

The optional long 5'-untranslated region of human ACAT1 mRNAs impairs the production of ACAT1 protein by promoting its mRNA decay

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We have previously reported that human ACAT1 mRNAs produce the 50 kDa protein using the AUG_{1397–1399} initiation codon, and also a minor 56 kDa isoform using the upstream in-frame GGC_{1274–1276} initiation codon. The GGC_{1274–1276} codon is located at the optional long 5'-untranslated region (5'-UTR, nt 1–1396) of the mRNAs. The DNA sequences corresponding to this 5'-UTR are located in two different chromosomes, 7 and 1. In the current work, we report that the optional long 5'-UTR significantly impairs the production of human ACAT1 protein initiated from the AUG_{1397–1399} codon, mainly by promoting its mRNA decay. The western blot analyses indicated that the optional long 5'-UTR potently impaired the production of different proteins initiated from the AUG_{1397–1399} codon, meaning that this impairing effect was not influenced by the 3'-UTR or the coding sequence of ACAT1 mRNA. The results of reverse transcription-quantitative polymerase chain reaction demonstrated that this 5'-UTR dramatically reduced the contents of its linked mRNAs. Analyses of the protein to mRNA ratios showed that this 5'-UTR mainly decreased its mRNA stability rather than altering its translational efficiency. We next performed the plasmid transfection experiments and used actinomycin D to inhibit transcription. The results showed that this 5'-UTR promoted its mRNA decay. Additional transfection and nucleofection experiments using RNAs prepared *in vitro* illustrated that, in both the cytoplasm and the nucleus of cells, the optional long 5'-UTR-linked mRNAs decayed faster than those without the link. Overall, our study brings new insight to the regulation of the human ACAT1 gene expression at the post-transcription level.

Keywords human ACAT1 mRNA; long 5'-UTR; mRNA stability; mRNA decay; ACAT1 protein production

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Introduction

Acyl-coenzyme A:cholesterol acyltransferase (ACAT) is an integral membrane protein mainly located in rough endoplasmic reticulum and catalyzes the formation of cholesteryl ester from cholesterol and long-chain fatty acyl-coenzyme A [1,2]. ACAT is one of the key enzymes participating in the cellular cholesterol homeostasis [3]. During the early stage of atherosclerosis disease, the excessive accumulation of cholesteryl esters produced by ACAT in macrophages leads to the formation of foam cells [4–6]. In a mouse model for Alzheimer's disease, the inhibition of ACAT activity significantly reduces the accumulation of amyloid plaques [7]. Thus, the ACAT activity is involved in various dyslipidaemia and in neurodegenerative diseases.

So far, two ACAT genes (*Acat1* and *Acat2*) have been identified in mammals. Compared with the liver- and intestine-specific expression of human ACAT2 gene [8–10], the human ACAT1 gene, identified initially by isolating ACAT cDNA K1 [11], is expressed ubiquitously in all the human tissues examined [1,2]. Human ACAT1 gene contains 18 exons (exons Xa, Xb, and 1–16). Interestingly, its genomic DNA is located in two different chromosomes (1 and 7): exons 1–16 are located in chromosome 1, whereas the exon Xa

(1279 bp) is located in chromosome 7 [12]. Up to now, the formation of exon Xb is still unclear. Four human ACAT1 mRNAs (7.0, 4.3, 3.6, and 2.8 kb), which are ubiquitously present in all the human tissues and cells examined, share the same sequence located in chromosome 1, but only the 4.3-kb mRNA contains the exon Xa, which is located in chromosome 7. Therefore, the human ACAT1 4.3-kb mRNA is produced from two different chromosomes by a novel RNA recombination event that presumably involves interchromosomal trans-splicing and the ACAT1 cDNA K1 is derived from this mRNA [12]. We further found that this mRNA could produce two isoforms, 50 kDa and 56 kDa, with different enzymatic activities from the AUG_{1397–1399} and its upstream in-frame GGC_{1274–1276} initiation codons [13,14], respectively. Thus, this mRNA contains an optional long 5'-untranslated region (5'-UTR). We also showed that in the optional long 5'-UTR, a RNA secondary structure (nt 1355–1384) can enhance the selection of the downstream AUG_{1397–1399} as initiation codon to produce the 50 kDa isoform [15], whereas two additional structures (nt 1255–1268 and nt 1286–1243) are required for the production of the minor 56 kDa isoform [14]. However, whether the entire optional long 5'-UTR of human ACAT1 mRNAs affects the production of ACAT1 50 kDa protein initiated from the AUG_{1397–1399} codon has not been examined.

Usually, the mature mRNA in eukaryotes has a tripartite structure consisting of 5'-UTR, coding region, and 3'-UTR, which plays crucial roles in the post-transcriptional regulation of gene expression. Messenger RNAs encoding proteins involved in developmental processes, such as growth factors, transcription factors, or proto-oncogene products, all of which need to be strongly and finely regulated, often have 5'-UTRs that are longer than average [16]. It is known that the features of 5'-UTRs may greatly affect the translational efficiency in a cap-dependent manner. The classical scanning model for translation initiation of ribosomes predicts that, in most natural eukaryotic mRNAs, translation initiates at the first AUG encountered by the 40S ribosomal subunit starting from the 5'-cap [17]. If a stable secondary structure ($\Delta G < -50$ kcal/mol) is located in the 5'-UTR, 17 nt downstream of the 5'-cap, the migration of 40S ribosomal subunit will be stalled [18,19]. However, in many cases, secondary structures or sequence elements in the 5'-UTR act as an internal ribosome entry site (IRES) to increase translational efficiency to some extent through the cap-independent mechanism [20,21].

RNA turnover plays important roles in controlling the post-transcriptional processes. Although the half-lives of many mRNAs may change in response to many factors including nutrient levels, cell growth rates, viral infection, exposure to toxins, and temperature shifts [22], the half-lives of most mRNAs are mainly determined by mRNA-degradation elements and mRNA-binding proteins [23]. The turnover of mRNAs is mostly regulated by *cis*-acting elements located in the 3'-UTR, such as the AU-rich elements (AREs), which promote mRNA decay in response to a variety of specific signals by a process referred to as ARE-mediated mRNA decay [24–26]. However, in some special cases [23], the *cis*-acting elements regulating the turnover of mRNAs are also found in the coding region and 5'-UTR. Several studies have indicated that the 5'-UTR regulates gene expression by altering mRNA stability, and RNA secondary structures located within the 5'-UTR play an important role in determining mRNA stability [27,28].

In the current work, we studied whether the optional long 5'-UTR of the human ACAT1 mRNAs may have an impact on the production of ACAT1 proteins. Our results revealed that this 5'-UTR can impair the production of different proteins, initiated from the AUG_{1397–1399} codon, mainly by promoting its mRNA decay.

Materials and Methods

Materials

Cell culture reagents and T4 DNA ligase were purchased from Invitrogen (Carlsbad, USA). All the restriction enzymes and agarose were from Promega (Madison, USA). Anti-rabbit and anti-mouse antibodies (IgGs) conjugated with horse-radish peroxidases (HRPs) were from Pierce (Rockford, USA). Actinomycin D was from Sigma–Aldrich (Milwaukee, USA). *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.

Cell culture and transfection

AC29, the mutant Chinese hamster ovary cells lacking the endogenous ACAT1 [29], was maintained in a basal Ham's F12 medium, supplemented with 10% fetal bovine serum and antibiotics, in a humid atmosphere of 5% carbon dioxide and 95% air at 37°C.

Transfection of plasmid was performed using FuGENE 6TM transfection reagent (Roche, Basel, Switzerland)

according to the manufacturer's instructions. Transfection of RNA was performed using LipofectamineTM 2000 (Invitrogen, Carlsbad, USA) or NucleofectorTM Kit T (Amaxa, Nattermannallee, Germany) according to the manufacturer's instructions.

Expression plasmids

The expression plasmid pcDNA3-K1 (pA1) containing the full human ACAT1 cDNA K1 sequence (1–4011 bp), which encodes 50 kDa and 56 kDa isoforms from the AUG_{1397–1399} and its upstream in-frame GGC_{1274–1276} initiation codons, respectively, was constructed as described in our previous work [13]. Then, the deletions of the 5'-UTR were obtained using the plasmid pA1. The fragments with 5'-region deletions (Δ 1–1289 and Δ 1–1390) were obtained by the one-step polymerase chain reaction (PCR) with the corresponding forward primers (1290F, 5'-AAAGGTACCGAGAGCT TCCCGGAGTCCG-3'; 1391F, 5'-AAGGTACCAATA CAATGGTGGGTGAAGA-3') and the common reverse primer (4011R, 5'-AAGGGCCCGTACAGACTCACT AGTC-3'), respectively. The mutation of the UAG stop codon from the GGC_{1274–1276} initiation codon was obtained by two-step PCR method described by Higuchi *et al.* [30] with the external and internal primer sets (A1F, 5'-AAGCTAGCGGGTAGAGACGGGGTTTCAC CG-3'/4011R, shown above, and M1F, 5'-ATATTT ATATATATCCAGTAGACCCCGAATTCGGGAGAG-3'/M1R, 5'-CTCTCCCGAATTCGGGGTCTACTGGATA TATATAAATAT-3'). All the deleted and mutated fragments amplified were inserted into the *KpnI* and *ApaI* sites of pcDNA3 (Invitrogen) to generate expression plasmids pA1d1, pA1d2, and pA1m, respectively.

The sequence encoding 3 × Flag was amplified from p3 × FLAG-CMV-14 expression vector (Sigma Aldrich, Milwaukee, USA) by PCR with the primer set (3FlagF, 5'-AGTGAACCGTCAGAA TTAAGC-3'/3FlagR, 5'-AAAGGGCCCATCACTACTT GTCATCGTC-3'). The amplified fragment was inserted into the *XbaI* and *ApaI* sites of pcDNA3 to generate vector p3Flag for the tagged expression by fusing 3 × Flag at COOH-terminal.

The partial human ACAT1 cDNA K1 sequence (1–1786 bp) containing the optional long 5'-UTR followed by the partial coding sequence, which encodes 25 kDa NH₂-terminal fragment of 56 kDa isoform from the GGC_{1274–1276} codon and 17 kDa NH₂-terminal fragment of 50 kDa isoform from the AUG_{1397–1399} codon, was amplified by PCR with the forward primer (A1F, shown above) and reverse primer (1786R, 5'-AAATCTAGAA

TCTAAGAGAGAGCGCCT-3'), and inserted into the *KpnI* and *XbaI* sites of the constructed vector p3Flag to generate expression plasmid pNTF for production of ACAT1 26 kDa NH₂-terminal fragment with 3 × Flag (ACAT1-NT-Flag26) from the GGC_{1274–1276} codon and ACAT1 18 kDa NH₂-terminal fragment with 3 × Flag (ACAT1-NT-Flag18) from the AUG_{1397–1399} codon. Then, using this constructed pNTF, two deletions (Δ 1–1289 and Δ 1–1390) in the 5'-UTR and one codon mutation (GGC_{1274–1276} to UAG) were performed by the one-step PCR with the corresponding forward primers (1290F, 1391F, and A1F, shown above) and the common reverse primer (1786R, shown above). All the amplified fragments with the deletions and mutation were inserted into the *KpnI* and *XbaI* sites of p3Flag to generate expression plasmids pNTFd1, pNTFd2, and pNTFm, respectively.

Using pNTF, pNTFd1, pNTFd2, and pNTFm, the partial ACAT1-coding sequence was replaced by the entire luciferase coding sequence (encoding luciferase, 550 amino acids with apparent molecular mass of 61 kDa) to obtain expression plasmids pA1L, pA1Ld1, pA1Ld2, and pA1Lm. First, the luciferase-coding sequence was amplified from pGL3-Basic (Promega) by PCR with primer set (FlucF, 5'-AAATCTAGAATGGAAGACGCCAAAACA TA-3'/FlucR, 5'-AAAGGGCCCTTACACGGCGATCTTT CC-3') and inserted into the *XbaI* and *ApaI* sites of pcDNA3 to generate the plasmid pFL. Then, the upstream sequences of the AUG_{1397–1399} codon in pNTF, pNTFd1, pNTFd2, and pNTFm were amplified with the corresponding forward primers (A1F, 1290F, and 1391F, shown above) and the common reverse primer (1396R, 5'-AAAT CTAGACATTGTA TTGTCTGAGGC-3'). Finally, these amplified fragments were individually inserted into the *KpnI* and *XbaI* sites of pFL to generate expression plasmids pA1L, pA1Ld1, pA1Ld2, and pA1Lm.

Restriction enzyme digestion and DNA sequencing confirmed all the constructed plasmids.

Preparation of protein samples and Western blot analysis

Cells were washed twice, scraped in ice-cold phosphate-buffered saline (PBS) and extracted on ice for 30 min in RIPA buffer containing protease inhibitor mixture (Sigma). Cell debris were removed by a spin at 16,000 g. Protein concentrations were determined using the BCA protein assay kit (Bio-Rad, Hercules, USA). For western blot analysis, 50 µg of total protein per lane was resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. After gel separation, the

proteins were transferred to nitrocellulose membranes. The membranes were treated at room temperature with 5% milk in TBST (50 mM Tris-HCl, pH 7.6, 0.15 M NaCl, and 0.05% Tween-20) for 3 h, and then incubated with anti-ACAT1 (dilution 1:1000; DM10) [31], anti- β -actin (dilution 1:10,000; AC-15, Sigma), anti-Flag (dilution 1:1000; M2, Sigma), and anti-luciferase (dilution 1:1000; Abcam, Cambridge, UK) antibodies for 3 h, respectively. After incubation with HRP-conjugated secondary antibodies for 1 h, the membranes were washed extensively with TBST and TBS (50 mM Tris-HCl, pH 7.6, and 0.15 M NaCl), respectively. The signals were developed using ECL western blotting detection reagent (Pierce).

Luciferase activity analysis

Cells were washed with PBS and lysed with $1 \times$ passive lysis buffer (Promega). Cell lysates were subjected to one freeze/thaw cycle at -80°C and room temperature, respectively, and assayed for luciferase activities using the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol. Assays were performed using an Auto Lumat BG-P luminometer (MGM Instrument Inc., Hamden, USA).

In vitro transcription

The linear templates were obtained by PCR using primer set (T7F, 5'-TAATACGACTCACTATAGGG-3'/pcR, 5'-TAATACGACTCACTATAGGG-3'), transcribed using T7 RNA polymerase in the presence of cap analog (Promega) by RiboMAXTM large-scale RNA production systems (Promega), and a ≥ 150 base poly(A) tail was added to the produced RNA by Poly(A) Tailing Kit (Ambion, Austin, USA).

Reverse transcription-qPCR analysis

Total RNA isolated using Trizol (Invitrogen, Carlsbad, USA) from transfected cells was pre-treated with RQ DNaseI (Promega) and reverse-transcribed using oligo(dT)₁₈ to obtain the relative cDNAs. The quantification of ACAT1-NT-Flag18 (ACAT-NT), luciferase (Luc) and neomycin (Neo) cDNAs from transcripts were done by qPCR using Brilliant SYBR Green qPCR Master Mix and Mx3005PTM instrument (Stratagene, La Jolla, USA). The primer sets for the ACAT1-NT, Luc, and Neo cDNAs were AF (5'-GCGCTCTCACAACTTT TCT-3')/AR (5'-AATCA CCGTCATGGTCTTTG-3'), LF (5'-TCAAAGAGGCGA ACTGTGTG-3')/LR (5'-GGTGTG GAGCAAGATGG AT-3'), and NF (5'-TGAATGAA

CTGCAGGAC GAG-3')/NR (5'-ATACTTTCTCGGCAG GAGCA-3').

Prediction of RNA secondary structures

The secondary structures of all the RNA in this study were predicted using the mFold program (version 3.2) developed by Zuker and Turner [32]. The portal for the mfold web server is <http://www.bioinfo.rpi.edu/applications/mfold>.

Other methods

Standard molecular biology techniques were performed according to the methods described by Sambrook *et al.* [33].

Results

The optional long 5'-UTR of human ACAT1 mRNAs impairs the production of different proteins initiated from the AUG₁₃₉₇₋₁₃₉₉ codon

It has been reported that the 50 kDa and 56 kDa isoforms with different enzymatic activities can be translated from the AUG₁₃₉₇₋₁₃₉₉ and its upstream in-frame GGC₁₂₇₄₋₁₂₇₆ initiation codons of human ACAT1 mRNA corresponding to cDNA K1 from different chromosomes, 7 and 1 [13]. Accordingly, this mRNA contains an optional long 5'-UTR (nt 1-1396) upstream to the AUG₁₃₉₇₋₁₃₉₉ codon. Our previous studies have revealed that the upstream and downstream RNA secondary structures in the vicinity of the GGC₁₂₇₄₋₁₂₇₆ codon are required for the production of 56 kDa isoform [14]. Therefore, we interestingly speculate if the optional long 5'-UTR of human ACAT1 mRNAs could affect the production of 50 kDa protein initiated from the AUG₁₃₉₇₋₁₃₉₉ codon, which is the main enzyme with high activity for the cellular cholesterol homeostasis.

To test this hypothesis, the full-length human ACAT1 cDNA K1 (ACAT1 1-4011) was mutated at the initiation codon GGC₁₂₇₄₋₁₂₇₆ with a stop codon UAG [Fig. 1(A), no. 2] to eliminate the production of the human ACAT1 56 kDa protein and the optional long 5'-UTR was deleted at two sites [$\Delta 1-1289$ and $\Delta 1-1390$, Fig. 1(A), nos. 3 and 4], respectively. The expression plasmids obtained were shown in Fig. 1(A). Western blot analysis showed that the production of ACAT1 50 kDa protein initiated from the AUG₁₃₉₇₋₁₃₉₉ codon was different among the expression plasmids [Fig. 1(B), top panel]. When the optional long 5'-UTR was deleted at two sites ($\Delta 1-1289$ and $\Delta 1-1390$), the amounts of ACAT1 50 kDa proteins initiated from the AUG₁₃₉₇₋₁₃₉₉ codon in nos. 3 and 4

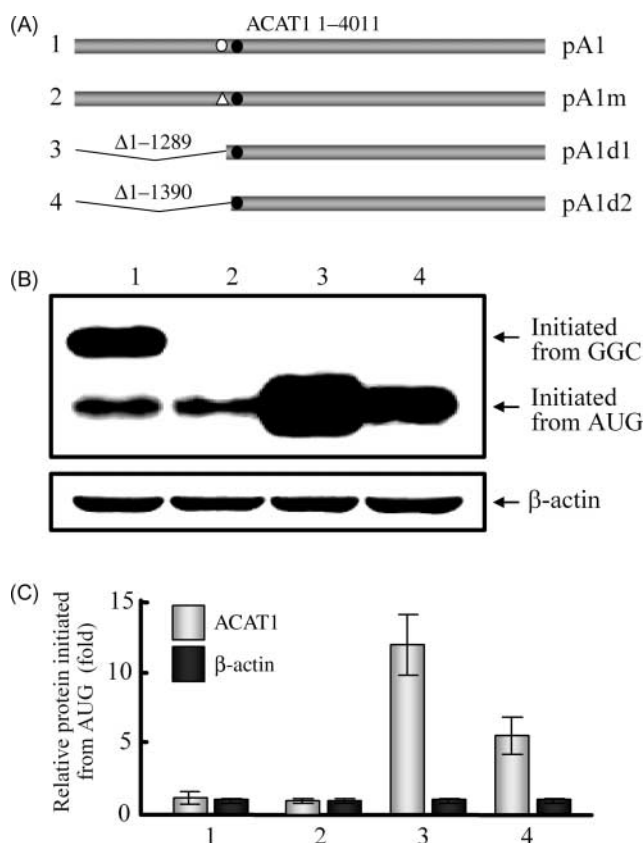


Fig. 1 Effect of the optional long 5'-UTR of human ACAT1 mRNAs on the production of the ACAT1 50 kDa protein initiated from the AUG₁₃₉₇₋₁₃₉₉ codon (A) Schematic representation of the entire human ACAT1 mRNAs with the wild-type (no. 1), mutated (no. 2), and truncated optional long 5'-UTRs (nos. 3 and 4). The GGC₁₂₇₄₋₁₂₇₆ initiation codon for ACAT1 56 kDa isoform was mutated to the UAG stop codon in pA1m and the optional long 5'-UTR was deleted in pA1d1 (Δ1-1289) and pA1d2 (Δ1-1390). Gray bar, ACAT1 mRNA sequence corresponding to the 1-4011 bp of the full-length cDNA K1 (ACAT1 1-4011); hollow circle, GGC₁₂₇₄₋₁₂₇₆ initiation codon; filled circle, AUG₁₃₉₇₋₁₃₉₉ initiation codon; hollow triangle, mutated UAG₁₂₇₄₋₁₂₇₆ stop codon. (B) Western blot analysis. Samples of lanes 1-4 were individually prepared from AC29 cells transiently transfected with the expression plasmids relative to nos. 1-4 depicted in (A). After a 24 h transfection, the lysates were prepared and immunoblotting was carried out with anti-ACAT1 antibodies and anti-β-actin antibody. Arrows indicated the positions of ACAT1 56- and 50 kDa proteins initiated from GGC₁₂₇₄₋₁₂₇₆ and AUG₁₃₉₇₋₁₃₉₉ codons, as well as the control β-actin protein, respectively. The experiments were repeated three times with similar results. (C) Relative ACAT1 proteins initiated from the AUG₁₃₉₇₋₁₃₉₉ codon. The intensities of ACAT1 50 kDa proteins initiated from the AUG₁₃₉₇₋₁₃₉₉ codon and the control β-actin proteins relative to lane 1-4 in (B) were quantified using the UVP Labwork software (UVP Inc., Upland, USA) for densitometric analysis and the protein amount of the cells transfected with pA1m was designated as the control one (1.0). Gray column, the relative ACAT1 proteins initiated from the AUG₁₃₉₇₋₁₃₉₉ codon; black column, the relative β-actin proteins. The data given are mean ± SD from three independent experiments.

were dramatically enhanced by about 11.8- and 5.5-folds [Fig. 1(B), nos. 3 and 4, top panel; Fig. 1(C), nos. 3 and 4, gray columns], respectively. As controls, the levels of β-actin proteins were not obviously altered [Fig. 1(B), bottom panel; Fig. 1(C), black columns]. These demonstrated that the optional long 5'-UTR could impair the production of ACAT1 50 kDa protein initiated from the AUG₁₃₉₇₋₁₃₉₉ codon. It should be noticed that, as shown in Fig. 1(B), the amount of the protein initiated from the AUG₁₃₉₇₋₁₃₉₉ codon in no. 3 was more than that in no. 4, indicating that both the nt 1-1289 and the nt 1-1390 sequences of this 5'-UTR could impair the production of ACAT1 protein initiated from the AUG₁₃₉₇₋₁₃₉₉ codon in different degrees. Consistent with our previous work [13], when the GGC₁₂₇₄₋₁₂₇₆ initiation codon was mutated to the UAG stop codon, the production of ACAT1 56 kDa isoform from this codon was eliminated, whereas the production of ACAT1 50 kDa protein initiated from the AUG₁₃₉₇₋₁₃₉₉ codon was not obviously altered [Fig. 1(B), nos. 1 and 2, top panel] and remained at low level.

To investigate the impairing effect of the optional long 5'-UTR, we further observed the production of the NH₂-terminal fragment of human ACAT1 protein. The new expression plasmids constructed contained the same 5'-UTRs upstream to the AUG₁₃₉₇₋₁₃₉₉ codon in the above ones except that followed by the partial human ACAT1-coding sequence (1397-1786 bp) with 3 × Flag sequence (3 × Flag) at the 3'-end [Fig. 2(A)]. Western blot results [Fig. 2(B) and (C)] showed that the pattern of the truncated ACAT1 proteins from the AUG₁₃₉₇₋₁₃₉₉ initiation codon was identical to that of the full-length ACAT1 proteins [Fig. 1(B) and (C)]. Likewise, both the nt 1-1289 and the nt 1-1390 sequences of this 5'-UTR could identically impair the production of the truncated ACAT1 proteins from the AUG₁₃₉₇₋₁₃₉₉ initiation codon [Fig. 2(B), nos. 7 and 8, top panel] and no obvious change of the β-actin protein control was individually observed [Fig. 2(B), bottom panel). The same results were obtained when an anti-Flag antibody (M2) was employed for western blot analysis (data not shown). These results mean that the 3'-UTR of the human ACAT1 mRNA does not influence the impairing effect of the optional long 5'-UTR on the ACAT1 protein production.

Also, we used the luciferase-coding sequence to replace the ACAT1-coding sequence [Fig. 2(D)], and detected whether the ACAT1-coding sequence can influence the impairing effect of the optional long 5'-UTR. The results of the western blot analysis [Fig. 2(E) and (F)] and the luciferase activity assay (data not shown) displayed that the luciferase protein production was consistent with the

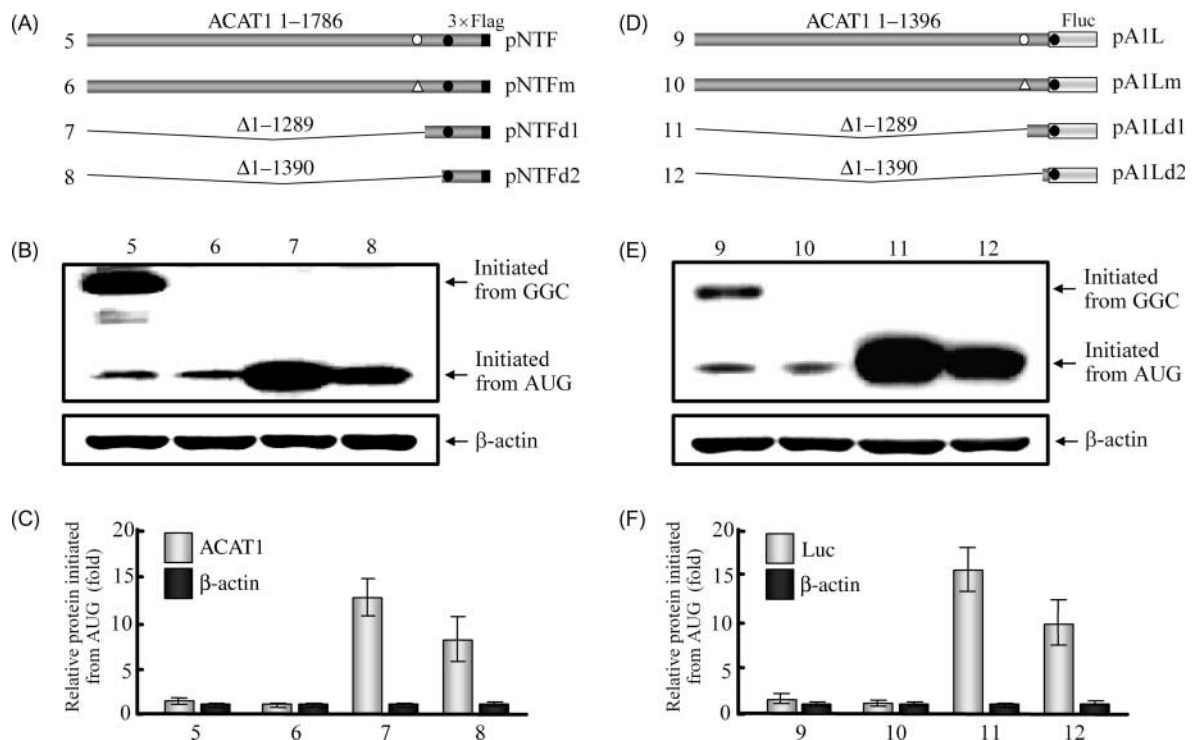


Fig. 2 Effect of the optional long 5'-UTR of human ACAT1 mRNAs on the production of different proteins from its linked mRNAs (A) Schematic representation of the partial human ACAT1 mRNAs with the wild-type (no. 5) and truncated optional long 5'-UTRs (nos. 7 and 8). The GGC₁₂₇₄₋₁₂₇₆ initiation codon for ACAT1 26 kDa NH₂-terminal fragment was mutated to the UAG stop codon in pNTFm and the optional long 5'-UTR was deleted in pNTFd1 (Δ 1-1289) and pNTFd2 (Δ 1-1390). Gray bar, ACAT1 mRNA sequence corresponding to the 1-1786 bp of the partial cDNA K1 (ACAT1 1-1786); black bar, fused in-frame 3 \times Flag coding sequence (3 \times Flag); hollow circle, GGC₁₂₇₄₋₁₂₇₆ initiation codon; filled circle, AUG₁₃₉₇₋₁₃₉₉ initiation codon; hollow triangle, mutated UAG₁₂₇₄₋₁₂₇₆ stop codon. (B) Western blot analysis. Samples of lane 5-8 were individually prepared from AC29 cells transiently transfected with the expression plasmids relative to nos. 5-8 depicted in (A). After a 24 h transfection, the lysates were prepared and immunoblotting was carried out with anti-ACAT1 antibodies and anti- β -actin antibody. Arrows indicated the positions of ACAT1 26- and 18 kDa NH₂-terminal fragments initiated from GGC₁₂₇₄₋₁₂₇₆ and AUG₁₃₉₇₋₁₃₉₉ codons, as well as the control β -actin protein, respectively. The experiments were repeated three times with similar results. (C) Relative ACAT1 proteins initiated from the AUG₁₃₉₇₋₁₃₉₉ codon. The intensities of ACAT1 18 kDa NH₂-terminal fragments initiated from the AUG₁₃₉₇₋₁₃₉₉ codon and the control β -actin proteins relative to lanes 5-8 in (B) were quantified using the UVP Labwork software (UVP Inc.) for densitometric analysis and the protein amount of the cells transfected with pNTFm was designated as the control one (1.0). Gray column, the relative ACAT1 proteins initiated from the AUG₁₃₉₇₋₁₃₉₉ codon; black column, the relative β -actin proteins. The data given are mean \pm SD from three independent experiments. (D) Schematic representation of the luciferase mRNAs linked with the wild-type (no. 9), mutated (no. 10) and truncated optional long 5'-UTRs (nos. 11 and 12) of human ACAT1 mRNAs. The GGC₁₂₇₄₋₁₂₇₆ initiation codon for ACAT1-luciferase-fused 69 kDa protein was mutated to the UAG stop codon in pA1Lm and the optional long 5'-UTR was deleted in pA1Ld1 (Δ 1-1289) and pA1Ld2 (Δ 1-1390). Gray bar, ACAT1 mRNA sequence corresponding to the 1-1396 bp of the partial cDNA K1 (ACAT1 1-1396); hatched bar, luciferase mRNA (Luc); others represent the same in (A). (E) Western blot analysis. Samples of lane 9-12 were individually prepared from AC29 cells transiently transfected with the expression plasmids relative to number 9-12 depicted in (D). After a 24 h transfection, the lysates were prepared and immunoblotting was carried out with anti-luciferase antibody and anti- β -actin antibody. Arrows indicated the positions of ACAT1-luciferase-fused 69 kDa protein and luciferase 61 kDa protein initiated from GGC₁₂₇₄₋₁₂₇₆ and AUG₁₃₉₇₋₁₃₉₉ codons, as well as the control β -actin protein, respectively. The experiments were repeated three times with similar results. (F) Relative luciferase proteins initiated from the AUG₁₃₉₇₋₁₃₉₉ codon. The intensities of luciferase 61 kDa proteins initiated from the AUG₁₃₉₇₋₁₃₉₉ codon and the control β -actin proteins relative to lane 9-12 in (E) were quantified using the UVP Labwork software (UVP Inc.) for densitometric analysis and the protein amount of the cells transfected with pA1Lm was designated as the control one (1.0). Gray column, the relative luciferase proteins initiated from the AUG₁₃₉₇₋₁₃₉₉ codon; black column, the relative β -actin proteins. The data given are mean \pm SD from three independent experiments.

ACAT1 protein production, indicating that the coding sequence of the human ACAT1 mRNA does not influence the impairing effect of the optional long 5'-UTR on the ACAT1 protein production.

These data confirm that the optional long 5'-UTR of human ACAT1 mRNAs potentially impairs the production of different proteins initiated from the AUG₁₃₉₇₋₁₃₉₉ codon, and this impairing effect is not influenced by the

3'-UTR or the coding sequence of human ACAT1 mRNA. In addition, the nt 1–1289 and the nt 1–1390 sequences of this 5'-UTR have impairing effects at different degrees [Fig. 1(B), nos. 3 and 4; Fig. 2(B), nos. 7 and 8; Fig. 2(E), nos. 11 and 12]. It will be very interesting to know how this 5'-UTR impairs the production of proteins.

The optional long 5'-UTR of human ACAT1 mRNAs mainly reduces its mRNA contents

The foregoing data have indicated that the mRNAs linked with the optional long 5'-UTR of human ACAT1 mRNAs can produce the proteins at low level. Since it

has been reported that the 5'-UTR can affect the stability and/or the translational efficiency of mRNA [16], we first tested the effect of this optional long 5'-UTR on the mRNA stability of its linked ACAT1 or luciferase mRNAs. The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed with the specific primer sets AF/AR, LF/LR, and NF/NR for cDNAs of ACAT1, luciferase, and control neomycin [Fig. 3(A) and (C)], respectively. The results revealed that the relative mRNAs with the deletion of the optional long 5'-UTR were increased by about 7.4- and 3.8-folds for ACAT1 [Fig. 3(B), nos. 7 and 8, gray columns] and about 8.3- and 4.8-folds for luciferase [Fig. 3(D), nos.

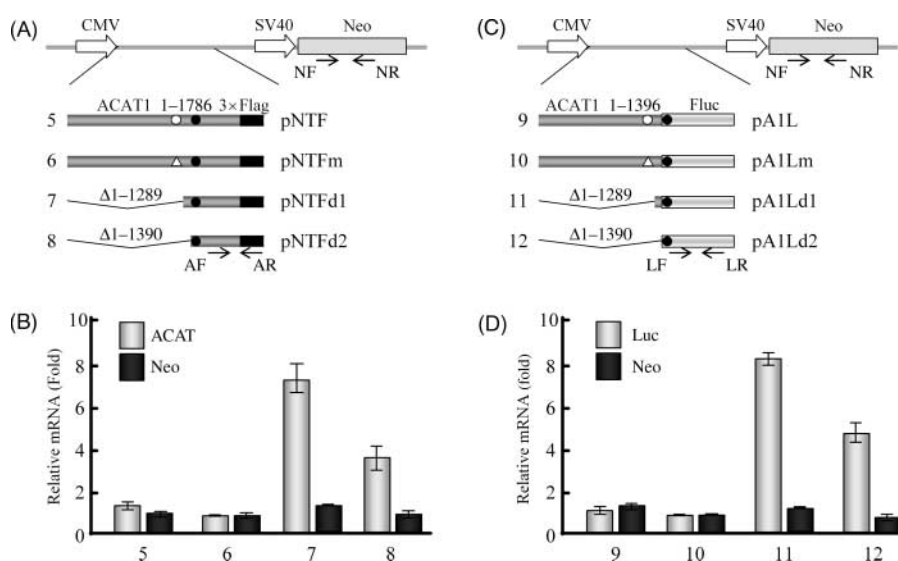


Fig. 3 The optional long 5'-UTR of human ACAT1 mRNAs reduces the contents of its linked mRNAs (A) Schematic representation of relative positions of the partial ACAT1 and neomycin cistrons, and the locations of two PCR primer sets. The partial ACAT1 mRNA sequences linked with the wild-type (no. 5), mutated (no. 6), and truncated optional long 5'-UTRs (nos. 7 and 8) at 5'-end and fused with the in-frame 3 × Flag coding sequence at 3'-end were transcribed under the control of the CMV promoter and at its downstream position, the neomycin mRNA under the control of the SV40 promoter. The primer set AF/AR for detecting the ACAT1 mRNA was located between the ACAT1 and 3 × Flag coding sequences, and the primer set NF/NR for detecting the neomycin mRNA was located in the neomycin coding sequence. Gray bar, ACAT1 mRNA sequence corresponding to the 1–1786 bp of the partial cDNA K1 (ACAT1 1–1786); black bar, fused in-frame 3 × Flag coding sequence (3 × Flag); hollow circle, GGC_{1274–1276} initiation codon; filled circle, AUG_{1397–1399} initiation codon; hollow triangle; mutated UAG_{1274–1276} stop codon. (B) Relative mRNA content. Samples of nos. 5–8 were individually prepared from AC29 cells transiently transfected with the expression plasmids relative to nos. 5–8 depicted in (A). After a 24 h transfection, the total RNAs were prepared and the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of ACAT1 and neomycin mRNAs was performed according to procedures described in the Materials and methods. The mRNA content of the cells transfected with pNTFm was designated as the control one (1.0). Gray column, the relative ACAT1 mRNAs; black column, the relative neomycin mRNAs. The data given are mean ± SD from three independent experiments. (C) Schematic representation of the relative positions of the luciferase and neomycin cistrons, and the locations of two PCR primer sets. The luciferase mRNA linked with the wild-type (no. 9), mutated (no. 10), and truncated optional long 5'-UTRs (nos. 11 and 12) were transcribed under the control of the CMV promoter and at its downstream position, the neomycin mRNA under the control of the SV40 promoter. The primer set LF/LR for detecting the luciferase mRNA was located in the luciferase-coding sequence and the primer set NF/NR for detecting the neomycin mRNA was located in the neomycin-coding sequence. Gray bar, ACAT1 mRNA sequence corresponding to the 1–1396 bp of the partial cDNA K1 (ACAT1 1–1396); hatched bar, luciferase mRNA (Luc); others represent the same in (A). (D) Relative mRNA content. Samples of nos. 9–12 were individually prepared from AC29 cells transiently transfected with the expression plasmids relative to nos. 9–12 depicted in (C). After a 24 h transfection, the total RNAs were prepared and RT-qPCR analysis of luciferase and neomycin mRNAs was performed according to the procedures described in the Materials and methods section. The mRNA content of the cells transfected with pA1Lm was designated as the control one (1.0). Gray column, the relative luciferase mRNAs; black column, the relative neomycin mRNAs. The data given are mean ± SD from three independent experiments.

11 and 12, gray columns] by comparing with those of the undeleted two [Fig. 3(B) and (D), gray columns; nos. 5, 6, 9, and 10], respectively. As controls, the contents of the neomycin mRNAs transcribed from the same plasmids were determined without alteration, which ruled out the transfection variation [Fig. 3(B) and (D), black columns]. Furthermore, as shown in Fig. 3(B) and (D), the relative mRNAs of ACAT1 or luciferase in nos. 7 and 11 were more than those in nos. 8 and 12, which indicated that both nt 1–1289 and nt 1–1390 sequences of the optional long 5'-UTR had reducing effect with different degrees, consistent with the observations at protein level [Fig. 2(B)–(F)]. These demonstrate that the optional long 5'-UTR of human ACAT1 mRNAs potentially reduces the contents of its linked mRNAs.

Then, the translational efficiencies were evaluated using the protein to mRNA ratio and the larger ratio represents the higher translational efficiency [34,35]. The relative ratios of both ACAT1 and luciferase proteins to mRNAs shown in Fig. 4 illustrated that the translational efficiencies with the deletions of the optional long

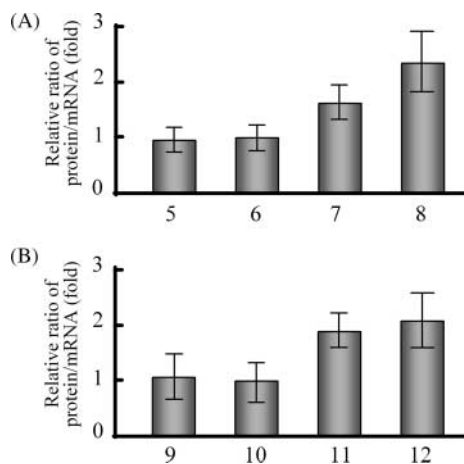


Fig. 4 Effect of the optional long 5'-UTR of human ACAT1 mRNAs on the translational efficiencies of its linked mRNAs (A) Translational efficiencies of the partial human ACAT1 mRNAs linked with the wild-type (no. 5), mutated (no. 6), and truncated optional long 5'-UTRs (nos. 7 and 8). The ratio represented the relative partial ACAT1 protein in Fig. 2(C) divided by the relative partial ACAT1 mRNA in Fig. 3(B), respectively. The translational efficiency of the mRNA derived from pNTFm (no. 6) was designated as the control one (1.0). (B) Translational efficiencies of the luciferase mRNAs linked with the wild-type (no. 9), mutated (no. 10), and truncated optional long 5'-UTRs (nos. 11 and 12) of human ACAT1 mRNA. The ratio represented the relative luciferase protein in Fig. 2(F) divided by the relative luciferase mRNA in Fig. 3(D), respectively. The translational efficiency of the mRNA derived from pA1Lm (no. 10) was designated as the control one (1.0). The data given are mean \pm SD from three independent experiments.

5'-UTR were increased by about 1.7- and 2.3-folds for ACAT1 [Fig. 4(A), nos. 7 and 8] and 1.8- and 2.0-folds for luciferase [Fig. 4(B), nos. 11 and 12] when compared with those of the undeleted two (Fig. 4, nos. 5, 6, 9, and 10), respectively, meaning that this 5'-UTR can decrease the translational efficiency.

Taken together, these data show that the nt 1–1289-deletion (Δ 1–1289) of the optional long 5'-UTR can increase by about 7.4- and 8.3-folds of the relative contents [Fig. 3(B), no. 7 and 3(D), no. 11] and about 1.7- and 1.8-folds of the relative translational efficiencies [Fig. 4(A), no. 7 and 4(B), no. 11] of the mRNAs, and the other deletion (Δ 1–1390) can increase by about 3.8- and 4.8-folds of the relative contents [Fig. 3(B), no. 8 and 3(D), no. 12] and about 2.3- and 2.0-folds of the relative translational efficiencies [Fig. 4(A), no. 8 and 4(B), no. 12] of the mRNAs, respectively. These results obviously indicated that the optional long 5'-UTR of the human ACAT1 mRNAs reduces its mRNA contents rather than its translational efficiencies. Due to the fact that the same CMV promoter was used to control transcription in these experiments, we postulate that the optional long 5'-UTR can impair the protein production mainly resulted from its mRNA decay.

The optional long 5'-UTR of the human ACAT1 mRNAs promotes its mRNA decay in cells

To test the above postulation, we examined the luciferase mRNA contents at different time points after transfection of the relative expression plasmids [Fig. 5(A), no. 10 or 11]. RT-qPCR results displayed that, during the extension of time, the optional long 5'-UTR-linked luciferase mRNA was increased very slightly [Fig. 5(B), no. 10], but the luciferase mRNA without the link was dramatically increased to reach a high level [Fig. 5(B), no. 11]. Moreover, the treatment of transcription inhibitor actinomycin D showed that, during the 4-h incubation, the optional long 5'-UTR-linked luciferase mRNA decayed by about 30%, much faster than the luciferase mRNA without the link [Fig. 5(C), nos. 10 and 11]. These results clearly indicated that the optional long 5'-UTR of human ACAT1 mRNAs promotes its mRNA decay.

In detail, we prepared two relative mRNAs with 5'-cap and 3'-poly(A) tail *in vitro* [Fig. 6(A)], and then transfected them into the cytoplasm by liposome [36,37] or nucleofected them into the nucleus by electroporation [38], respectively. RT-qPCR results showed that the optional long 5'-UTR-linked luciferase mRNA decayed by about 41% in cytoplasm [Fig. 6(B), A1Lm] and about 30% in nucleus [Fig. 6(C), A1Lm], which are

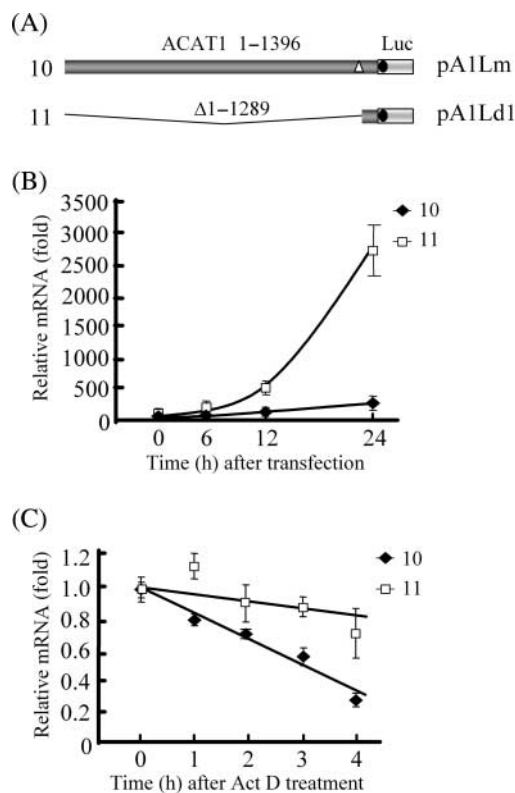


Fig. 5 The optional long 5'-UTR of human ACAT1 mRNAs promotes the decay of its linked mRNAs (A) Schematic representation of luciferase mRNAs linked with the mutated (no. 10) and truncated optional long 5'-UTRs (no. 11) of human ACAT1 mRNA. The GGC₁₂₇₄₋₁₂₇₆ initiation codon was mutated to the UAG stop codon in pA1Lm and the optional long 5'-UTR was deleted (Δ1-1289) in pA1Ld1. Gray bar, ACAT1 mRNA sequence corresponding to the 1-1396 bp of the partial cDNA K1 (ACAT1 1-1396); hatched bar, luciferase mRNA (Luc); filled circle, AUG₁₃₉₇₋₁₃₉₉ initiation codon; hollow triangle; mutated UAG₁₂₇₄₋₁₂₇₆ stop codon. (B) Relative mRNA content. Samples of number 10 and 11 were individually prepared from AC29 cells transiently transfected with the expression plasmids relative to nos. 10 or 11 depicted in (A). After 4 h of transfection, the total RNAs were prepared at the points of time indicated. reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of luciferase and GAPDH mRNAs was performed according to procedures described in the Materials and methods. Hollow square, the relative luciferase mRNAs of the cells transfected with pA1Lm; filled diamond, the relative luciferase mRNAs of the cells transfected with pA1Ld1. (C) Relative mRNA content. Samples of nos. 10 and 11 were individually prepared from AC29 cells transiently transfected with the expression plasmids relative to nos. 10 or 11 depicted in (A). After a 24 h transfection, the cells were treated with actinomycin D (5 μg/ml) and the total RNAs were prepared at the points of time indicated. RT-qPCR analysis of luciferase and GAPDH mRNAs was performed according to procedures described in the Materials and methods. Hollow square, the relative luciferase mRNAs of the cells transfected with pA1Lm; filled diamond, the relative luciferase mRNAs of the cells transfected with pA1Ld1. The luciferase mRNA contents were normalized to the GAPDH mRNA contents for all samples and the relative luciferase mRNA at 0 h was designated as the control one (1.0). The data given are mean ± SD from three independent experiments.

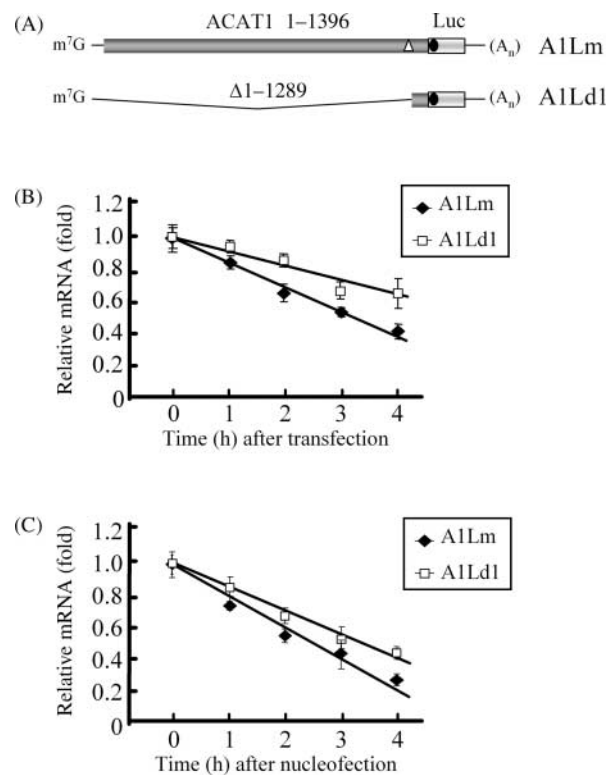


Fig. 6 The mRNA linked with the optional long 5'-UTR of human ACAT1 mRNAs decays faster in both cytoplasm and nucleus (A) Schematic representation of *in vitro* prepared luciferase mRNAs linked with the mutated (A1Lm) and truncated optional long 5'-UTRs (A1Ld1) of human ACAT1 mRNA. The GGC₁₂₇₄₋₁₂₇₆ initiation codon was mutated to the UAG stop codon in A1Lm and the optional long 5'-UTR was deleted (Δ1-1289) in A1Ld1. The mRNA A1Lm and A1Ld1 were transcribed, capped, and poly-adenylated *in vitro*. Gray bar, ACAT1 mRNA sequence corresponding to the 1-1396 bp of the partial cDNA K1 (ACAT1 1-1396); hatched bar, luciferase mRNA (Luc); filled circle, AUG₁₃₉₇₋₁₃₉₉ initiation codon; hollow triangle; mutated UAG₁₂₇₄₋₁₂₇₆ stop codon. (B) Relative mRNA content. The *in vitro* prepared mRNAs relative to A1Lm and A1Ld1 depicted in (A) are transiently transfected into the cytoplasm of AC29 cells according to the procedures described in the Materials and methods. After 4 h, the total RNAs were prepared at the points of time indicated. reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of luciferase and GAPDH mRNAs was performed according to the procedures described in the Materials and methods. Hollow square, the relative luciferase mRNAs of the cells transfected with A1Lm; filled diamond, the relative luciferase mRNAs of the cells transfected with A1Ld1. (C) Relative mRNA content. The *in vitro* prepared mRNAs relative to A1Lm and A1Ld1 depicted in (A) are transiently nucleofected into the nucleus of AC29 cells according to procedures described in the Materials and methods. After 4 h, the total RNAs were prepared at the points of time indicated. RT-qPCR analysis of luciferase and GAPDH mRNAs was performed according to procedures described in the Materials and methods. Hollow square, the relative luciferase mRNAs of the cells nucleofected with A1Lm; filled diamond, the relative luciferase mRNAs of the cells nucleofected with A1Ld1. The luciferase mRNA contents were normalized to the GAPDH mRNA contents for all the samples and the relative luciferase mRNA at 0 h was designated as the control one (1.0). The data given are mean ± SD from three independent experiments.

much faster than those of the luciferase mRNA without the link [Fig. 6(B) and (C), A1Ld1]. These demonstrate that the optional long 5'-UTR-linked mRNAs, which were prepared *in vitro*, decayed both in the cytoplasm and nucleus of the cells. It is confirmed that the optional long 5'-UTR of the human ACAT1 mRNAs promotes its mRNA decay in cells.

Discussion

In this study, we find that the optional long 5'-UTR of human ACAT1 mRNAs can impair the production of ACAT1 protein mainly by promoting its mRNA decay. First, we test whether the optional long 5'-UTR affects the production of ACAT1 protein initiated from the AUG₁₃₉₇₋₁₃₉₉ codon. The western blot results indicate that the production of human ACAT1 50 kDa protein initiated from the AUG₁₃₉₇₋₁₃₉₉ codon is dramatically increased when the optional long 5'-UTR is deleted (Fig. 1). By deleting the partial human ACAT1-coding sequence at the 3'-region and replacing the entire human ACAT1-coding sequence with the luciferase-coding sequence, the optional long 5'-UTR can also impair the production of proteins from the AUG₁₃₉₇₋₁₃₉₉ codon (Fig. 2). These indicate that the impairing effect of this 5'-UTR on the protein production is not influenced by the 3'-UTR or the coding sequence of human ACAT1 mRNA. Further experiments reveal that this 5'-UTR mainly promotes its mRNA decay besides influencing its translational efficiency. Collectively, this work brings forward a new insight to the regulation of the human ACAT1 gene expression at the post-transcription level.

Recently, more and more evidences revealed that expression regulation of genes at post-transcriptional levels is crucial for all organisms, from bacteria to mammals [39]. The post-transcriptional regulation determines the fate of the RNAs, including their subcellular localization, stability, and the efficiency of their translation [16]. This is controlled by specific RNA-binding proteins and/or non-coding RNAs, which bind to specific sequences or structural elements in the RNAs and thereby regulating functions of mRNA subsets [40]. In the current work, we find that the decay of the human ACAT1 mRNA can be obviously promoted by its optional long 5'-UTR. Our work implied that the optional long 5'-UTR of the human ACAT1 mRNAs may regulate the mRNA stability by certain *cis*-acting sequences or elements that interact with *trans*-acting proteins and/or non-coding RNAs. Further investigations are needed to test this possibility.

In this work, the optional long 5'-UTR of human ACAT1 mRNAs can also decrease its translational efficiency. Generally, most 5'-UTRs can affect the translational efficiency of their mRNAs [16,17]. Kozak's work [19] has indicated that the secondary structures from a 5'-UTR can result in at least four-fold decrease of the translational efficiency. However, the optional long 5'-UTR can only lead to the 2.3-fold decrease of the translational efficiency of human ACAT1 mRNA. Hence, the decreasing effect of the optional long 5'-UTR on the translational efficiency does not reach the level reported by Kozak, suggesting that this 5'-UTR might contain some activating elements increasing the translational efficiency besides the decreasing effect of Kozak's structures. It is consistent with our previous study that the IRES exists in the optional long 5'-UTR of human ACAT1 mRNAs, and induces the cap-independent ribosome initiation to increase translational efficiency to some extent [14].

It has been reported that the RNA secondary structures in 5'-UTR can affect its mRNA stability [41,42]. We find that the human ACAT1 mRNA can be destabilized by its optional long 5'-UTR, in which there are multiple predicted RNA secondary structures (Fig. 7, RNA secondary structures I–VIII). Previously, we revealed that the RNA secondary structure VIII can enhance the selection of the downstream AUG₁₃₉₇₋₁₃₉₉ as an initiation codon to produce the human ACAT1 50 kDa isoform [15], and the upstream VI and downstream VII of the GGC₁₂₇₄₋₁₂₇₆ initiation codon are required for the

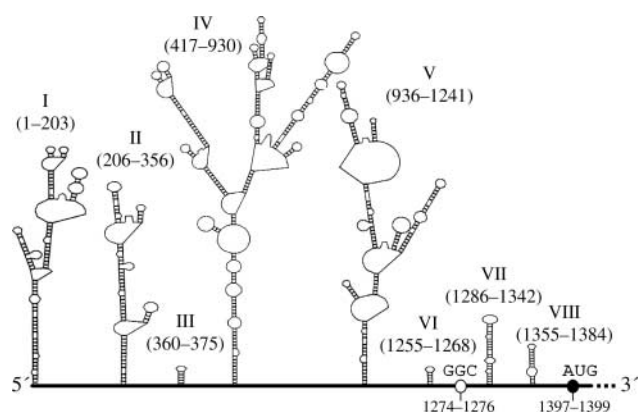


Fig. 7 The predicted RNA secondary structures in the optional long 5'-UTR of human ACAT1 mRNAs. Predicted RNA secondary structures in the optional long 5'-UTR of human ACAT1 mRNAs. Eight RNA secondary structures were labeled with I–VIII and the relative regions were indicated in parentheses, respectively. Hollow circle, GGC₁₂₇₄₋₁₂₇₆ initiation codon; filled circle, AUG₁₃₉₇₋₁₃₉₉ initiation codon.

production of human ACAT1 56 kDa isoform [14]. Interestingly, the results in this work show that the nt 1–1289 sequence containing the RNA secondary structures I–VI in the optional long 5'-UTR can destabilize the human ACAT1 mRNA [Fig. 3(B), comparing nos. 5 and 6 with no. 7], but the nt 1290–1390 sequence containing the other VII and VIII exhibits the stabilizing effect [Fig. 3(B), comparing no. 7 with no. 8] in the absence of the RNA secondary structures I–VI. It is suggested that the RNA secondary structures I–VI might recruit certain proteins or non-coding RNAs as mRNA destabilizing factors to promote its mRNA decay, whereas the RNA secondary structures VII and VIII might serve as the mRNA-stabilizing elements. More importantly, the nt 1–1279 and 1290–1390 sequences in the optional long 5'-UTR (nt 1–1396) of the human ACAT1 mRNA corresponding to cDNA K1 are from different chromosomes, 7 and 1 [12]. Thus, we propose that they could regulate the human ACAT1 gene expression by destabilizing or stabilizing their mRNAs under different conditions, respectively, which might provide a more elaborate regulation of ACAT1 activity for cellular cholesterol homeostasis.

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