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-splcing 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 isozyme. To our knowledge, our result provides the first mammalian example that a trans-splliced mRNA produces a functional protein. Acyl-coenzyme A:cholesterol acyltransferase (ACAT) 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

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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 ACAT1orf have been produced (10). When AC29 cells were transfected with the plasmid that encodes 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 against surfactant protein D (SP-D) (against the 78-kDa glucose-regulated protein GRP78, an endoplasmic reticulum marker), rhodamine-conjugated donkey anti-goat IgG, fluorescein-conjugated goat anti-ti-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 ProteinTM standards (prestained, all blue) were from Bio-Rad. The TaqDNA 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% CO2, 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 × 106 cells per 60-mm dish in 5 ml of 1640 medium containing 10% FBS were grown for 2 days in medium containing PMA (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 ACAT1orf have been produced (10). When AC29 cells were transfected with the plasmid that encodes 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.
pcDNA3-K1D2m58, respectively. The ACAT1 cDNA K1 fragment (nt 1304–1786) with the first three ORF-ATG codons deleted with GCC, plus an additional ATG codon inserted upstream to the first mutagen GCC TAG, was amplified by reverse PCR as described above, using the internal oligonucleotide set (M51F, 5'-CTCA-GACATACATATGGCGTGTGGAAGAGGA3'/M51R, 5'-CTCTTCT- TACACCCAGCAGTATGTTGGTCTAG3') and common LDM/HAAH oligonucleotides. After digestion with Xhol and XbaI, the PCR products were individually inserted into the Xhol and XbaI sites of pcDNA3 vector to obtain the expression plasmids pcDNA3-K1D2m6, 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 pcDNA3-K1D2m6 to generate pcDNA3-K1D4m6. 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-Alpha) at C terminus of human ACAT1 proteins were generated by PCR, using the forward oligonucleotide (5'-CAACATATATGTTG- GTTAGCATATAC-3') with NdeI sites (italics) and reverse oligonucleotide (5'-GAAGACCCTTGACAGGAGGTGAGATCTCATATG- GGTTAAGACGTTAAACGCAAAGACATGTC-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 XhoI, and ligated into pcDNA3-K1D2m6, pcDNA3-K1D2m58, pcDNA3-K1D4, and pcDNA3-K1D4m6 to generate the expression plasmids pcDNA3-K1D2HA, pcDNA3-K1D2m6HA, pcDNA3-K1D2m58HA, pcDNA3-K1D4HA, and pcDNA3-K1D4m6mHA, as shown in Fig. 6A. 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 oligonucleotides (primers underlined) coding for FLAG octapeptide. The amplified fragments were digested with Bsu36I and XbaI of pBSK-K1 and pcAND2-Flag. All plasmids were confirmed by restriction enzyme digestion confocal microscope at 0.36 optical section (Eclipse TE300, Nikon Instruments, Tokyo). The GST-ACAT1exonX plasmid was used to express the fusion protein. The GST-ACAT1exonX fusion protein was affinity-purified by using the GST-ACAT1exonX 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 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.

Double Immunofluorescence Staining—Using ACAT2 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 rhodamine-conjugated donkey anti-goat IgG diluted at 1:500 and fluorescein-conjugated goat anti-rabbit IgG diluted at 1:500 as the secondary antibodies. After carefully washing out the non-reacted secondary antibodies, the specimens were mounted using Dako fluorescent mounting medium (Dako, Carpinteria, CA) and viewed under a laser scanning confocal microscope at 0.36 μm/optical section (Eclipse TE300, Nikon Instruments, Tokyo).
The Chimeric ACAT1 mRNA Produces a Novel Isoenzyme

**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 an 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 protein. 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).

Exon 1–16 of the ACAT1 cDNA is composed of 1.7 kb and contains the full-length ACAT1 cDNA K1 (4011 bp) is predicted to encode a protein of 131 amino acids. 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.

**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.
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The translation initiation site for 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 AUG1397–1399 of human ACAT1 cDNA K1 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 ORF fusion protein plasmid containing the first predicted 40 aa, extending from the N-terminal of ACAT1 ORF (diagrammed in Fig. 4A), 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. 4B, left panel), whereas both the 50- and 56-kDa ACAT1 proteins could be detected with the antibody DM10 (Fig. 4B, 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. 4C, 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 AUG1297–1399 of ACAT1 ORF. The TAG mutations are indicated in Fig. 5A 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.
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. 5B, right panel). These results imply that the GGC1274–1277 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. The purification method described under “Experimental Procedures” was used. The transfection and Western blot experiments were repeated three times with similar results.

**FIG. 5**. The GGC1274–1277 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 ATG1397–1399 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 tryptic. 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 tryptic and iodoacetamide treatments. The theoretical N-terminal peptide is GTPNSGELPGVDPAGC*SVTASR (underlined), and its molecular weight is 2242.0768. Asterisks indicate modifications by iodoacetamide. Arrows indicate trypsin cleavage sites.

**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-KID4) 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.
ACAT1-NTP-FLAG. In addition, peptides with molecular weights of 1710.179, 1850.391, and 2023.421 were also obtained (Fig. 5D). These values are consistent with the molecular weights of predicted smaller tryptic peptides (1710.9649, 1850.391, and 2023.421) 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 56-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
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 56-kDa ACAT1 protein (viewed in green). The 50-kDa protein is expressed in AC29 cells by transfecting cells with the expression plasmid 5 KID4 described in Fig. 2A. 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.

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 56-kDa ACAT1 protein (viewed in green). The 50-kDa protein is expressed in AC29 cells by transfecting cells with the expression plasmid 5 KID4 described in Fig. 2A. 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.
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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. 7A, the 50-kDa ACAT1 signal is shown in Fig. 7D, and the GRP78 signals are shown in Figs. 7B and 7E. 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 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.
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/ml. 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) μg 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-ACAT1 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.

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 (ACAT1ORF). 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 (ACAT1ORF), 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 ACAT1ORF. 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-
splicing have been reported (reviewed in Ref. 17). A noted example was by Caudeliva 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 isoenzymes 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 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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REFERENCES

1. Chang, T. Y., Chang, C. C. Y., and Cheng, D. (1997) Annu. Rev. Biochem. 66, 613–638
2. Buhman, R. F., Arcada, M., and Farese, R. V., Jr. (2000) Biochim. Biophys. Acta 1529, 142–154
3. Rudel, L., Lee, R., and Cockman, T. (2001) Curr. Opin. Lipidol. 12, 121–127
4. Chang, T. Y., Chang, C. C. Y., Lin, S., Yu, C., Li, B. L., and Miyazaki, A. (2001) Curr. Opin. Lipidol. 12, 289–296
5. Chang, C. C. Y., Huh, H. Y., Cadigan, K. M., and Chang, T. Y. (1993) J. Biol. Chem. 268, 20747–20755
6. Lin, S., Cheng, D., Liu, M. S., Chen, J., and Chang, T. Y. (1999) J. Biol. Chem. 274, 23276–23285
7. Lin, S., Lu, X., Chang, C. C. Y., and Chang, T. Y. (2003) Mol. Biol. Cell 14, 2447–2459
8. Chang, T. Y., Chang, C. C. Y., Lu, X. H., and Lin, S. (2001) J. Lipid Res. 42, 1933–1938
9. Oelkers, Behari, A., Creelman, D., Billheimer, J. T., and Sturley, S. L. (1998) J. Biol. Chem. 273, 26765–26771
10. Chang, C. C. Y., Chen, J., Thomas, M. A., Cheng, D., Del Priore, V. A., Newton, R. S., Pape, M. E., and Chang, T. Y. (1995) J. Biol. Chem. 270, 28532–28540
11. Chang, C. C. Y., Sakashita, N., Ornvold, K., Lee, O., Chang, E., Dong, R., Lin, S., Lee, C. Y. G., Strum, S., Kashyap, R., Fung, J., Farese, R. V., Jr., Patouill, J. F., Delon, A., and Chang, T. Y. (2000) J. Biol. Chem. 275, 20853–20859
12. Sakashita, N., Miyazaki, A., Takeya, M., Horieuchi, S., Chang, C. C. Y., Chang, T. Y., and Takahashi, K. (2000) Am. J. Pathol. 156, 227–236
13. Song, B. L., Qi, W., Yang, X. Y., Chang, C. C. Y., Zhu, J. Q., Chang, T. Y., and Li, B. L. (2001) Biochim. Biophys. Res. Commun. 282, 580–588
14. Sakashita, N., Miyazaki, A., Chang, C. C. Y., Morgaelli, P., Chang, T. Y., Nakamura, O., Kiyota, E., Hakamata, H., Satoh, M., Tamagawa, H., Horieuchi, S., and Takeya, M. (2003) Lab. Invest. 83, 1–13
15. Li, B. L., Li, X. L., Duan, Z. J., Lee, O., Lin, S. M., Ma, Z. M., Chang, C. C. Y., Yang, X. Y., Park, J. P., Mohandas, T. K., Noll, W., Chan, L., and Chang, T. Y. (1999) J. Biol. Chem. 274, 11060–11071
16. Yu, C., Zhang, Y., Lu, X., Chang, C. C. Y., and Chang, T. Y. (2002) Biochemistry 41, 3762–3769
17. Maniatis, T., and Tasic, B. (2002) Nature 418, 236–243
18. Tsuchiyru, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., and Tada, K. (1980) Int. J. Cancer 26, 171–176
19. Tsuchiyru, S., Kobayashi, Y., Goto, Y., Okumura, H., Nakae, S., Konno, T., and Tada, K. (1982) Cancer Res. 42, 1530–1536
20. Cheng, W., Kleveland, K. V., and Abumrad, N. A. (1995) Am. J. Physiol. 269, E504–E508
21. Hibuchi, R., Krumbel, B., and Saiki, R. K. (1988) Nuclear Acids Res. 16, 7351–7367
22. Liu, J., Streiff, R., Zhang, Y. L., Vestal, R. E., Spence, M. J., and Biggs, M. R. (1997) J. Lipid Res. 38, 2035–2048
23. Peterson, G. L. (1977) Anal. Biochem. 83, 346–356
24. Chang, C. C. Y., Doubilet, G. M., and Chang, T. Y. (1986) Biochemistry 25, 1093–1099
25. Lee, O., Chang, C. C. Y., Lee, W., and Chang, T. Y. (1998) J. Lipid Res. 39, 1722–1727
26. Sambrook, J., Frisch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Yu, C., Chen, J., Lin, S., Liu, J., Chang, C. C. Y., and Chang, T. Y. (1999) J. Biol. Chem. 274, 36139–36145
28. Yang, L., Chen, J., Chang, C. C. Y., Yang, X. Y., Wang, Z. Z., Chang, T. Y., and Li, B. L. (2004) Acta Biochim. Biophys. Sin. 36, 259–268
29. Ohno, K., Fukushima, M., Fujiswa, M., and Narumiya, S. (1988) J. Biol. Chem. 263, 19764–19770
30. Zhang, F., Klawon, N., and Lukacs, G. L. (1989) Nature Struct. Biol. 5, 80–83
31. Dern, R., Reuter, G., and Loewendwein, A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9724–9729
32. Caudeliva, C., Serra, D., Miliar, A., Codony, C., Asins, G., Bach, M., and Hegardt, F. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12185–12190
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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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