Acyl-CoA:cholesterol acyltransferase (ACAT) 1 is an intracellular enzyme present in a variety of human and other animal tissues. It catalyzes the formation of cholesteryl esters from cholesterol and long-chain fatty acyl coenzyme A (for a review, see Ref. 1). In addition to storing cholesterol intracellularly, ACAT plays important physiological roles, including hepatic lipoprotein assembly, dietary cholesterol absorption, and ste-roidogenesis (for reviews, see Refs. 2–4). Under pathological conditions, accumulation of cholesteryl esters produced by ACAT is characteristic of foam cell formation in atherosclerotic lesions (for a review, see Ref. 5; also see Ref. 6). For these reasons, ACAT has been a pharmaceutical target for developing cholesterol-lowering and/or anti-atherosclerosis agents (for a review, see Ref. 7). The human ACAT-1 cDNA was cloned by a somatic cell and molecular genetic approach. Chinese hamster ovary (CHO) cell mutants lacking ACAT activity (including clone AC29) were isolated (8); subsequent stable transfection experiments showed that human genomic DNAs complemented the ACAT deficiency in AC29 cells (9). A 1.2-kb human genomic DNA fragment was cloned from the stable transfec-
tants. This fragment (designated as G2 DNA) led to the eventual cloning of a full-length human ACAT cDNA K1 (4011 bp in length). Expression of this cDNA, designated as ACAT-1, in AC29 cells complemented the ACAT deficiency of the mutant (10). Additional results showed that expressing this cDNA in insect cells, which do not contain endogenous ACAT-like activity, produced high levels of ACAT activity in vitro, confirming that this cDNA encodes the catalytic component of ACAT enzyme (11). The coding region of the ACAT gene has been mapped to chromosome 1q 25 (12). Protein sequence analysis revealed the ACAT-1 protein as a hydrophobic protein containing multiple transmembrane domains and sharing several peptide regions in common with other acyltransferases (10). Re-
combinant human ACAT-1 protein expressed in CHO cells has been purified to homogeneity; the homogeneous ACAT-1 protein remains catalytically active and uses cholesterol as a sub-
strate in a highly cooperative manner (13). Homologues of human ACAT-1 cDNA have also been cloned from other species (reviewed in Ref. 1), including two yeast homologues (14, 15). Disruption of the ACAT-1 gene in mice has been reported (16); the ACAT-1 gene-deficient mice exhibit marked reduction in cholesteryl ester levels in only selective tissues and not in all the tissues examined. These and other results led to the mo-
lecular cloning of ACAT-2 cDNA (17–19). The predicted amino acid sequence of ACAT-2 is homologous but distinct from that of ACAT-1. The physiological roles of ACAT-1 and ACAT-2 in various tissues of different species are currently under intense investigation by several laboratories. In humans, immu-
nodepletion experiments suggest that the ACAT-1 protein plays major catalytic roles in hepatocytes, adrenal glands, macrophages, and kidneys, but not in the intestines (20).

The 4.0-kb human ACAT-1 cDNA contains a single open reading frame of 1.65 kb. It also contains an unusually long 5′-untranslated region (5′-UTR; 1396 bp) and 965 bp of 3′-untranslated region. Using the coding region as probe, North-
An ACAT-1 mRNA Produced from Two Different Chromosomes

**EXPERIMENTAL PROCEDURES**

Isolation and Characterization of the Human ACAT Genomic DNAs—A 1.2-kb human ACAT-1 genomic DNA fragment (designated as G2 DNA), known to contain exonic human ACAT-1 sequences, has been previously isolated from CHO cell transfectants that stably express human ACAT activity (10). DNA sequencing revealed that G2 DNA contained 1139 bp. The sequence includes the 79-bp exon 8, a 668-bp intron 8, an 82-bp exon 9, and a 310-bp piece of intron 9 (result not shown). Within intron 8, a pair of oligomers has been found to give rise to a single 411-bp PCR product when either the G2 DNA or the total human genomic DNA was used as the template. The sequence for the forward primer is 5'-GGGACCCAGGCTTCGTTG-3'; the sequence for the reverse primer is 5'-GAATTAAAGGAAAGCGACGAAC-3'. This pair of oligomers was sent to Genomic Systems, Inc. (St. Louis, MO) for PCR screening the human genomic DNA P1 library (human DNA fragments cloned in the BamHI site of p1 vector pAHS1SacB II). Three positive P1 clones have been isolated (P1 733, 734, and 774). Detailed analyses revealed that these overlapping P1 clones contain the human ACAT-1 protein coding regions, but they did not contain the unusually long 1289-bp region located at the 5'-UTR of human ACAT cDNA K1. To isolate additional P1 clone(s) that cover this region, two additional primers were selected from the 5'-UTR of human ACAT cDNA K1; the sequence for the forward primer is 5'-GGGACCCAGGCTTCGTTG-3' (human ACAT cDNA K1 nt 982–997), and the sequence for the reverse primer is 5'-GATAACCCACTGGAAG-3' (human ACAT cDNA K1 nt 1181–1196). Using the second pair of primers, further screening (done at Genomic Systems) yielded a single P1 clone (P1 4651). The P1 clones were restriction-mapped by digestion with a combination of restriction endonucleases, electrophoresed on 1% agarose gels, and stained by ethidium bromide. The sizes of digested fragments were determined by comparison with DNA size standards from Life Technologies, Inc. The sizes of larger fragments (larger than 4 kb) were confirmed by estimating the sizes of their composite fragments after subcloning and further digestion. Appropriate restriction fragments were subcloned into linker sites of pBluescript vectors (Stratagene) for sequencing. DNA sequencing was performed using P1 clones and/or their subcloned fragments as the templates. Sequencing was according to the double-stranded DNA cycle sequencing system kit, and Tag DNA polymerase was supplied by Life Technologies, Inc. Exon/intron junctions were determined by using sequencing primers corresponding to nucleotide sequences of the human ACAT cDNA K1 (10).

Determination of the 5'-Ends of Human ACAT-1 mRNAs—Rapid amplification of the 5' cDNA ends (5'-RACE) was performed using the 5'-SLIC (single strand ligation to single strand cDNA) protocol (27, 28). Total RNAs used as templates were prepared from HeLa cells, human U937 cells, or various native human tissues as indicated, with TRIzol reagent (Life Technologies, Inc.). First strand cDNA synthesis was performed using SUPERScript II reverse transcriptase. The reaction conditions were as described in the instruction manual provided by Life Technologies, Inc.; the primer used was ACAT specific reverse primer 5'-ACCCACACCATTATCATAA-3', located at human ACAT cDNA K1 nt 1670–1655. The conditions for ligating the oligonucleotide to the single strand cDNA were set according to the protocol described (28). The sequence of the anchor oligonucleotide used was a 28-mer: 5'-CGTCGACTATAGAGGCGCCGACGTCTT-3'. After the ligation, PCR amplification was performed with 2-μl aliquots of the terminated ligation mixtures. For PCR, the primer specific for the anchor primer was 5'-AAAGCTTGGCCGGCTGCTCTAGTGACGGC-3'; the primer specific for the human ACAT-1 cDNA was the reverse primer 5'-CGGACGGCGACTCT-3' (designated as ACAT22; located at human ACAT cDNA K1 nt 1574–1595). The reactions were run as follows: cycle 1, 5 min at 94°C; cycles 2–32, 45 s at 94°C, 45 s at 54°C, and 90 s at 72°C. To analyze the size of the PCR products, a 20-μl aliquot of the PCR reaction product was electrophoresed through a 1% agarose gel and stained with ethidium bromide; λ DNA fragments were used as the ladder. The PCR products were subcloned into pBluescript SK+ vector or T-vector and sequenced. For each cloned PCR product, approximately 150 bp from each end were sequenced.

**RNase Protection Assay—**For the RNase protection assay, a 365-nt...
radiolabeled antisense riboprobe was synthesized by in vitro transcription of the cloned human ACAT cDNA fragment (nt 1113–1374, encompassing part of exon Xb, all of exon Xa, and part of exon 1) in the presence of [α-32P]CTP. The transcription reaction with T7 RNA polymerase was performed in vitro using a kit from Promega. Total RNAs isolated from human fetal brain or fetal kidney, at 100 μg each, were hybridized to radiolabeled riboprobe (2 × 10^6 cpm) at 42° for 14 h. The hybridized samples were digested with 1000 units of RNase T1 at 37° for 15 min. After ethanol precipitation, samples were analyzed by electrophoresis followed by autoradiography.

**Primer Extension Analysis**— Primer extension experiments were carried out using Superscript™II RNase H reverse transcriptase from Life Technologies, Inc. In accordance with the instruction manual, 1 pmol of 5'-end-labeled human ACAT-1 specific reverse primer (5'-ATCCACGACCTTTAGGAGGCGC-3'; human ACAT cDNA K1 nt 92–71) was hybridized to 2 μg of human liver poly(A) RNA at 70 °C for 10 min. The reverse transcription reaction was performed at 42 °C for 45 min. The product was analyzed with a sequencing gel.

**Luciferase Activity Assays Using the P1 or P7 Promoter of the Human ACAT-1 Gene**— Various fragments within the 6603/65 region containing the P1 promoter were isolated from a subclone of P1 774. Similarly, various fragments within the -612/+150 region containing the P7 promoter were isolated from a subclone of P1 4651. These fragments were subcloned into the multiple cloning site of the luciferase reporter gene vector pXPl (29) or vector pGL2-E (Promega). Constructs containing the promoters were identified by restriction enzyme analysis and by sequencing. Transfection was performed by either the calcium phosphate method (30) or the LipofectAMINE method (Life Technologies, Inc.). The hybridization solution contained 0.2 g of Cot-1 DNA (Life Technologies), and 30 μg of biotin-labeled DNA (Life Technologies, Inc.) in 15 μl of Hybridil VII (Oncor)/slide. The probe mixture was heat-denatured at 70 °C for 5 min and allowed to preanneal at 37 °C for 2 h. Chromosome preparations on slides were conditioned prior to hybridization in a 30-min, 37 °C bath in 2× SSC, followed immediately by dehydration at room temperature in 70, 80, and 95% EtOH (2 min each), and air-dried. The slides were then denatured in 70% formamide, 2× SSC at 70 °C for 5 min, followed by serial dehydration at room temperature. Hybridization was carried out for 18 h in a moist 37 °C chamber. Slides were washed in 50% formamide, 2× SSC at 37 °C for 30 min, followed by 2× SSC at 37 °C for 10 min. Slides were further washed three times at room temperature in phosphate-buffered detergent prior to signal detection. Hybridized DNA was detected with avidin-fluorescein isothiocyanate, followed by a single round of amplification according to the supplier’s instructions (Oncor). FISH signals were captured using a monochromatic CCD camera mounted on a Zeiss epifluorescence microscope with a LUDL filter wheel and a fixed multi-band pass beam splitter using MacProbe software (PSI, Houston, TX). The P1 774 insert, containing the exon 1–15 region and 3'-UTR of the human ACAT-1 gene, was mapped to chromosome 1q 25 (result not shown), confirming our early report with the cDNA fragment as probe (12). Using the P1 4651 insert as probe, analysis of 25 metaphases showed that 14 had four signals, nine had three signals, and two had two signals at 7q31.3. No background signals (sites with >2 signals) were observed. Four signals are expected in a metaphase under conditions of fully efficient hybridization and signal detection, representing four copies of the locus, two on each of the replicated homologues. These results allow localization of the human ACAT-1 exon Xb region to band 7q31.3.
The DNA sources for PCR analyses shown in Fig. 9 were genomic DNAs prepared from human fibroblasts, mice A9 cells, mouse A9 cells containing human chromosome 1 (A9–1), or mouse A9 cells containing human chromosome 7 (A9–7). The mouse A9 cells containing human chromosome 1 or chromosome 7 were purchased from the Coriell Institute for Medical Research (Camden, NJ). The details for conducting the PCR described in Fig. 11 are described in the figure legend. Other Methods—Standard molecular biology techniques were performed according to methods described by Sambrook et al. (32). Adult and fetal human tissues were obtained from national hospitals in Shanghai, China. Consents were obtained from donors or their relatives for removal of tissues for research purposes. All human tissues were of donor transplantation quality. The tissues were rapidly frozen and stored in liquid nitrogen until use. For preparation of poly(A)^+ RNAs serving as templates for RT-PCR experiments described in Fig. 1, the total RNAs from the indicated human tissues were prepared using guanidinium thiocyanate extraction followed by centrifugation in cesium chloride solution (32). The poly(A)^+ RNAs were isolated from the total RNAs by the oligo(dT)-cellulose affinity column chromatography procedure (32). CHO cells, human breast cancer cells, melanoma cells, and A293 cells were grown in appropriate media as described previously (24). Stable transfectants of CHO cells were selected by growing cells in G418 according to procedures previously described (10). Cloning of individual transfectants with cloning rings was carried out according to procedures previously described (10). For measurement of cholesterol ester biosynthesis in intact cells, the [3H]oleate pulse in intact cells followed by lipid analysis was according to procedures previously described (33).

RESULTS

Presence of the Long 5'-UTR in Human ACAT-1 mRNA—The human ACAT cDNA K1 contains an unusually long 5'-UTR (1396 bp). Within this region, an EcoRI site (nt 1282–1287) is present. To rule out the possibility that nucleotides 1–1289 of human ACAT cDNA K1 may be a ligament artifact produced during cDNA synthesis in vitro, RT-PCR experiments were performed using poly(A)^+ RNAs isolated from adult human brain, intestine, or liver as templates. The oligonucleotides relative to human ACAT cDNA K1 nt 982–997 served as the forward primer and nt 1395–1410 as the reverse primer. These two primers are located in regions flanking the EcoRI site described above. With these templates, a single 429-bp PCR product was obtained (Fig. 1A). Direct sequencing of the PCR product revealed the sequence to be identical to that found in human ACAT cDNA K1. Part of the PCR product sequence is shown in Fig. 1B. Additional experiments gave the same result. poly(A)^+ RNAs from human liver tumor Hep G-2 cells or human monocytic THP-1 cells were used as the templates. Also, numerous control experiments showed that when poly(A)^+ RNAs from mouse liver or yeast tRNA was used as template, no PCR products of discrete size were detected. The positive results and the negative control experiments described above have been consistently reproduced in T. Y. Chang’s laboratory (Hanover, NH) and B. L. Li’s laboratory (Shanghai, China). Together, these data show that the 5'-UTR present in human ACAT cDNA K1 is a genuine exonic region contiguous with the human ACAT-1 coding sequence present in an authentic ACAT-1 mRNA.

Multiple Human ACAT-1 mRNAs—Using the entire human ACAT-1 coding region as probe, previous experiments have suggested the existence of multiple human ACAT-1 mRNAs (with sizes of approximately 7.0, 4.3, 3.6, and 2.8 kb). To examine the origin of these mRNAs, we prepared various riboprobes derived from small segments of the human ACAT cDNA K1, covering various parts of the 5'-untranslated, coding, and 3'-untranslated regions (lengths and locations of these probes are indicated in the Fig. 2 legend). These probes were labeled and hybridized individually with poly(A)^+ RNAs from human melanoma cells. The results (Fig. 2) show that the short 5'-UTR sequence (exon 1; recognized by probe 5'U1), the various coding regions (recognized by probes Cod1 to -4), and the proximal portion of the 3'-untranslated region (recognized by probe 3'U1) were present in all four types of human ACAT-1 mRNAs. In contrast, the long 5'-UTR (recognized by probes 5'U2 and 5'U3) of human ACAT cDNA K1 is present only in the 4.3-kb human ACAT-1 mRNA Lane 4 of Fig. 2 also shows that probes 5'U2 and 5'U3 crosshybridized with an additional 2.3-kb RNA species. Since this species was not recognized by probes within the human ACAT-1 coding region, the significance of this observation is unknown at present.

Cloning the Genomic DNA of the Human ACAT-1 Gene—The human genomic DNA inserts in four P1 clones (P1 733, 734, 734, and 4651) were isolated (see “Experimental Procedures” for isolation). They were subjected to restriction mapping, plasmid subcloning, and DNA sequencing analysis. The relationship between the human ACAT cDNA K1 and the four human ACAT-1 genomic DNA fragments inserted in P1 clones is shown in Fig. 3. Three of these inserts (P1 733, 734, and 774) were found to be partially overlapping (Fig. 3). The P1 774 insert contains the short 5'-UTR sequence, the entire coding region, and the 3'-UTR (designated as exons 1–16) of the human ACAT-1 gene. The P1 4651 insert contains only the long 5'-UTR (designated as exon Xa) and not the short 5'-UTR or the coding region of the human ACAT-1 gene. The sequence CCGAATTCCG (designated as exon Xb) found in human ACAT cDNA K1 nt 1280–1289 and the 4.3-kb ACAT-1 mRNA has not been located within these genomic clones at present.

To test the biological activity, DNAs prepared from clone P1 774 were co-transfected along with the plasmid DNA pcDNAneo to perform stable transfection experiments, using the ACAT-deficient CHO cell mutant (clone AC29) as the recipient. The resultant uncloned transfected cells (designated as uncloned P1F) were assayed for ability to synthesize cholesterol esters in intact cells. The value was compared with values obtained in AC29 cells and in 25RA cells (the parental CHO cells of AC29 cells) (8, 34). The results (Table I) indicate that the P1 774 insert was able to stimulate cholesterol ester synthesis in AC29 cells. Control experiments indicate that the P1 vector without the insert along with pcDNAneo did not stimulate cholesterol ester synthesis in the AC29 cells (results not shown). The P1F transfected populations were selected by their growth resistance to the drug G418 present in the medium. The drug G418 selected for clones that maintained the pcDNAneo plasmid in their cell genomes. However, each of these clones might lose the P1 774 insert during continuous cell growth. We therefore cloned a number of stable transfected cells from the uncloned P1F cell population and assayed each of these clones for its ability to synthesize cholesterol esters. The results (Table I) indicate that the cloned P1F transfected population could be divided into two categories; clones...
PIF 1–4 showed near full complementation of ACAT deficiency, while clones P1F 7–12 showed no complementation. We next performed Southern analysis and found that the P1 774 insert was present in clones P1F 1–4 but absent in clones P1F 7–12 (result not shown). These results suggest that the P1 774 insert contains all of the elements necessary for expressing the human ACAT-1 gene in CHO cells.

The restriction map of the human genomic DNA insert in clone P1 774 is shown in Fig. 4A. Sequencing analysis shows that this insert contains the exonic sequences (exons 1–16) present in human ACAT cDNA K1 nt 1290–4011; however, it does not contain the first 1289-nt sequence present at the long 5'-UTR of human ACAT cDNA K1. The restriction map of the insert in clone P1 4651 is shown in Fig. 4B. Sequencing analysis shows that this insert contains 1277 (present as a single uninterrupted sequence, exon Xa) of the first 1279 nt at the 5'-end of human ACAT cDNA K1. The first two nucleotides (two guanines, found in nt 1 and 2 of human ACAT cDNA K1) were not found in the genomic DNA. This difference may be the result of a mRNA capping event coupled with the addition of an extra guanine at the 5'-end by reverse transcriptase during the cDNA synthesis step. Other possibilities also exist. In the human ACAT cDNA K1 sequence, a 10-bp sequence CCGAATTCGG (exon Xb) is present immediately downstream from the 1279 bp (the cDNA sequence flanking the 10-bp sequence is shown in Fig. 1). Repeated cloning and sequencing work has indicated that the exon Xb sequence has not been localized downstream to the exon Xa sequence in the P1 4651 insert or upstream from the exon 1 sequence in the P1 774 insert. Furthermore, the restriction enzyme

![Fig. 4. Restriction maps of human ACAT-1 gene. A, P1 774; B, P1 4651. Abbreviations for various restriction enzymes are indicated. Lengths were drawn to scale. The locations of early introns (introns 1–3) and early exons within these inserts were indicated.](image)

**TABLE II**

| Exon | Size  | 5' splice sequence | Intron size | 3' splice sequence | Amino acid interrupted |
|------|-------|-------------------|-------------|-------------------|------------------------|
|      | bp    |                   | kb          |                   |                        |
| Xa   | 1277  | CCAGGGCACCC       | ND          | ????????????     | CCGAA                  |
|      | 10    | TTAGGGGG         | ND          | tgggcgcag         | GAGAGTTCG             |
| 1    | 99    | CGGGCTCTAGG      | 7.90        | tttctcttag        | ACAATAAGATG           |
| 2    | 126   | CCTTAGTAATG      | 20.35       | cttccctctag       | GTGGAGTATGA           |
| 3    | 59    | AGAGCCGAGAG      | 11.80       | aaaaatcttag       | GAATTTGAGGG           |
| 4    | 152   | ATAGACCCG        | 2.21        | ccctctctctag      | GTGGTTGAGAG           |
| 5    | 60    | CTCTGTATATTC     | 1.45        | acatcttacag       | TGAACGGCTT            |
| 6    | 108   | ATGAAGAGAGAG     | 1.51        | ttctctctctag      | GTGTGTGCTT            |
| 7    | 283   | ATTAGGAGCCG      | 0.82        | gtttgttgtgag      | ATGCCTTTGG            |
| 8    | 79    | GGAACATGGAG      | 0.67        | cttctctctctag     | GCACGGTTG              |
| 9    | 82    | GCTATCCCCCAGG    | 0.65        | ttcctctctctag     | GATGACTGGCCC          |
| 10   | 46    | GTTGACCCAGG      | 1.32        | atctctctctag      | GAGAGGCTT              |
| 11   | 130   | ATCTGAGGAGG      | 2.65        | tttctctctctag     | GTTGTTGCTG             |
| 12   | 98    | GTTCTCTAGAGG     | 1.16        | ttcctctctctag     | GATTGGTGAGAG           |
| 13   | 99    | CTCTTCCTGGG      | 1.35        | tttctctctctag     | TGTGTGTG               |
| 14   | 136   | TTCTGTGAGA       | 0.89        | ctatctctctctag    | TGGTGTTGCA            |
| 15   | 146   | TCTGAAAACTG      | 2.06        | cttctctctctag     | GGTGGTTGGA            |
| 16   | >1018 | TGGGATATGGCCGCCC | ND          | ND                 | ND                     |

* The 10-bp sequence designated as exon Xb, CCGAATTCGG, present immediately downstream from exon Xa in cDNA K1, has not been located at present.

* The number 1018 is calculated from 4011 bp of human ACAT-1 cDNA K1, which does not contain the poly(A) tail at its 3'-end (boldface and underlined) (10).

* The flanking sequence of exon Xa has not been found at present.
analyses showed that the 3'-end of P1 4651 insert (more than 20 kb upstream from exon 1; Fig. 4B) and the 5'-end of P1 774 insert (Fig. 4A) do not overlap with each other.

Exon/Intron Organization—Restriction fragments of the P1 774 insert and the P1 4651 insert were subcloned and sized. The sizes of the introns were estimated by compiling the sizes of the subcloned fragments; for larger fragments (greater than 4 kb), the fragments were further subcloned and sized to confirm the size estimation. The human genomic DNA fragments containing the exonic sequences present in the 4.0-kb human ACAT cDNA K1 were sequenced to localize exons. All exon-flanking regions were sequenced bidirectionally. Table II provides available exon-intron boundary nucleotide sequences, exon lengths, sizes of the respective intervening introns, and the amino acid interrupted by each intron. The exonic sequences in the human genomic DNA were in almost complete agreement with the published human ACAT cDNA K1 sequence (10). Repeated sequencing of the human ACAT-1 genomic DNA revealed that two errors were made in the previous assignment of the cDNA sequence. The nucleotides coding amino acid 207 should be GCC coding alanine instead of CGC coding arginine as previously published; also, the nucleotides coding amino acid 475 should be CTC instead of CTG (the coding amino acid at 475 remains as leucine). With the exception of intron 3, all introns determined begin with a 5'-GT and conclude with a 3'-AG terminus. However, intron 3 was found to contain a rare 5'-GC splice donor junction. The sequence immediately downstream from the 1277-bp exon Xa (aagctcaaaaa) and the sequence immediately upstream from the 99-bp exon 1 (ttggccgcaag) do not conform to any known intronic sequences specific for RNA splicing reactions. Other salient features of the human ACAT genomic structure include protein coding beginning in exon 2 and terminating in exon 16. Introns 1–3 are large, each longer than 7 kb. The nucleotide sequences flanking exon Xb in the genomic DNA have not been identified at present.

Identification of the P1 and P7 Promoters—The 648- and the 612-bp genomic DNA fragments flanking exon 1 or exon Xa of the human ACAT-1 gene have been subcloned and sequenced (Fig. 5). To test the promoter activities, these two fragments were each ligated into the luciferase reporter gene vector pXP-1 or pGL2-E in forward orientation. The resultant plasmids were each transiently co-transfected into the CHO cell line AC29 or the human cell line A293. The results show that both fragments can promote the expression of the luciferase reporter gene (result not shown). The sequences in these two fragments have been designated as the P1 and P7 promoters. The P1 promoter sequence in the first 648 bp flanking the exon 1 is rich in GC content (Fig. 5A). Neither a typical TATA box nor a typical CCAAT box was found. There is a potential Sp1-binding site; this is consistent with other TATA-less promoters, in which transcription initiation directed by these templates is critically dependent on Sp1 (35). This region also contains a 9-bp sequence for the B1 transcription enhancer element within the apo-E promoter (36); a 6-bp sequence required for transcription of the HMG-CoA reductase gene (37); and the 11-bp motif (in inverted orientation) known to bind the transcription factor NF-κB, found within the promoter of the TNF-α gene (38). In the P7 promoter, within the first 612-bp genomic DNA fragment flanking the optional exon Xa (Fig. 5B), a typical TATA box is also absent. However, two copies of a typical CCAAT box are found in this region. Other potential binding sites for various transcription factors include Sp1, Ap2, and TFIIId (in inverted orientation) (39); the insulin enhancer sequence is also present (40).
An ACAT-1 mRNA Produced from Two Different Chromosomes

Human ACAT-1 mRNA—5'-RACE was performed using the 5'-SLIC strategy (described under “Experimental Procedures”). Total RNAs used as templates were prepared from HeLa cells, human U937 cells, or various native human tissues as indicated. After ligation reaction with the anchor oligonucleotide, a human ACAT-1-specific primer (ACAT22, located in human ACAT cDNA K1 nt 1359–1374) was used as the anchor oligonucleotide, a human ACAT-1-specific primer (ACAT22, located in human ACAT cDNA K1 nt 1359–1374) was used as the 5'-reverse primer in PCR experiments (Fig. 6A). For C, lane 1, HeLa cell total RNA was used; lanes 2–5 were performed as negative controls; in lane 2 or 3, HeLa cell total RNA was replaced with yeast tRNA or double distilled H2O; in lane 4, HeLa cell total RNA without the 5'-SLIC reaction was directly subjected to PCR amplification; in lane 5, the ligation product using HeLa cell total RNA was subjected to PCR amplification with an irrelevant primer set (ACAT30, ACAT29), which was located within human ACAT-1 intron 1. Lane M, 1-kb DNA ladder from Life Technologies, Inc. For D, lane 1, total RNAs prepared from human fetal kidney were used as the templates; lane 2, total RNAs prepared from fetal brain tissue were used as templates. Lane M, DNA ladders (Sino-American Biotech Co.).

The 5'-end of the human ACAT-1 mRNA that contains exon Xa was also determined by primer extension analysis, using specific reverse primer located at human ACAT cDNA K1 nt 92–71 as probe (see “Experimental Procedures”). As shown in Fig. 7, the size of the main product is 91 nt, which is one nucleotide shorter than the 5'-sequence starting from nt 92 present in the human ACAT cDNA K1. This difference can be accounted for by an extra guanine present at the 5'-end of the cDNA; this nucleotide may be added by the reverse transcriptase action during the cDNA synthesis step. The results of the primer extension experiment essentially corroborate with the results of the 5'-RACE experiments, indicating that the 5'-end of human ACAT cDNA K1 is full-length. The transcription start sites for the two different promoters are indicated by an asterisk in Fig. 5, A and B.

Mapping the Exon 1–16 Region and the Optional Exon Xa Region of the Human ACAT-1 Gene—Restriction enzyme analyses have shown that the 3'-end of P1 4651 and the 5'-end of P1 774 do not overlap with each other (Fig. 4). This raises the possibility that the chromosomal locations of these two genomic DNA segments may be very distant from each other. To test this possibility, we performed FISH experiments to metaphase chromosomes and found that the P1 774 insert, containing the exon 1–16 region of the human ACAT-1 gene, is mapped to chromosome 1q 25 (results not shown), confirming our earlier report (12). To our surprise, the P1 4651 insert, containing the optional exon Xa region of the human ACAT-1 gene, is mapped to chromosome 7q 31.3 (Fig. 8). To test the fidelity of the FISH results, we next performed PCR analysis using genomic DNAs prepared from human cells, mouse cells, or human mouse somatic hybrids as templates. Two sets of primers were used (described in Fig. 9, A and B). The first set contained sequences flanking...
TGCA 1

FIG. 7. Primer extension analysis. The human ACAT-1 specific reverse primer 5'-ATCCAGACCTTTAGAGGACGG-3' (human ACAT cDNA K1 nt 92–71) was used as probe; the human liver mRNA was used as the template. In a separate experiment, the same primer was used to produce a sequencing ladder, using human ACAT genomic DNA inserted into P1 4651 as the template. Both the primer extension product and the sequence ladder were analyzed by a sequencing gel. In this gel, TGCA lanes on the left show the sequencing ladder; lane 1 on the right shows the primer extension products. The estimated sizes of the two primer extension products are as indicated by arrows.

FIG. 8. In situ hybridization of biotin-labeled human ACAT genomic DNA inserted into P1 4651 to human metaphase chromosomes. The left panel shows an ideogram of human chromosome 7. The right panel shows a digital image of human ACAT genomic DNA inserted into P1 4651 to partial metaphase chromosomes of a normal individual. Arrows indicate the probe signal at q31.3. No signal was detected at other chromosome locations.

The 385 bp within the exon Xa region (forward primer designated as K1T7D, 5'-GGAGTACGTCTACTC-3'; reverse primer designated as C1–7, 5'-GATAACCGACTTGA-3'). The second set contained sequences flanking the 1064-bp region within the intron 1/exon 2 junction (forward primer designated as ACAT 30, 5'-GCACTTGTAGATAC-3'; reverse primer designated as C1–5, 5'-CCATTACCTAGGTTCTC-3'). The results showed that when the first primer set was used (Fig. 9C, left), a single 385-bp PCR product was obtained when genomic DNAs from human fibroblasts (lane HF) or mouse A9 cells containing human chromosome 7 (lane A9–7) were used as the templates; no PCR product was found when genomic DNAs from mouse A9 cells containing human chromosome 1 (lane A9–1) or from the mouse A9 cells (lane Mouse) were used as templates. These results were further validated by sequencing analysis, which revealed that the 385-bp PCR product shown in lane A9–7 is identical to the sequence found in the corresponding region in human genomic P1 4651 insert. Conversely, when the second primer set was used (Fig. 9C, right), a single 1064-bp PCR product was obtained when genomic DNAs from human fibroblasts (lane HF) or mouse A9 cells containing human chromosome 1 (lane A9–1) were used as templates; no PCR product was found when genomic DNAs from mouse A9 cells containing human chromosome 7 (lane A9–7) or from the mouse A9 cells (lane Mouse) were used as templates. Again, these results were further validated by sequencing analysis, which revealed that the 1064-bp PCR product shown in lane A9–1 is identical to the sequence found in the corresponding region in the human genomic P1 774 insert. Along with Fig. 8, these results indicate that the exon 1–16 region of the human ACAT-1 gene is located in chromosome 1, while the optional exon Xa region of the human ACAT-1 gene is located in chromosome 7.

RNase Protection Assay—to verify the existence of the two types of human ACAT-1 transcripts from the two promoters (P1 and P7) as well as the existence of the mRNA that contains the exon Xa and Xb, we performed an RNase protection assay. A uniformly labeled 365-nt antisense riboprobe was synthesized by in vitro transcription of the cloned human ACAT-1 cDNA fragment (nt 1113–1374, encompassing 167 nt at the 5'-end of exon Xa, the 10 nt in exon Xb, and 85 nt at the 5'-end of exon Xb (Fig. 10A)). This probe was annealed to RNAs prepared from human fetal brain, human fetal kidney, or yeast and then digested with RNase. When human RNAs were used, three major protected fragments of 262, 167, and 85 bp in length were found (Fig. 10B, lanes 1 and 2). If yeast tRNA was used in hybridization, no protected band of discrete size was detectable (Fig. 10B, lane 3). These results indicate that three different protected bands can be detected. Additional experiments obtained the same three protected fragments if RNAs from adult human tissues were analyzed by the same assay described here (results not shown).

Attempts to Detect the Chimeric DNA Present in the Human Genome at Low Level through DNA Rearrangement—it remains possible that the long 5'-UTR (exons Xa, Xb, and 1) present in the 4.3-kb ACAT-1 mRNA is the result of transcription from a chimeric DNA present in the human genome; this chimeric DNA might be produced through DNA rearrangement by certain novel mechanism(s). Such an event, if occurring at the low frequency, would not be detectable by the gene mapping methods employed in Figs. 8 and 9. To test this possibility, we designed a PCR experiment that is sensitive enough to detect the chimeric DNA. The results are described in Fig. 11; the diagram on the right in A predicts that if the hypothetical chimeric DNA existed in the human genome, then a distinct 1388-bp PCR product should have been obtained by using as much as 1000 ng of human genomic DNA serving as the template (B, lane 11); in contrast, the control experiments showed that we could detect the 1388-bp PCR product if 0.002 ng of the plasmid DNA ACAT cDNA K1, which contains contiguous sequences of exon Xa, exon Xb, and exon 1, was used as the template (lane 14). Further demonstration of the sensitivity and fidelity of our PCR method, various additional control experiments were also planned. The left and middle diagrams of Fig. 11A illustrate the expected sizes of the PCR products in various control lanes. The results show that under the PCR conditions employed, we could detect the appropriate PCR products by using as little as 0.1 ng of human genomic DNA serving as the template (lanes 13–11 and 6–8). The results of additional control experiments (shown in lanes 5 and 10) have demonstrated the fidelity of the PCR primers used in lanes 1–6 and 6–8. Together, the results show that the 4.3-kb ACAT-1 mRNA is not the result of transcription from a chimeric DNA present at low level.

Dinucleotide Repeat Polymorphism in the Human ACAT-1 Gene—As shown in Fig. 12, intron 12 of the human ACAT-1 gene contains a GT repeat (14 repeats of GT repeats 28 bp GT) that is underlined (underlined sequences) away from the intron 12/exon 13 (underlined sequences) junction. To examine the polymorphism of this repeat, genomic DNAs from 372 individuals were used to perform PCR using two primers flanking the GT repeat region (boldface and italic sequences). The major allele, containing 14 GT repeats, was confirmed by sequencing. We next attempted to use this marker to correlate possible variation in ACAT-1 gene expression with occurrence in carotid artery atherosclerosis in 145 subjects (41).
We studied the GT allele distribution among cases and controls and found that the GT allele distribution among the cases, controls, and total sampling population (372 individuals) are remarkably similar (results not shown). The diagram (Fig. 13) shows the distribution of the GT repeats within the total sampling population in this study.

**DISCUSSION**

Four ACAT-1 mRNAs of discrete sizes (7.0, 4.3, 3.6, and 2.8 kb) have been seen consistently in various human cells and tissues (10, 21, 22, 24). Northern analyses suggest that these mRNAs share the same coding region. In addition, using poly(A)⁺ mRNAs from human HepG2 cells as template and primers flanking the entire human ACAT-1 coding region, Matsuda et al. (22) showed that the RT-PCR analysis consistently yielded only one detectable product of 1.7 kb, representing the size of the full-length coding region. These data strongly suggest that the multiple mRNAs produced from the human ACAT-1 gene contain the same coding region. The 3.6- and 2.8-kb mRNAs contain the exon 1 sequence followed by the coding sequence (exons 2–15). The 4.3-kb mRNA contains exons 1–15 and an additional long 5’-UTR 1289 nt in length, designated as exon Xa and Xb. The 7.0-kb mRNA contains the exon 1 sequence, the coding sequence, but lacks the long 5’-UTR of human ACAT-cDNA K1, we believe that they are produced by the P1 promoter. The difference in length between the 3.6- and the 2.8-kb mRNA may be caused by the difference(s) in their 3’-UTRs. The longest human ACAT-1 mRNA (7.0 kb) is also consistently detected as a minor species; it comprises approximately 5–15% of total transcripts in most tissues or cell lines examined thus far. At present, we are unable to determine its origin. Since it lacks exon Xa, this large RNA species may be an incompletely spliced intermediate. Other possibilities also exist.

The 4.3-kb mRNA contains the optional exon Xa (1279 bp)
and exon Xa (10 bp), followed by exon 1 as its long 5′-UTR. For exon Xb, 1277 of the first 1279 bp in the 5′-UTR of human ACAT cDNA K1 have been located as a single uninterrupted sequence in the human genomic P1 4651 insert. Using this human genomic P1 4651 insert, the 612-bp genomic sequence immediately upstream from the 1277-bp exon Xb sequence has been subcloned, sequenced, and identified as the P7 promoter region of the human ACAT-1 gene. The 5′-sequence in the human ACAT-1 cDNA K1 has not been found in the cloned genomic DNAs.

Gene mapping analyses by FISH experiments and PCR analyses of human-mouse somatic cell hybrids indicate that the exon 1 region the coding region and the P1 promoter region of the human ACAT-1 gene is located in chromosome 1, while the P7 promoter and the optional exon Xa region are located in chromosome 7. These results suggest that the 4.3-kb mRNA is produced from two different chromosomes. To test the validity of this interpretation, we performed RNase protection assays. Three protected fragments of 85, 167, and 262 bp, have been detected, leading to the following interpretation. The 85-bp band comes from the transcript by the P1 promoter; the 167-bp band comes from the transcript by the P7 promoter; and the 262-bp band comes from the 4.3-kb mRNA. Together, these results show that the 4.3-kb mRNA is produced from two different chromosomes. We also considered the alternative interpretation that the 4.3-kb mRNA may be a transcript of a chimeric DNA present at low levels in the human genome; the chimeric DNA would be produced by certain novel cellular mechanism(s). However, this interpretation is not supported by the results reported in Fig. 11.

The result of the RNase protection assay suggests that the 4.3-kb ACAT-1 mRNA is produced by a novel RNA recombination event that takes place between the two discontinuous RNAs transcribed by the P1 promoter and the P7 promoter. The molecular nature of the recombination event is not clear at present. It may involve trans-splicing reactions. Trans-splicing is a posttranscriptional event that joins two discontinuous transcripts intermolecularly to produce a mature mRNA species (for reviews, see Refs. 46–48). While there are ample examples of trans-splicing that occur in lower organisms (including trypanosomes, nematodes, and plants), to our knowledge, only a few potential examples have been reported in mammalian species. Perbal and colleagues (49–51) showed that in chicken and human thymic cells, the expression of a mature c-myb protooncogene mRNA may require the intermolecular linkage of coding sequences localized to two different chromosomes. Sullivan et al. (52) showed the possible existence of a mRNA in fetal rat liver resulting from linkage of coding sequences from the androgen-binding protein gene, localized in chromosome 10, and the histidine decarboxylase gene, localized in chromosome 3. It should be pointed out that in these studies, the existence of two precursor transcripts that were required to produce the mature mRNA by trans-splicing was never demonstrated by RNase protection assays. No further progress could be found in the literature following these initial studies, which were published in the early 1990s. Eul et al. (53) showed that a pre-mRNA transcribed from a SV 40 viral DNA can form a mature mRNA species via trans-splicing in intact mammalian cells as well as in HeLa cell nuclear extracts in vitro. This report shows that the biochemical machinery required to produce trans-splicing is present in extracts of mammalian cells. These investigators used viral pre-mRNA, not cellular pre-mRNA, as the substrate. More recently, Caudevilla et al. (54) demonstrated that natural trans-splicing of pre-mRNAs of a cellular gene (carnitine octanoyltransferase) does occur in vitro. The 4.3-kb ACAT-1 mRNA described in this report may be the result of trans-splicing. However, we note that there is not an obvious 5′-splice site present at the junction of exon Xb and the RNA immediately downstream from it. Therefore, if the 4.3-kb ACAT-1 mRNA is produced through trans-splicing reactions, the detailed molecular mechanism involved must deviate from that of the normal RNA trans-splicing reactions demonstrated in the literature. The most intriguing and puzzling aspect of our finding is that the 10-bp palindromic sequence in human ACAT-1 cDNA K1 nt 1280–1289, designated as exon Xb, has not been found within the genomic clones. It is
possible that this sequence may play important role(s) in a novel trans-splicing reaction. Further investigations are needed to clarify these issues. In addition, further investigations are needed to evaluate the physiological significance of the 4.3-kb ACAT-1 mRNA.

The finding that human ACAT-1 gene contains two different promoter elements, with the P1 promoter located within the genomic region immediately upstream from transcription start sites, bears resemblance to a number of other mammalian genes. Further investigations are needed to clarify these issues. In addition, further investigations are needed to evaluate the physiological significance of the 4.3-kb ACAT-1 mRNA.

**Fig. 11.** Attempts to detect the hypothetical chimeric DNA at low level by PCR. Diagrams in A illustrate the predicted results by PCR. The actual results are shown in B. DNAs in hypothetical quantities from different sources as indicated were used as the template for the PCR experiment. 1000, 100, or 0 ng of genomic DNA from HEK293 cells (lanes 11–13) or 0.002 ng of the plasmid DNA containing the full-length sequence human ACAT cDNA K1 (lane 14), was used as the template; the primer set, L8 (GTA GAG ACG GGG TTT CAC CG; located in exon Xa, nt 3–22 of cDNA K1) and DP2 (CGG CAG CGG GCA CTT CGG CCA A; located in exon 1, 1353–1374 nt of cDNA K1), was used. Lane M, PCR size markers. 100, 10, 0.1, or 0 ng of genomic DNA of human cell HEK293 (lanes 1–4) or 1.0 ng of P1 DNA 4651 known to contain the uninterrupted exon Xa of human ACAT-1 (lane 5) was used as the template; the primer set, K1T7D (GGC AGT AGA CTC ATC T; located at exon Xa, nt 812–827 of cDNA K1) and C1–7 (GAT AAC CCA CTG GAA G; located at exon Xa, nt 1181–1196 of cDNA K1), was used. 100, 10, 0.1, or 0 ng of genomic DNA of human cell HEK293 (lanes 6–9) or 0.002 ng of the plasmid DNA containing the full-length sequence human ACAT cDNA K1 (lane 10), was used as the template; the primer set, ACAT30 (GCA CTT AGT AGA TAC T; located at human ACAT intron 1) and C1–5 (CCA TTA CTA GGT GTC T; located at human ACAT exon 2, nt 1500–1515 of cDNA K1), was used. PCR experiments were performed in a 100-μl volume containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 200 μM each dNTP, 400 nM each specific primer, and 5 units of Taq polymerase. For lanes 1–5, the reactions were run as follows: cycle 1, 3 min at 94 °C; cycles 2–32, 45 s at 94 °C, 40 s at 51 °C, and 50 s at 72 °C; cycle 33, 10 min at 72 °C. For lanes 6–10, the annealing temperature was reduced to 49 °C; for lanes 11–14, the annealing temperature was raised to 61 °C; other PCR conditions used were the same as described for lanes 1–5. After PCR, the products were analyzed along with size markers on 1.2% agarose gel.

**Fig. 12.** Sequence of the (GT)n microsatellite-containing region within intron 12 of the ACAT-1 gene. The sequences of GT repeat (boldface type) and exon 13 are underlined. The two boldface and italic sequences are the two primers used to analyze the polymorphism of this repeat sequence among the human population.

Total (372) # of GT repeats

**Fig. 13.** Frequency distribution of the (GT)n alleles within the human ACAT-1 gene in a sample of 372 individuals. Allele designations correspond to the number of dinucleotide repeats.
An ACAT-1 mRNA Produced from Two Different Chromosomes

genes reported (55–57). Our studies using various deletion constructs (results not shown) suggested that the two promoters may contain different cis-acting elements, implying that the two human ACAT-1 promoters may be controlled by different trans-acting factors under different physiological conditions. This possibility will be pursued in the future.

A GT dinucleotide polymorphism has been identified within intron 12. We attempted to use this marker to correlate possible variation in the human ACAT-1 gene expression with occurrences in carotid artery atherosclerosis and found that the distribution of the GT repeats within the cases and the controls in our sampling population is identical. Since our sampling population is small and geographically restricted, our results cannot be definitely generalized. In the future, the human ACAT-1 gene polymorphic marker reported here may prove useful for correlating variation of human ACAT-1 gene expression with various genetic abnormalities in lipid metabolism and in human diseases.

This is the first report describing the genomic organization of ACAT genes. The implication that the 4.3-kb human ACAT-1 mRNA is derived from two discontinuous precursor RNAs produced from two different chromosomes provides a novel system for further studies on RNA recombination reactions in mammalian species. In addition, other results described here will be useful for further studies on the regulation of the human ACAT-1 gene and its products.

Acknowledgments—We thank Drs. Dan Schroen and Matt Vincenti for providing advice for using the pPP1 vector for ACAT promoter analysis. We also thank Nancy Nutile-McMeneny, Chris LaPointe, Eddie Wong, and Charlie Coon for participating in certain stages of this work. We acknowledge critical comments made by the reviewers of this manuscript and thank Dr. Charles Cole for stimulating discussion and helpful advice. The oligonucleotides used in this work were synthesized by Sushma Rampal at Beckman Instruments using the Beckman Oligo 1000 synthesizer and at the Shanghai Institute of Biochemistry, China.

REFERENCES

1. Chang, T. Y., Chang, C. C. Y., and Cheng, D. (1997) Annu. Rev. Biochem. 66, 613–638.
2. Suckling, K. E., and Stange, E. F. (1985) J. Lipid Res. 26, 647–671.
3. Dietzcy, J. M., Turley, S. D., and Spady, D. K. (1993) J. Lipid Res. 34, 1637–1659.
4. Wilson, M. D., and Rudel, L. L. (1994) J. Lipid Res. 53, 493–955.
5. Tabas, I. (1995) Curr. Opin. Lipidol. 6, 260–268.
6. Miyazaki, A., Sakashita, N., Lee, O., Takahashi, K., Horisuchi, S., Hakamata, H., Morganelli, P. M., Chang, C. C. Y., and Chang, T. Y. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 1568–1574.
7. Krause, B. R., and Bocan, T. M. A. (1995) in ACAT Inhibitors: Physiologic Mechanisms for Hypolipidemic and Anti-atherosclerotic Activities in Experimental Animals (Ruffolo, R. R. J., and Hollinger, M. A., ed.) pp. 173–198, CRC Press, Inc., Boca Raton, FL.
8. Cadigan, K. M., Heider, J. G., and Chang, T. Y. (1988) J. Biol. Chem. 263, 274–282.
9. Cadigan, K. M., Chang, C. C. Y., and T. Y. C. (1989) J. Cell. Biol. 108, 2201–2219.
10. Chang, C. C. Y., Huh, H. Y., Cadigan, K. M., and Chang, T. Y. (1993) J. Biol. Chem. 268, 20747–20755.
11. Cheng, D., Chang, C. C. Y., Qu, X., and Chang, T. Y. (1995) J. Biol. Chem. 270, 683–692.
12. Chang, C. C. Y., Noll, W., Nutile-McMeneny, N., Lindsay, E. A., Baldini, A., Chang, W., and Chang, T. Y. (1994) Somatic Cell Mol. Genet. 20, 71–74.
13. Chang, C. C. Y., Lee, C. Y. G., Chang, E. T., Cruz, J. C., Levesque, M. C., and Chang, T. Y. (1998) J. Biol. Chem. 273, 35132–35143.
14. Yang, H., Bard, M., Bruner, D. A., Gleeson, A., Deckelbaum, R. J., Aljinovic, G., Pohl, T. M., Rothstein, R., and Sturley, S. L. (1996) Science 272, 1353–1356.
15. Yu, C., Kennedy, N. J., Chang, C. C. Y., and Rothblatt, J. A. (1996) J. Biol. Chem. 271, 24157–24161.

An ACAT-1 mRNA Produced from Two Different Chromosomes

11071