of $ACAT1_{ORF}$ (diagrammed in Fig. 4*A*), fused in-frame at the C-terminal of the bacterial protein GST. This plasmid was used to express the fusion protein in *E. coli*. The expressed protein was gel-purified and used to produce antibodies in rabbits. The resultant antisera (designated as DM58) were affinity-purified by using the fusion protein (described under "Experimental Procedures"). We next expressed the 56-kDa ACAT1 and the 50-kDa ACAT1 in AC29 cells by performing transfection studies, using the ACAT1 plasmids 1 and 5 described in Fig. 2. Western analysis of transfected cell extracts showed that only the 56-kDa ACAT1 protein could be detected with antibody DM58 (Fig. 4*B*; *left panel*), whereas both the 50- and 56-kDa ACAT1 proteins could be detected with the antibody DM10 (Fig. 4*B*, *right panel*). In addition, we also performed transfection studies using the ACAT1-NTP plasmids 6 and 10 described in Fig. 3. As shown in Fig. 4*C*, only the larger ACAT1-NTP (25 kDa) could be detected with antibody DM58, whereas both the 25- and 17-kDa ACAT1-NTP could be detected with the antibody DM10. These results indicate that the 56-kDa ACAT1 protein contains an additional N-terminal amino acid sequence upstream of the N-terminal of the 50-kDa ACAT1 that can be detected with the antibody DM58.

The Translation Initiation Site for the 56-kDa ACAT1 Protein—To determine the translation initiation site for the 56-kDa ACAT1 protein, we performed site-specific mutagenesis experiments, using either the full-length ACAT1 construct K1D2 (Fig. 2) or the ACAT1-NTP construct D2 (Fig. 3) as the template and introduced a series of stop codons (TAG) in the nucleotides region between 1268 and 1396 (131 nucleotides) that is upstream to the initiation codon $AUG_{1397–1399}$ of $ACAT1_{ORF}$. The TAG mutations are indicated in Fig. 5*A* as *black triangles*. These constructs were individually transfected into AC29 cells, and the expressed products were analyzed by Western blotting. The results show that, when K1D2 was used as the template, m41 or m12 did not abolish the expression of the 56-kDa protein, whereas m6, m2, m37, or m38 did (Fig. 5*B*;

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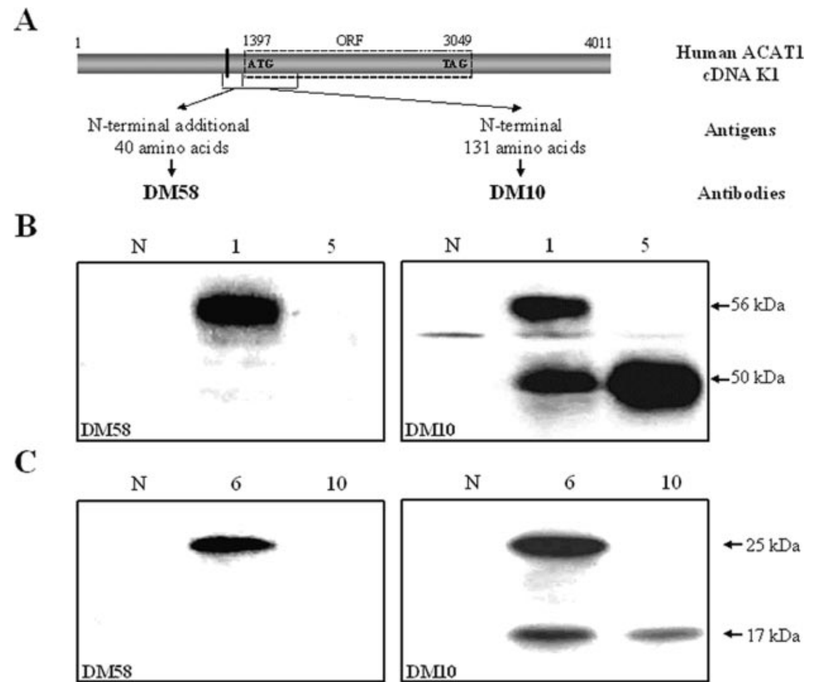

FIG. 4. **The specificity of the anti-ACAT1 antibody DM58 prepared against the 40-amino acid peptide upstream to the N-terminal of $ACAT1_{ORF}$.** *A*, diagram illustrating the locations of the ACAT1 peptides used as antigens to generate specific anti-ACAT1 polyclonal antibodies DM10 and DM58. *B* and *C*, Western analysis of translation products in AC29 cells. The cells were transfected with ACAT1 expression plasmids number 1 (pcDNA3-K1) or number 5 (pcDNA3-K1D4) diagrammed in Fig. 2, or with ACAT1-NTP expression plasmids number 6 (pcAND) or number 10 (pcAND4) diagrammed in Fig. 3, respectively. The control (*lane N*) was used with AC29 cells transfected with the expression vector pcDNA3 only. The expressed human ACAT1 proteins (*lanes 1* and *5* in *B*) or ACAT1-NTPs (*lanes 6* and *10* in *C*) were detected by antibody DM58 (*left panels*, final concentration: 2.5 μg/ml) or by antibody DM10 (*right panels*, final concentration: 0.5 μg/ml) as indicated. The DM58 antibody was raised and affinity-purified as under "Experimental Procedures." The transfection and Western blot experiments were repeated three times with similar results.

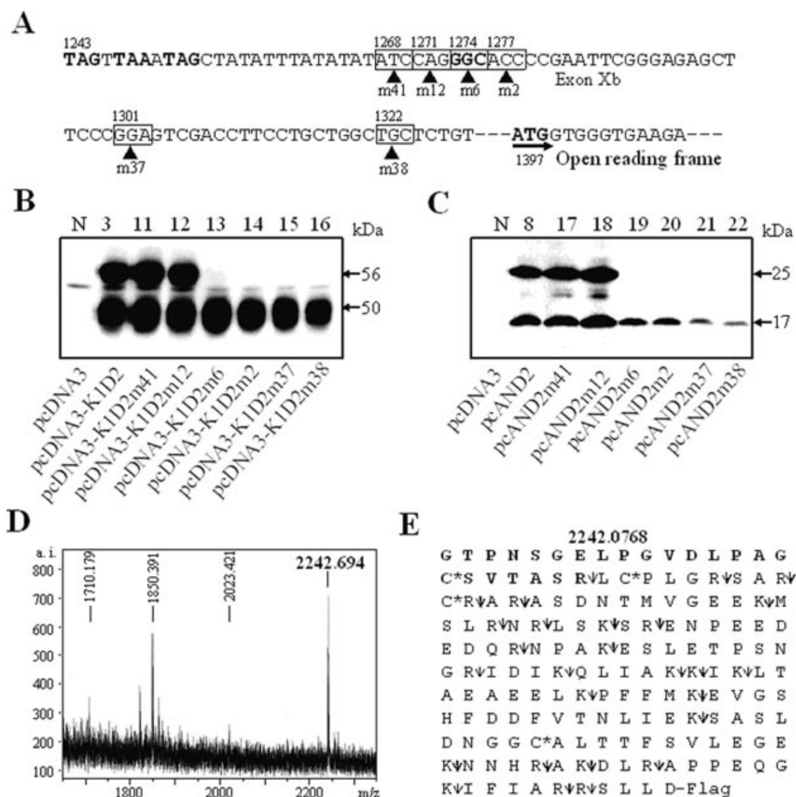

FIG. 5. **The $GGC_{1274-1276}$ located in the exon Xa is the translation initiation codon for the 56-kDa human ACAT1 protein.** *A*, schematic diagram to demonstrate the successively engineered codon mutations located upstream of the $ATG_{1397-1399}$ codon of cDNA K1. Individual mutations, replacing a given codon (*boxed*) to the stop codon TAG, were indicated as *black triangles*. *B*, analysis of the gene products of wild-type and various mutant K1D2 plasmids as indicated, expressed in AC29 cells, by Western blotting using the antibody DM10. *C*, analysis of the gene products of wild-type and various mutant D2 plasmids as indicated in AC29 cells, by Western blotting using the antibody DM10. The results shown are representative of three separate experiments. *D*, MALDI-TOF MS analysis of ACAT1-NTP-FLAG. The purified ACAT1-NTP-FLAG protein, described under "Experimental Procedures," underwent in-gel digestion with trypsin. The peptide mixture was desalted, treated with iodoacetamide, and then analyzed by MALDI-TOF MS under "Experimental Procedures." Mass spectra were recorded in the positive mode. *E*, theoretical analysis of the ACAT1-NTP-FLAG peptides after the trypsin and iodoacetamide treatments. The theoretical N-terminal peptide is GTPNSGELPGVDLPAGC*SVTASR (*underlined*), and its molecular weight is 2242.0768. *Asterisks* indicate modifications by iodoacetamide on cysteines. *Arrows* indicate trypsin cleavage sites.

left panel). Likewise, when D2 was used as the template, m41 or m12 did not abolish the expression of the 25-kDa protein, whereas m6, m2, m37, or m38 did (Fig. 5*B*, *right panel*). These results imply that the $GGC_{1274-1277}$ codon may be the translation initiation codon for the 56-kDa human ACAT1 protein. GGC codes for glycine, thus, the 56-kDa ACAT1 uses an unusual non-AUG codon for translation initiation. To test the validity of this finding by using a different approach, we purified the fusion protein ACAT1-NTP-FLAG expressed in transfected AC29 cells by using ANTI-FLAG® M2 affinity gel chromatography, followed by SDS-PAGE. The fusion protein, migrated as a single 25-kDa band, was in-gel-digested with trypsin, then treated with iodoacetamide. The peptide mixture was analyzed by MALDI-TOF mass spectrometry. The result showed (Fig. 5*D*) that the molecular weight of the largest tryptic fragment of ACAT1-NTP-FLAG is 2242.694 (*bold number*, Fig. 5*D*). This value is consistent with the molecular weight (2242.0768) of the predicted tryptic peptide sequence GTPNSGELPGVDLPAGC*SVTASR (*underlined* in Fig. 5*E*; the *asterisk* represents Cys modified by iodoacetamide) from

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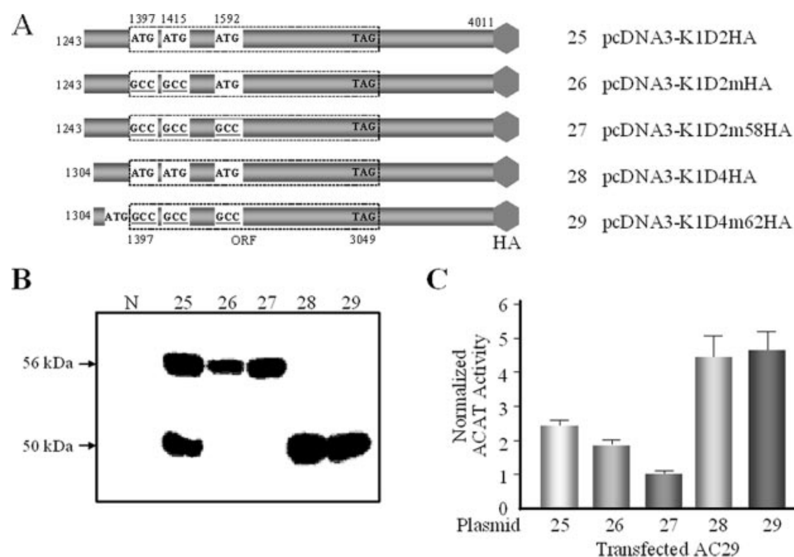

FIG. 6. **Enzymatic activities of human ACAT1s.** *A*, schematic diagram of various expression plasmids 25–29 (*25*, pcDNA3-K1D2HA; *26*, pcDNA3-K1D2mHA; *27*, pcDNA3-K1D2m58HA; *28*, pcDNA3-K1D4HA; and *29*, pcDNA3-K1D4m62HA) used in transfections to determine ACAT enzyme activities. The predicated ORF of ACAT1 cDNA K1 is *boxed in* with a *dotted line*. *B*, Western analysis with anti-HA antibody of cell extracts after cells were transiently transfected with one of the expression plasmids 25–29 as indicated in *lanes 25-29*, or with pcDNA3 only to serve as control as indicated in *lane N*. *C*, normalized ACAT enzyme activities present in cells expressing the 56- and 50-kDa protein (*lane 25*), or expressing the 56-kDa protein only (*lanes 26* and *27*), or expressing the 50-kDa protein only (*lanes 28* and *29*). ACAT activity determination was described under "Experimental Procedures." The data represent one of three separate experiments with similar results.

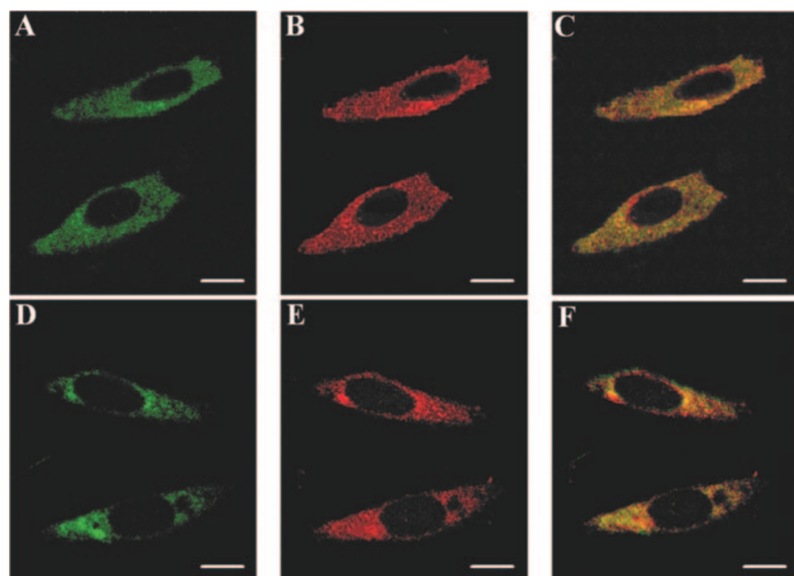

FIG. 7. **Colocalization of the 56-kDa ACAT1 or the 50-kDa ACAT1 with the ER marker GRP78, as revealed by double immunofluorescence stainings of transfected AC29 cells.** *A*, the signals for the 56-kDa ACAT1 protein (viewed in *green*). The 56-kDa protein is expressed in AC29 cells by transfecting cells with the expression plasmid 23 (pcDNA3-K1D2m) that bears the first two ATG to GCC mutations of $ACAT1_{ORF}$. It does not contain the HA tag at its C terminus as plasmid 26 KID2mHA (which is shown in Fig. 6). *B*, the signals for GRP78 (viewed in *red*). *C*, the *overlay* of *panels A* and *B*, suggesting extensive colocalization of the *green* and *red signals*. *D*, the signals for the 50-kDa ACAT1 protein (viewed in *green*). The 50-kDa protein is expressed in AC29 cells by transfecting cells with the expression plasmid 5 K1D4 described in Fig. 2*A*. *E*, the signals for GRP78 (viewed in *red*). *F*, the *overlay* of *panels D* and *E*, suggesting extensive colocalization of the *green* and *red signals*. The methods for performing double immunofluorescence are described under "Experimental Procedures." *Scale bars*, 10 μm.

ACAT1-NTP-FLAG. In addition, peptides with molecular weights of 1710.179, 1850.391, and 2023.421 were also obtained (Fig. 5*D*). These values are consistent with the molecular weights of predicted smaller tryptic peptides (1710.9649, 1849.8966, and 2023.1298) from ACAT1-NTP-FLAG. Together, these results show that the first amino acid of the 56-kDa human ACAT1 protein is glycine, encoded by the non-ATG codon GGC.

The 56-kDa ACAT1 Protein Alone Is Enzymatically Active—At this point, all the ACAT1 constructs described either produce both the 56-kDa protein and the 50-kDa protein, or produce only the 50-kDa protein. To determine whether the 56-kDa protein alone is enzymatically active, one must create one or more constructs that produce only the 56-kDa protein. In $ACAT1_{ORF}$, two ATG codons exist near its N-terminal, encoding the Met residues at positions 1, and 7, respectively. We had earlier showed that each of these two Mets can serve as an initiation codon to produce the 50-kDa protein (28). To eliminate these two ATGs from serving as initiation codon(s), we used the plasmid K1D2 (described in Fig. 2) as the template, performed site-specific mutagenesis experiments, and produced three additional constructs in pcDNA3 (plasmids 25–27; Fig. 6). For reliable protein detection by Western analysis, we engineered the additional nucleotide sequences for the HA tag at the C termini of these constructs. We have previously shown that the HA tag inserted at the C terminus of ACAT1 protein has little effect on its enzyme activity (6). Plasmid 25 serves as the unmutated control. Plasmid 26 lacks the first 2 ATG codons, whereas plasmid 27 lacks the first three ATG codons in $ACAT1_{ORF}$; the relevant ATGs were altered to GCCs, encoding Ala. Plasmids 28 and 29 (Fig. 6) were constructed such that they only produce the 50-kDa ACAT1 protein. We then individually transfected these plasmids into AC29 cells for expression. Afterward, the sizes and the levels of the expressed ACAT1 proteins were determined by Western blotting using the HA antibody, and the ACAT enzyme activity was determined. The Western analyses (Fig. 6*B*) show that, as expected, plasmid 25 produces both the 56- and 50-kDa proteins, whereas plasmids 26 and 27 produce only the 56-kDa protein. Plasmids 28 and 29 produce only the 50-kDa protein. The ACAT activity analysis (Fig. 5*C*) shows that the 56-kDa protein alone (*lanes 26* and *27*) is enzymatically active. Its normalized activity is ~30% of the activity of the 50-kDa ACAT1 protein.

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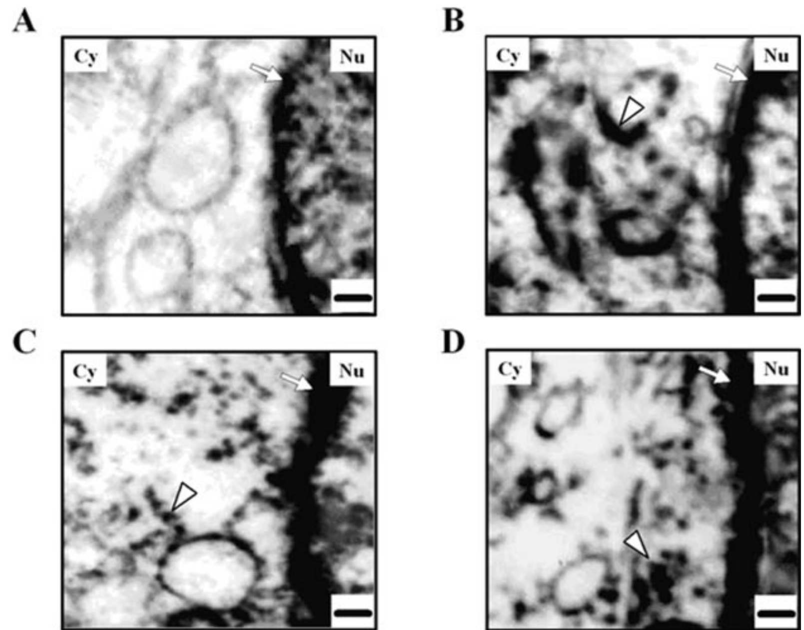

FIG. 8. **Localizations of the 56-kDa ACAT1 and the 50-kDa ACAT1 in transfected cells as studied by immunoelectron microscopy.** Anti-ACAT1 antibody DM10 were used. AC29 cells were transfected with one of the expression plasmids (3, 23, or 5) as indicated, or with the expression vector pcDNA 3 to serve as negative control. *A*, pcDNA3 only; *B*, plasmid 3; *C*, plasmid 23; and *D*, plasmid 5. *Open arrowheads*, tubular ER region; *open arrows*, nuclear membrane; *Cy*, cytosol; *Nu*, nucleus. Size of *scale bars* is 0.1 μm. The details for immunoelectron microscopy were described under "Experimental Procedures."

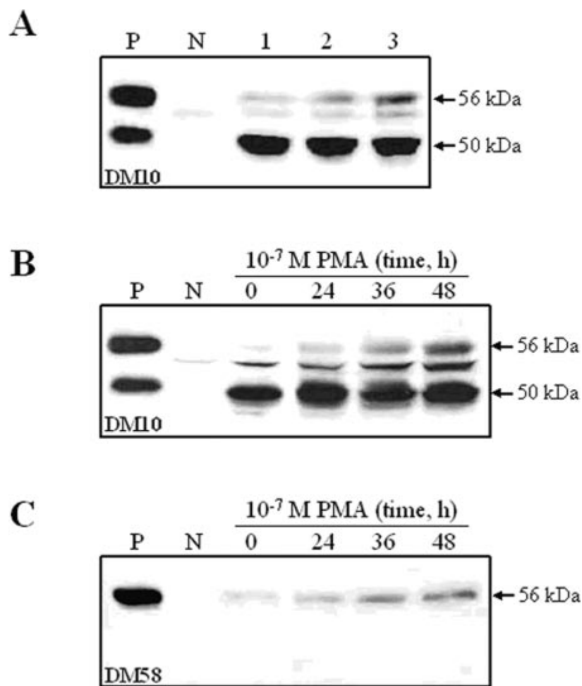

FIG. 9. **Endogenous expression of the 56-kDa ACAT1 in THP-1 macrophages.** *A*, THP-1 cells treated with PMA for 48 h were lysed in 10% SDS at 37 °C for 1 h (*lane 1*), 2 h (*lane 2*), or 3 h (*lane 3*), respectively. The cell lysates were freshly prepared for Western analysis. The blot was developed with anti-ACAT1 antibody DM10 at 0.5 μg/ml. *B* and *C*, staggered THP-1 cells were treated with PMA for 0, 24, 36, or 48 h as indicated, and then lysed in 10% SDS at 37 °C for 3 h. Two parallel gels were run; one developed with anti-ACAT1 antibody DM10 at 0.5 μg/ml (*panel B*), and the other with antibody DM58 at 2.5 μg/ml (*panel C*). The positive control (*P*) was prepared by using extracts of AC29 cells transfected with the expression plasmid 3 described in Fig. 2*A*. The negative control (*N*) was prepared by using extracts of AC29 cells transfected with pcDNA3 only. The experiments were repeated three times with similar results.

The 56-kDa hACAT1 Is Also Localized in the ER—We had previously shown that the 50-kDa ACAT1 protein is mainly localized in the endoplasmic reticulum (10, 12). To compare the subcellular localization of the 56- and the 50-kDa ACAT1s, we performed transient transfections in AC29 cells, using either plasmid K1D2m, which only expresses the 56-kDa ACAT1, or plasmid K1D4, which only expresses the 50-kDa ACAT1. We then performed double immunofluorescence experiments in fixed intact cells, using antibody DM10 to visualize the ACAT1 protein (in *green*), and the anti-GRP78 to visualize the resident endoplasmic reticulum marker GRP78 (in *red*). The staining patterns were examined under laser scanning confocal microscopy. The 56-kDa ACAT1 signal is shown in Fig. 7*A*, the 50-kDa ACAT1 signal is shown in Fig. 7*D*, and the GRP78 signals are shown in Figs. 7*B* and 7*E*. Merging the signals in *panels A* and *B* gives the signals in *panel C*. Merging the signals in *panels D* and *E* gives the signals in *panel F*. Overlap between the *green signal* and the *red signal* creates the *yellow signal*. The results show that both the 50-kDa protein and the 56-kDa protein extensively overlap with the GRP78 signal. Thus, similar to the 50-kDa ACAT1, the 56-kDa ACAT1 protein is also mainly localized in the ER. We had previously showed by immunoelectron microscopy that, in human macrophages, the 50-kDa ACAT1 is mainly located in the rough ER (12). In our current work, we used the same antibody DM10 and performed immunoelectron microscopy to study the subcellular localization of ACAT1 in transfected AC29 cells. The results are shown in Fig. 8. *Panel B* represents cells expressing both the 50- and 56-kDa ACAT1s, *panel C* represents cells expressing the 56-kDa ACAT1 only, and *panel D* represents cells expressing the 50-kDa ACAT1 protein only. The negative control (*panel A*) shows that no immunoreactivity occurred in pcDNA3-transfected AC29 cells. The results show that both the 50- and 56-kDa ACAT1 are mainly distributed in the tubular ER regions near the nuclei.

The 56-kDa ACAT1 Protein Is Present in Human THP-1 Macrophages and in Human Monocyte-derived Macrophages—Using antibodies DM10 and DM58, we performed Western analyses on cell extracts prepared from a variety of human cells, including hepatocytes, fibroblasts, A293 cells, HepG2 cells, HeLa cells, and CaCo2 cells, and looked for the presence of the 50- and 56-kDa ACAT1 protein. The results show that although the presence of the 50-kDa ACAT1 is always clearly detectable in all the cell types examined, the presence of the 56-kDa protein is either absent or is barely detectable (data not shown). One exception has been made: the 56-kDa protein can be clearly demonstrated in cell extracts prepared from the phorbol ester-activated THP-1 macrophages, as shown in Fig.

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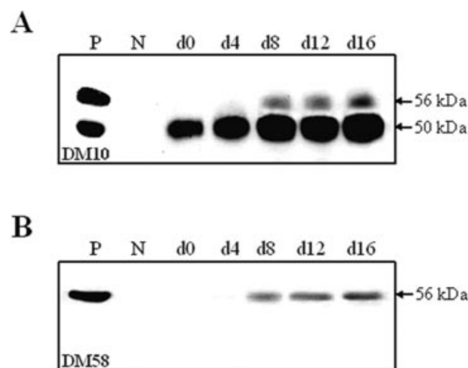

FIG. 10. **Expression of ACAT1 proteins in human monocyte-derived macrophages.** Human monocytes were cultured at 6 million/100-mm dish for various days as indicated (*d0*, *d4*, *d8*, *d12*, and *d16*); cell lysates were freshly prepared (by incubating in 10% SDS at 37 °C for 3 h) for Western analysis. Western blots were developed with anti-ACAT1 antibody DM10 at 0.5 μg/ml (*A*) or with antibody DM58 at 2.5 μg/ml (*B*). The experiments were repeated three times with similar results.

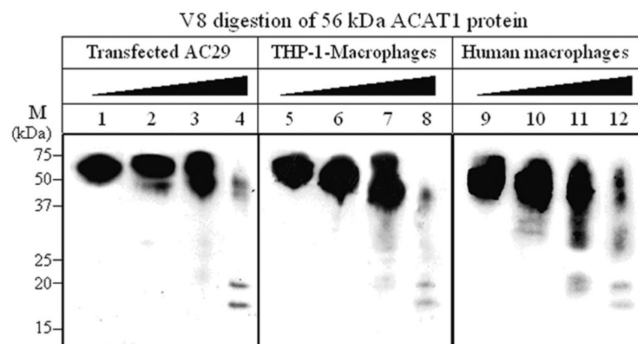

FIG. 11. **Limited proteolysis analysis of the 56-kDa ACAT1 protein.** Whole cell extracts of AC29 cells transfected to express the 56-kDa ACAT1, or THP-1 macrophages, or human blood monocyte-derived macrophages (*d12*) as indicated were solubilized by 10% SDS, with final protein concentration at ~3 μg/μl. 100 μg of protein lysates from transfected AC29 cells, 600 μg of protein lysates from THP-1 macrophages, and 600 μg of protein lysates from human monocyte-derived macrophages were loaded per lane and analyzed by 12% SDS-PAGE. After electrophoresis, the gels near the 56-kDa region were cut. Slices of gel cubes were loaded into the stacking wells of a 15% polyacrylamide gel. 20 μl of 20% glycerol mix, followed by 10 μl of 10% glycerol mix containing 0.005 (*lanes 1*, *5*, and *9*), 0.05 (*lanes 2*, *6*, and *10*), 0.5 (*lanes 3*, *7*, and *11*), or 5 (*lanes 4*, *8*, and *12*) mg of V8 protease was overlaid on the gel slices. The samples were in-gel-digested for 2 h, separated by electrophoresis, and transferred to membranes. The anti-ACAT-1 antibody DM10 (with final concentration of 0.5 μg/ml) was used to analyze the partial proteolytic patterns. The experiments were repeated two times with similar results.

9*A*. Curiously, as shown in *lanes 1–3* of Fig. 9*A*, we find that the intensity of the 56-kDa ACAT1 increases when the cell extracts are incubated in 10% SDS at 37 °C from 1 to 3 h. It is possible that the 56-kDa ACAT1 protein may form insoluble aggregate(s) with other cellular material *in vitro* and may require longer incubation in SDS for its solubilization. THP-1 cell is a monocytic cell line. Upon treating with the phorbol esters (PMA), these cells differentiate and become macrophage-like cells within 1 or 2 days. We monitored the expression of the 50- and 56-kDa ACAT1 in THP-1 cells for various time points up to 48 h, after the cells were exposed to PMA. The results (Fig. 9, *B* and *C*) show that the intensities of both the 50- and 56 kDa ACAT protein steadily increase with time after PMA treatment. The intermediate band detected in Fig. 9 (*A* and *B*) with DM10 is most likely a nonspecific signal recognized by the DM10 antibodies. This signal can be observed occasionally. (For example, this signal can be seen in immunoblots using the AC29 cell extracts, shown in Fig. 2). In results not shown, immunofluorescence experiments using the antibody DM58 show the endogenous 56-kDa ACAT1 protein present in THP-1 macrophages is mainly localized in the ER. We next found that the 56-kDa ACAT1 is also present in human monocyte-derived macrophages; the amount of the 56-kDa ACAT becomes clearly detectable after the blood-borne monocytic cells are differentiated into mature macrophages (by incubating cells in culture for 8 days or longer) (Fig. 10, *A* and *B*). The presence of the 56-kDa ACAT1 in human macrophages is demonstrated by Western blotting. It is possible that the 56-kDa protein band detected by these methods is not ACAT1, but a protein that contains the same antigenic sites as the 50-kDa ACAT1 and the 56-kDa ACAT. To rule out this possibility, we performed SDS-PAGE, using extracts prepared from transfected cells that express the recombinant 56-kDa ACAT1 protein, or from THP-1 macrophages, or from the human monocyte-derived macrophages, and isolated the 56-kDa protein bands. We then carried out limited proteolysis of these protein bands using V8 protease, and compared their partial digestion patterns by SDS-PAGE followed by Western blotting using anti-ACAT1 antibody DM10. Many investigators have used this method to probe the structural differences between closely related proteins (for a few examples, see Refs. 29–30). The results showed that partial proteolytic patterns of these samples prepared from three different sources were indistinguishable (Fig. 11). Thus, the 56-kDa protein recognized by the anti-ACAT1 antibodies in THP-1 macrophages and in human monocyte-derived macrophages (right) is the same as the 56-kDa ACAT1 protein expressed in AC29 cells.

DISCUSSION

We had previously reported that one of the four ACAT1 mRNAs, the 4.3-knt mRNA, contains an optional long 5′-UTR, and is probably produced by interchromosomal *trans*-splicing of two discontinuous pre-mRNAs (15). The function of the chimeric mRNA remained unknown. Our current studies show that *in vitro*, the 4.3-knt chimeric ACAT1 mRNA can be translated to produce a novel ACAT1 isoform, in addition to the normal ACAT1 ($ACAT1_{ORF}$). This isoform has an apparent molecular mass of 56 kDa on SDS-PAGE and is ~6 kDa larger than the molecular mass of the normal ACAT1 ($ACAT1_{ORF}$), which is translated from the ACAT1 mRNAs that do not contain the optional long 5′-UTR. The 56-kDa ACAT1 contains additional amino acids that extend from the N-terminal of the $ACAT1_{ORF}$. To produce the 56-kDa ACAT1, the 3′ region of exon Xa, the Xb sequence, and exons 1–16 are all required. Unlike the ACAT1 exons 1–16, which are located in chromosome 1, the ACAT1 Xa sequence is located in chromosome 7. The glycine codon GGC located in exon Xa is shown to be the initiation codon for translating the 56-kDa ACAT1. Taking these results together, we conclude that the functional significance of the chimeric ACAT1 mRNA is to increase the ACAT enzyme diversity by producing a novel ACAT1 isozyme. The location of exon Xb (10 bp) is unknown at present. We speculate that exon Xb may be produced during the mRNA *trans*-splicing reaction. Other possibilities cannot be ruled out at present.

As reviewed by Maniatis and Tasic (17), five types of RNA *trans*-splicing events have been observed in the animal kingdom, including spliced leader addition *trans*-splicing, exon duplication, intergenic *trans*-splicing, intragenic *trans*-splicing, and interchromosomal *trans*-splicing. A predicted biological function of RNA *trans*-splicing is that it increases protein diversity. Recently, this predicted function was demonstrated experimentally in the model organism *Drosophila* (31). In mammalian systems, only a few studies observing RNA *trans*-

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splicing have been reported (reviewed in Ref. 17). A noted example was by Caudevilla *et al.* (32), who reported that, in rat liver cells, there exist carnitine octanoyltransferase mRNA variants with duplication of exons 2 and 3; these variants are produced by mRNA *trans*-splicing. However, the functional significance of these mRNA variants is not known. To our knowledge, our current result provides the first mammalian example that a functional protein, the 56-kDa ACAT1, can be produced from *trans*-spliced mRNA. To test the possibility that the *trans*-spliced ACAT1 mRNA might also exist in mammalian species other than human, we have performed various 5′-RACE experiments, designed to examine the 5′-UTR elements of the ACAT1 mRNAs present in mouse, rat, and rabbit. The results obtained thus far provide no evidence to support the possibility that *trans*-spliced ACAT1 mRNA may also exist in these species (results not shown). Thus, *trans*-spliced ACAT1 mRNA may only occur in primates or in humans only.

In various human tissues examined, ACAT1 mRNAs are present in relatively low abundance (5). The chimeric ACAT1 mRNA that produces both the 50- and the 56-kDa protein constitutes less than 20% of the total ACAT1 mRNAs. Other ACAT1 mRNAs (that do not contain the optional 5′-UTR) comprise the majority of the total ACAT1 mRNAs. The 50-kDa ACAT1 itself is a relatively sparse protein in most human cells and tissues examined (10, 11). The scarcity of the 56-kDa ACAT1 protein is probably the main reason why it has been difficult to demonstrate its presence in native human cells and tissues. We have produced the polyclonal antibody DM58 that specifically recognize the 56-kDa ACAT1 but not the 50-kDa ACAT1. Using antibodies DM58 and DM10 (which recognize both the 50-kDa ACAT1 and the 56-kDa ACAT1) as tools in parallel Western blots, thus far we are able to demonstrate the presence of the 56-kDa ACAT1 protein in PMA-activated THP-1 macrophages, and in human monocyte-derived macrophages; these cells express relatively abundant ACAT1 messages (33), (34). The 56-kDa ACAT1 may also be present in other human tissues and cells, and we are currently investigating this possibility in our laboratories.

The results of this study add to the growing list of ACAT isozymes that can be found in various human tissues (4, 14). The biological function of the 56-kDa ACAT1 is currently unknown. Our current results show that, when expressed alone, similar to the 50-kDa ACAT1, the 56-kDa ACAT1 is also located in the ER, and is also enzymatically active. Its activity is ~30% that of the 50-kDa ACAT1 protein. The 50-kDa ACAT1 protein forms homotetramers in intact cells and *in vitro* (27, 35). Thus, it is possible that, when present in the same cell, the 56-kDa ACAT1 and the 50-kDa ACAT1 may form hetero-oligomeric complex(es), with the 56-kDa protein serving as an endogenous inhibitor of the 50-kDa ACAT1. In addition, our current result suggests that only limited cell types/tissues express the 56-kDa protein. Thus, the mode(s) of regulation of the 56-kDa protein at the transcriptional and/or post-transcriptional levels may be very different from that of the 50-kDa ACAT1 protein. These are intriguing possibilities that require further investigations in the future.

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Human Acyl-Coenzyme A:Cholesterol Acyltransferase 1 (*acat1*) Sequences Located in Two Different Chromosomes (7 and 1) Are Required to Produce a Novel ACAT1 Isoenzyme with Additional Sequence at the N Terminus

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