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A Stable Upstream Stem-loop Structure Enhances Selection of the First 5′-ORF-AUG as a Main Start Codon for Translation Initiation of Human ACAT1 mRNA

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Abstract Human ACAT1 cDNA K1 was first cloned and functionally expressed in 1993. There are two adjacent in-frame AUG codons, $AUG_{1397–1399}$ and $AUG_{1415–1417}$, at 5′-terminus of the open reading frame (ORF, nt 1397–3049) of human ACAT1 mRNA corresponding to cDNA K1. In current work, these two adjacent in-frame AUGs at 5′-terminus of the predicted ORF (5'-ORF-AUGs) as start codons for translation initiation of human ACAT1 mRNA were characterized in detail. Codon mutations indicated that both of these two adjacent 5′-ORF-AUGs can be selected as start codons but the first 5′-ORF-$AUG_{1397–1399}$ is a main start codon consistent with that of the predicted ORF of human ACAT1 mRNA. Further deletion and mutation analyses demonstrated that a stable upstream stem-loop structure enhanced the selection of the first 5′-ORF-$AUG_{1397–1399}$ as a main start codon, in addition to upstream nucleotide A in the –3 position, which is a key site of Kozak sequence. In addition, result of ACAT1 enzymatic activity assay showed no obvious difference between these two ACAT1 proteins respectively initiated from the two adjacent 5'-ORF-AUGs. This work showed that a stable upstream stem-loop structure could modulate the start codon selection during translation initiation of mRNAs that contain adjacent multi-5′-ORF-AUGs.

Key words ACAT1; start codon; Kozak sequence; stem-loop structure

Acyl-coenzyme A:cholesterol acyltransferase (ACAT) is an integral membrane protein, which is mainly located in rough endoplasmic reticulum (ER), and is responsible for catalyzing the intracellular formation of cholesteryl ester from cholesterol and long-chain fatty acyl-coenzyme A [1,2]. Human ACAT1 cDNA K1 was firstly cloned and functionally expressed in 1993 [3]. Further studies with specific anti-ACAT1 antibody (DM10) illustrated that one major 50 kD ACAT1 protein was expressed in various human cells, tissues and transfected AC29 cells [4–6]. The human ACAT1 mRNA sequence corresponding to the cDNA K1 ORF region contains two adjacent in-frame AUG codons, $AUG_{1397–1399}$ and $AUG_{1415–1417}$ at 5′-terminus (5′-ORF-AUGs), which may be regarded as start codons from fourteen AUG codons in the whole ORF for translation initiation of human ACAT1 mRNA. So far, no experimental evidence shows that translation of the major 50 kD ACAT1 protein is initiated from the 5′-ORF-AUGs.

Mammalian mRNA usually initiates translation from the first 5′-ORF-AUG codon, according to the ribosome scanning model, which is ensured by its optimal context motif conforming to all or part of Kozak sequence GCCRCCaugG (R=purine) [7]. The most highly conserved

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The abbreviations used are: ACAT, acyl-coenzyme A:cholesterol acyltransferase; kb, kilobase(s); bp, base pair(s); cDNA, complementary DNA; nt, nucleotide(s); ORF, open reading frame; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis

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position within Kozak sequence is the purine (usually A) in the –3 position (3 nt before the codon AUG which is numbered +1 to +3). Mutations affecting A in the –3 position (A^{-3}) strongly impair initiation *in vivo* and *in vitro* [8]. Furthermore, the G in the +4 position (G^{+4}) is also highly conserved and contributes strongly, especially in the absence of A^{-3} [9]. In addition, the rest of the GCCRCCaugG motif varies and contributes partially for translation initiation, especially when both the purine in the –3 position and G in the +4 position are lacking [7].

It is also shown that the stable stem-loop structure downstream to AUG codon can increase the translation efficiency from this AUG codon, especially the AUG codon in a suboptimal context motif [10]. A likely explanation for enhancing effect of a downstream stem-loop structure is that the downstream secondary structure can slow ribosome scanning, thereby providing more time for recognition of the upstream AUG codon and preventing possible leaking scanning from the AUG codon in the suboptimal context motif.

Meanwhile, translation initiation is usually down-regulated with a stable upstream stem-loop structure [11, 12]. On one hand, a stable upstream stem-loop structure can impair translation by preventing the ribosomal 40S subunits from engaging to mRNA when the secondary structure occurs near to the 5′-end cap region. On the other hand, a stable upstream stem-loop structure can presumably weaken the migrating ribosomal subunit at this secondary structure, preventing the ribosome from accessing to the downstream AUG codon.

So far, little is known about how the upstream stem-loop structure modulates the start codon selection of the mRNA containing adjacent multi-5′-ORF-AUGs. In the current work, we demonstrated that both of the two adjacent 5′-ORF-AUGs of human ACAT1 mRNA could be used as start codons for translation initiation but the first 5′-ORF-$AUG_{1397–1399}$ was a main start codon. More interesting, it was also elucidated that a stable upstream stem-loop structure modulated selection of the first 5′-ORF-$AUG_{1397–1399}$ as a main start codon for translation initiation of human ACAT1 mRNA.

Materials and Methods

Materials

Cell culture reagents and T4 DNA ligase were purchased from Life Technologies (Rockville, USA). All the restriction enzymes and agarose were from Promega. Anti-rabbit IgG conjugated with HRP was from Pierce (Rock-ford, USA). ECL detection reagent was from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). *Taq* DNA polymerase and dNTPs were from Sino-American Biotech (Shanghai, China). All the oligonucleotides were synthesized with an automated DNA synthesizer at Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Cell culture

The ACAT-deficient mutant cell line (AC29, [10]) derived from CHO was maintained in a basal Ham's F12 medium, supplemented with 10% fetal bovine serum (FBS) and antibiotics, in a humid atmosphere of 5% CO_2 and 95% air at 37 °C.

Expression plasmids

The partial human ACAT1 cDNA K1 sequence (1304–1786 bp) was amplified by PCR using the forward (LDSF, 5′-AGGGCTCGAGGTCGACCTTCCTGCTG-3′) and reverse (HAHR, 5′-AAGCGACTCTAGAGGATCGATC-3′) primers that contain *Xho*I or *Xba*I site (underlined). The amplified fragments (about 500 bp) were then individually purified, digested with *Xho*I and *Xba*I, and inserted into the *Xho*I and *Xba*I sites of pcDNA3 (Invitrogen) with in-frame downstream stop codon TAG to generate expression plasmid for human ACAT1 N-terminal product, named pcNLD13.

Either or both of the two adjacent AUG codons at 5′-terminus of the open reading frame (ORF) of human ACAT1 mRNA corresponding to cDNA K1 were mutated by a modified two-step PCR method described by Higuchi *et al.* [13]. Briefly, the first reaction was performed by using the above forward primer LDSF hybridized to 5′-region of the partial human ACAT1 cDNA K1 sequence (1304–1786 bp) inserted into the above constructed expression plasmid, and an internal reverse primer for the desired mutation by mismatched base(s). The second reaction was performed by using an internal forward primer for the same desired mutation by mismatched base(s) and the above reverse primer (HAHR) hybridized to the 3′-end of the partial human ACAT1 cDNA K1 sequence inserted into the above constructed expression plasmid. These two overlapping DNA fragments generated by the above different PCRs are "fused" by denaturing and annealing in a subsequent primer extension reaction. Finally, the "fusion" and extension product was amplified by PCR using LDSF as forward primer and HAHR as reverse primer. An additional AUG codon was inserted directly upstream to the two mutated ORF-AUG codons

at 5′-terminus of the open reading frame (ORF) of human ACAT1 mRNA corresponding to partial cDNA K1 (1304–1786 bp) by the two-step PCR. The final PCR products containing the mutated codon(s) were digested with *Xho*I and *Xba*I and inserted into the *Xho*I/*Xba*I sites of pcDNA3 vector to generate expression plasmid containing partial ACAT1 cDNA K1 sequence (1304–1786 bp) with mutant codon(s) replacing either or both of the first two ORF-AUG codons for human ACAT1 N-terminal product. The related internal reverse/forward primers used and the relevant plasmid names were listed in Table 1.

The other mutations and deletions of partial human ACAT1 cDNA K1 sequence (1304–1786 bp) were also achieved by using the modified strategy of two-step PCR as above. The final PCR products of partial human ACAT1 cDNA K1 sequence (1304–1786 bp) containing deletion or mutation were digested with *Xho*I and *Xba*I and inserted into the *Xho*I/*Xba*I sites of pcDNA3 vector to generate expression plasmid for the N-terminal region of human ACAT1. The relative internal reverse and forward primers listed in Table 2 and 3 were respectively used to generate different deletions (Δ1340–1396, Δ1355–1384 and Δ1362–1379) and mutations (the nucleotide A_{1394} replaced respectively by U, substitution and disruption of the

Table 1 Internal reverse (R)/forward (F) primers for codon mutations and relevant plasmids

Plasmid name	Sequences of internal reverse (R) and forward (F) primers	
pcNLD13M45	R	5′-CTTCTCTTCACCCACGGCTGTATTGTCTGAG-3′
	F	5′-CTCAGACAATACAGCCGTGGGTGAAGAGAAG-3′
pcNLD13M46	R	5′-CTTCTCTTCACCCACCTATGTATTGTCTGAG-3′
	F	5′-TCAGACAATACATAGGTGGGTGAAGAGAAG-3′
pcNLD13M47	R	5′-GTTTCTTAGAGAGGCCTTCTCTTCACCCACGGCTGTATTGTCTGAG-3′
	F	5′-CTCAGACAATACAGCCGTGGGTGAAGAGAAGGCCTCTCTAAGAAACCGGCTG-3′
pcNLD13M48	R	5′-GTTTCTTAGAGAGGCCTTCTCTTCACCCAC-3′
	F	5′-GGTGAAGAGAAGGCCTCTCTAAGAAACCGGCTG-3′
pcNLD13M51	R	5′-GTTTCTTAGAGAGGCCTTCTCTTCACCCACGGCCATTGTATTGTCTGAG-3′
	F	5′-CTCAGACAATACAATGGCCGTGGGTGAAGAGAAGGCCTCTCTAAGAAACCGGCTG-3′

The mutated codons were underlined.

Table 2 Internal reverse (R)/forward (F) primers for deletions and relevant plasmids

Plasmid name	Sequences of internal reverse (R) and forward (F) primers	
pcNLD14	R	5′-TTCACCCACCATGGAAGCGGTCACAGAG-3′
	F	5′-GTGACCGCTTCCATGGTGGGTGAAGAG-3′
pcNLD15	R	5′-TGTCTGAGGCCCTTCGGCCAAGAG-3′
	F	5′-CTCTGGGCCGAAGGGCCTCAGACAATAC-3′
pcNLD16	R	5′-TGTATTGTCTGAAAGAGGGCAGAGCCGGGAAG-3′
	F	5′-CTCTGCCCTCTTTCAGACAATACAATGGTG-3′

Table 3 Internal reverse (R)/forward (F) primers for nucleotide mutations and relevant plasmids

Plasmid name	Sequences of internal reverse (R) and forward (F) primers	
pcNLD13M39	R	5′-CACCCACCATTGAATTGTCTGAAGCCCGCGCCCGGCAG-3′
	F	5′-GCCTCAGACAATTCAATGGTGGGTGAAG-3′
pcNLD13M54	R	5′-TCTGAGCGCCGCGGGCGGCAGCGCCCACTTCGCGCAAGAGGGCAGAGCCGGGAAG-3′
	F	5′-CTCTTGCGCGAAGTGGGCGCTGCCGCCCGCGGCGCTCAGACAATACAATGGTGGGTG-3′
pcNLD13M55	R	5′-TCTGAGTTCCGCGTTCGGCAGCGGGCACTTCGGCCAAGAGGGCAGAGCCGGGAAG-3′
	F	5′-CTCTTGGCCGAAGTGCCCGCTGCCGAACGCGGAACTCAGACAATACAATGGTGGGTG-3′

The mutated codons were underlined.

upstream stem-loop structure from 1355 to 1384).

All the deletion and mutation plasmids were confirmed by restriction enzyme digestion and DNA sequencing. For enzymatic activity assays, the expression plasmids (pcDNA3A1D4, pcDNA3A1D4M60, pcDNA3A1D4-M61, and pcDNA3A1D4M51) containing partial human ACAT1 cDNA K1 sequence (1304–4011 bp) including the whole ORF sequence (1397–3049 bp) were constructed by substitution of the *Bsu*36I/*Xba*I ACAT1 cDNA fragment (1454–4011 bp) for the same *Bsu*36I/*Xba*I ACAT1 cDNA fragment (1454–1786 bp) in above constructed expression plasmids (pcNLD13, pcNLD13M45, pcNLD13M48, pcNLD13M51, respectively) containing the partial ACAT1 cDNA K1 sequence (1304–1786 bp).

Transfection

The constructed expression plasmids were individually transfected into AC29 cells by using the method of calcium phosphate co-precipitation described by Liu *et al*. [14] with slight modifications. Briefly, cells were seeded at density of 5×10^5 cells per 60 mm dish in 5 ml of culture medium containing 10% FBS for 36 h. Cells were then sequentially cultured with fresh medium containing 10% FBS for 2 h and medium without FBS for 30 min before transfection. 1.2 ml of transfection reagent was firstly prepared by gently mixing equal volume of DNA/calcium ions (15 μg DNA for per 60 mm dish and 125 mM calcium ions at final concentration) and HeBS (final concentration: 21 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM glucose), then added into medium and incubated with cells at 37 °C for 9 h. After that cells were washed once with PBS, replaced with fresh medium containing 10% FBS, and cultured for another 48 h.

Preparation of protein samples and Western blot analysis

Cells were harvested and lysed with 10% SDS in 50 mM Tris (pH 7.5), 1 mM EDTA (pH 7.5), 50 mM DTT, plus protease inhibitor cocktail (Sigma), incubated at 37 °C and sheared with a syringe fitted with an 18-gauge needle to homogeneity. Protein concentrations of the cell lysates were determined by a modified Lowry method [15]. The protein samples were then subjected to SDS-PAGE. After gel separation, the proteins were transferred to nitrocellulose membrane. The membrane was treated successively 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 2 h, with affinity purified rabbit anti-ACAT1 antibody (DM10) for 3 h, and with HRP-conjugated goat anti-rabbit antibody for 1 h. After incubation, the membrane was washed extensively with TBST and TBS (50 mM Tris-HCl, pH 7.6, and 0.15 M NaCl), respectively. The immunoreactive band was visualized by using ECL detection reagent.

Enzymatic activity assay

AC29 cells were individually transfected with pcDNA3 and plasmids expressing the human ACAT1 mRNA relative to number 1 to 4 in Fig. 5(A). [^{3}H]-oleate pulse in intact cells and Western blot with a specific anti-ACAT1 antibody DM10 were performed after the transfection. The [^{3}H]-oleate pulse assay, measuring [^{3}H]-cholesteryl ester formation by ACAT, was performed essentially as described previously [5,16]. ACAT1 activities were normalized by protein amounts demonstrated on the parallel Western blot analysis with DM10.

Other methods

Standard molecular biology techniques were performed according to the methods described by Sambrook *et al*. [17].

Results

Translation initiation of two adjacent 5′-ORF-AUGs in human ACAT1 mRNA

Human ACAT1 cDNA K1 was first identified from a human macrophage cDNA library by Chang and colleagues [4]. With an in-frame stop codon UAA located at 150 nucleotides upstream to the predicted ORF [Fig. 1 (A)], there are two adjacent in-frame AUG codons (named as 5′-ORF-$AUG_{1397–1399}$ and 5'-ORF-$AUG_{1415–1417}$, respectively) at 5′-terminus of the predicted open reading frame (ORF, nt 1397–3049) of mRNA corresponding to human ACAT1 cDNA K1. Sequence analysis [Fig. 1(B)] shows that the first 5′-ORF-$AUG_{1397–1399}$ is flanked with nucleotides A^{-3} (3 nt before the codon AUG which is numbered 1 to 3) and G^{+4} (the the nucleotide after the codon AUG which is numbered 1 to 3), which are exactly consistent with those of Kozak sequence [7], while the flanking nucleotide U^{+4} of second 5′-ORF-$AUG_{1415–1417}$ is not consistent with that of Kozak sequence. This suggests that both of these two adjacent 5′-ORF-AUGs may be used as start codons for translation initiation of human ACAT1 mRNA but the first 5′-ORF-$AUG_{1397–1399}$ is more favorable to be selected as a start codon according to Kozak sequence.

To identify the start codon for translation initiation,

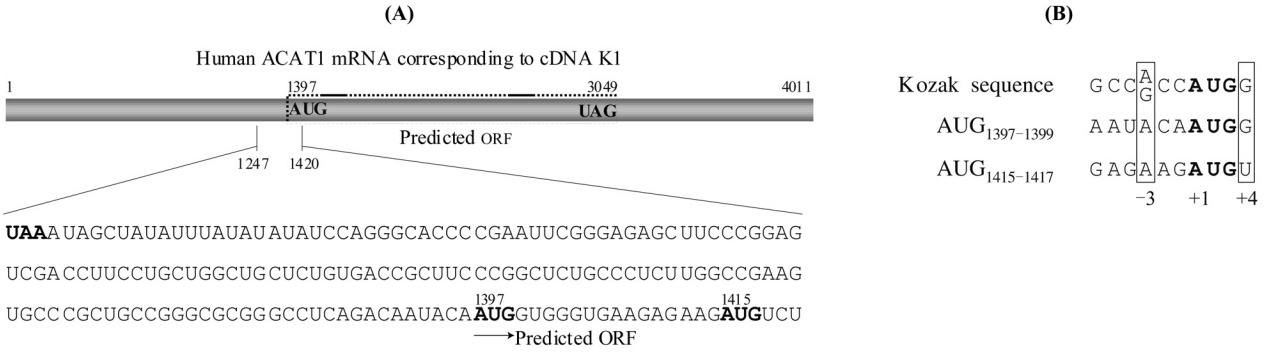

Fig. 1 Upstream and 5′-terminal sequence of the predicted ORF of human ACAT1 mRNA

(A) Schematic diagram of human ACAT1 mRNA (nt 1–4011) corresponding to cDNA K1 [3], and the upstream and 5′-terminal sequences (nt 1247–1420) of the predicted ORF (nt 1397–3049). The in-frame two adjacent 5′-ORF-AUGs (AUGs at 5′-terminus of the predicted ORF) and upstream UAA codons are in bold. (B) Comparison among flanking sequences of two adjacent 5′-ORF-AUGs in human ACAT1 mRNA and Kozak sequence [7]. The AUGs are in bold. The nucleotides of AUG are named +1 to +3, and the upstream positions –3 and downstream +4 are boxed.

either or both of these two adjacent 5′-ORF-AUGs of human ACAT1 mRNA were replaced by codon GCC or UAG [Fig. 2(A)]. The plasmids expressing the partial human ACAT1 mRNAs (nt 1304–1786) with or without relevant mutation(s) were constructed and individually transfected into AC29 cells for the transient expression assays. The total proteins of the transfected AC29 cells were used for Western blot analysis with a specific anti-ACAT1 antibody (DM10). When both of two adjacent 5′-ORF-AUGs in the partial human ACAT1 mRNA (nt 1304–1786) are present [Fig. 2(A), number 1], two special protein bands with sizes of 17 kD (more than 95%) and 16 kD (less than 5 %) human ACAT1 N-terminal products (ACAT1-NTPs) were observed [Fig. 2(B), lane 1]. When the second 5′-ORF-$AUG_{1415–1417}$ is maintained and the first 5′-ORF-$AUG_{1397–1399}$ was displaced by GCC [Fig. 2(A), number 2] or UAG [Fig. 2(A), number 5], only special 16 kD ACAT1-NTP was examined [Fig. 2(B), lanes 2 and 5], suggesting that the second 5′-ORF-$AUG_{1415–1417}$ is used as a start codon for translation initiation of 16 kD ACAT1-NTP. If the second 5′-ORF-$AUG_{1415–1417}$ was displaced by GCC, only special 17 kD ACAT1-NTP initiated from the first 5′-ORF-$AUG_{1397–1399}$ was detected [Fig. 2(B), lane 3]. It was understandable that neither of 17 kD or 16 kD ACAT1-NTP could be observed [Fig. 2(B), lane 4] with both 5′-ORF-AUGs displaced by GCC [Fig. 2(A), number 4].

From these results, it was concluded that both of the two adjacent 5′-ORF-AUGs in human ACAT1 mRNA could be used as start codons for translation initiation, but the first 5′-ORF-$AUG_{1397–1399}$ was selected as a main start codon (more than 95%) for ACAT1 expression. The very

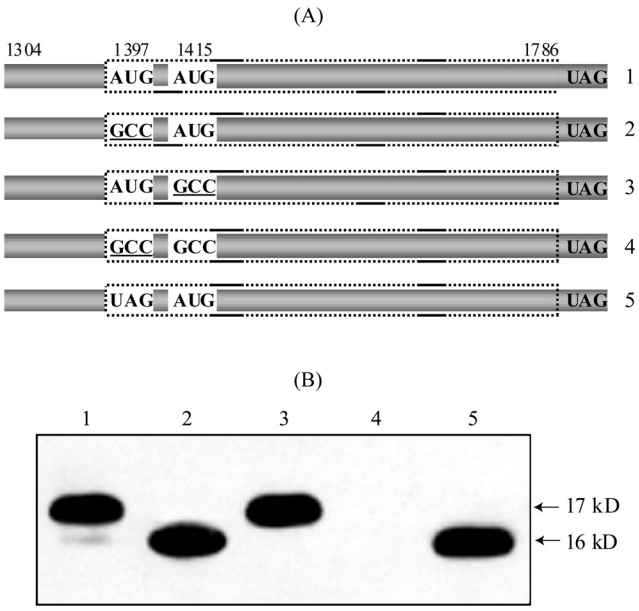

Fig. 2 Both of two adjacent 5′-ORF-AUGs as start codons for translation initiation of human ACAT1 mRNA

(A) Schematic diagram of the partial human ACAT1 mRNAs (nt 1304–1786, Number 1 to 5) corresponding to cDNA K1 sequences respectively inserted into the expression plasmids pcNLD13, pcNLD13M45, pcNLD13M48, pcNLD13M47 and pcNLD13M46. In number 2 to 5 (mutants), either or both of two adjacent 5'-ORF-AUGs (bold) shown in number 1 (wild type) were replaced by the mutated GCC or UAG (bold and underlined). (B) Western blot analysis. Samples of lane 1 to 5 were individually prepared from AC29 cells transfected with the plasmids expressing wild type and mutants relative to number 1 to 5 in above A. The detailed experimental performances are described under "Materials and Methods".

different efficiencies of translation initiated from these two adjacent 5′-ORF-AUGs [Fig. 2(B), lane 1] suggested that

the upstream sequence might contribute to selection of the first 5′-ORF-AUG$_{1397–1399}$ as a main start codon by affecting ribosome scanning.

Enhancement of a stable upstream stem-loop structure on selection of the first 5′-ORF-AUG as a main start codon

Therefore, the effect of the upstream sequence [Fig. 3 (A)] on the first 5′-ORF-AUG$_{1397–1399}$ was further studied by mutation and deletion. Mutating [Fig. 3(B), number 2] the A$_{1394}$ at the –3 position of the first 5′-ORF-AUG$_{1397–1399}$ to pyridine U reduced the expression of 17 kD ACAT1-NTP with the increased expression of 16 kD ACAT1-NTP [Fig. 3(C), lane 2] to about 37% of total 17 and 16 kD ACAT1-NTPs [Fig. 3(D), lane 2], indicating that the A$_{1394}$ at the -3 position, which is the same site exactly as Kozak sequence, plays an important role in selection of the first 5′-ORF-AUG$_{1397–1399}$ as a main start codon for translation initiation of human ACAT1 mRNA. Deletion of nucleotides 1340 to 1396 including the nucleotide A$_{1394}$ [Fig. 3 (B), number 3] further increased the expression of 16 kD ACAT1-NTP [Fig. 3(C), lane 3] to about 60% of total ACAT1-NTPs [Fig. 3(D), lane 3], showing that in addition to the A$_{1394}$ at the –3 position, existence of another factor in this deleted sequence promoted selection (about 20%) of the first 5′-ORF-AUG$_{1397–1399}$ as a main start codon.

So, the possible modulation of nucleotides 1340 to 1396 on the start codon selection from the two adjacent 5′-ORF-AUGs was further detected. Computer analysis showed a stem-loop structure [nt 1355–1384, Fig. 4(A)] located in the region of nucleotides 1340 to 1396, and then the effect of the predicated upstream stem-loop structure on selection of the first 5′-ORF-AUG$_{1397–1399}$ as a main start codon was investigated. Mutation by interconversion of G-C pairs [Fig. 4(B), number 2], which maintained the predicated stem-loop structure, did not change the expression of 17 and 16 kD ACAT1-NTPs [Fig. 4(C,D), comparing lane 2 with lane 1]. When introducing four unpaired A nucleotides

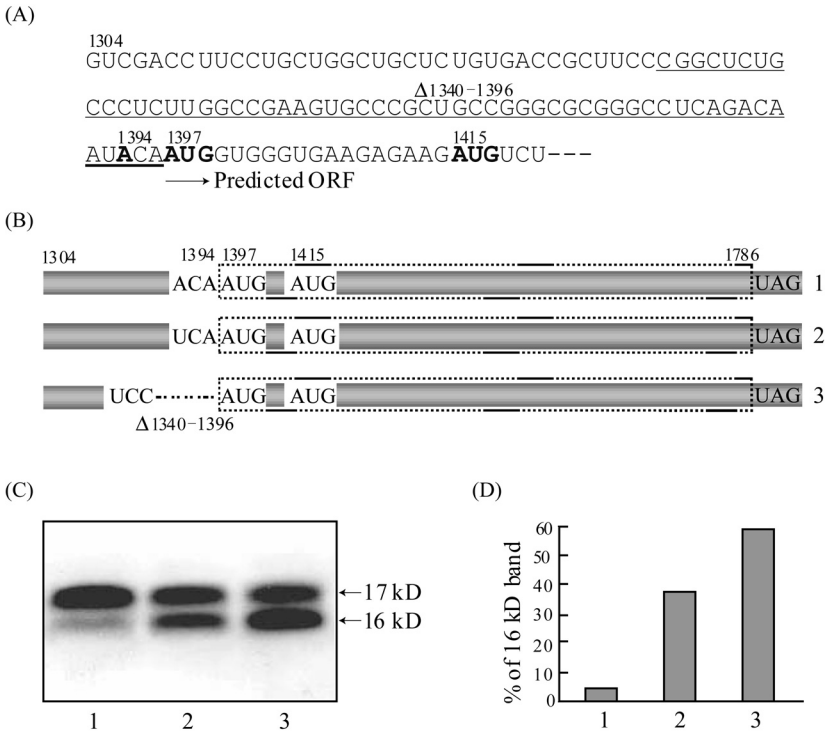

Fig. 3 Effect of the nucleotide at the –3 position upstream of start codon on translation initiation

(A) Partial sequence of ACAT1 mRNA (nt 1304–1420). The nucleotide A at the 1394 position and two adjacent 5′-ORF-AUG codons of human ACAT1 mRNA are in bold. The underlined sequence (nt 1340–1396) is designed to be deleted in experiment. (B) Schematic diagram of the partial ACAT1 mRNAs (nt 1304–1786, Number 1 to 3) corresponding to cDNA K1 sequences respectively inserted into the expression plasmids pcNLD13, pcNLD13M39 and pcNLD14. Number 1, wild type; Number 2, mutant by the nucleotide U (underlined) replaced to A at the 1394 position; Number 3, deletion (dotted line for Δ1340–1396). (C) Western blot analysis. Samples of lane 1 to 3 were individually prepared from AC29 cells transfected with the plasmids expressing wild type and mutants relative to number 1 to 3 in above B. The detailed experimental performances are described under "Materials and Methods". (D) Percentage of the expressed 16 kD ACAT1 N-terminal product. Intensities of the 16 kD protein bands shown in above C are compared with the total intensities of both two protein bands (16 kD and 17 kD), which are determined by using the UVP Labwork software (UVP Inc.).

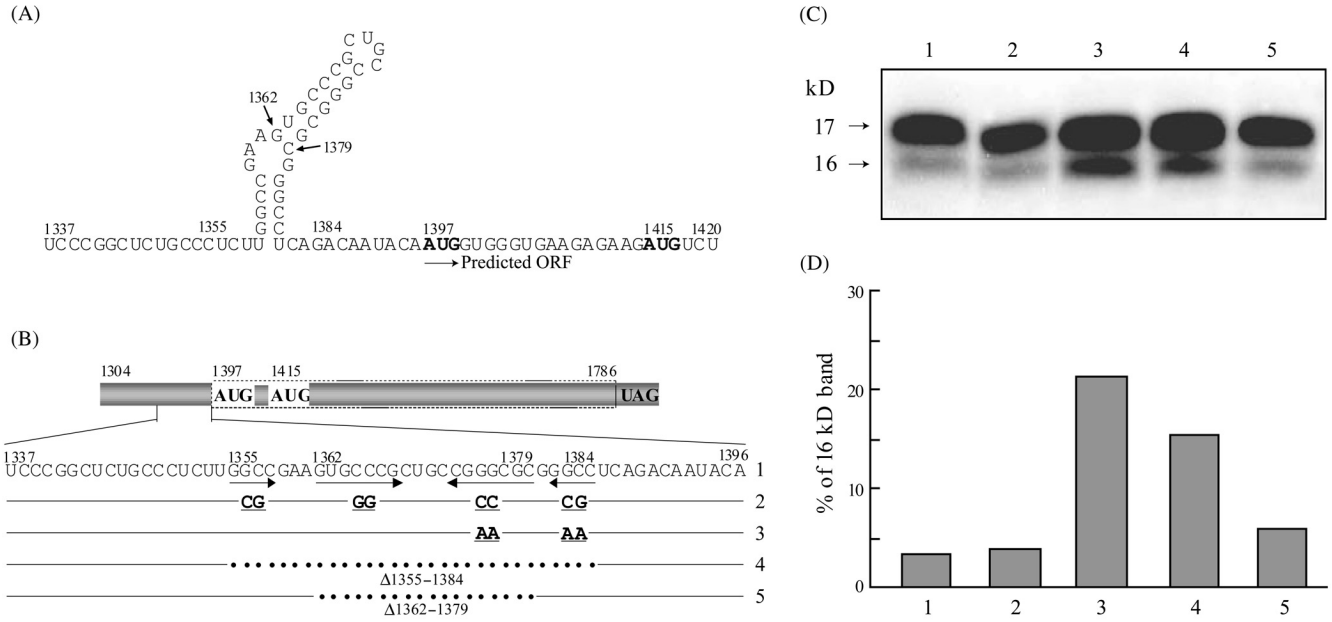

Fig. 4 Effect of the stem-loop structure upstream of start codon on translation initiation

(A) Predicted stem-loop structure (nt, 1355–1384) upstream of start codon in the partial ACAT1 mRNA (nt 1337–1420). The two adjacent 5′-ORF-AUG codons of human ACAT1 mRNA are in bold. (B) Schematic diagram of the partial ACAT1 mRNAs (nt 1337–1420, Number 1 to 5) corresponding to cDNA K1 sequences respectively inserted into the expression plasmids pcNLD13, pcNLD13M54, pcNLD13M55, pcNLD16 and pcNLD15. The complementary stem sequences of the stem-loop structure (nt 1355–1384) are indicated by the oppositely oriented arrows. Number 1, wild type; Number 2 and 3, different mutations was introduced in the stem-loop structure; Number 4 and 5, different deletions (dotted lines for Δ1355–1384 and Δ1362–1379). Sequences that were not altered in the mutants are indicated as continuous lines. (C) Western blot analysis. Samples of lane 1 to 5 were individually prepared from AC29 cells transfected with the plasmids expressing wild type and mutants relative to number 1 to 5 in (B). The detailed experimental performances are described under "Materials and Methods". (D) Percentage of the expressed 16 kD ACAT1 N-terminal product. Intensities of the 16 kD protein bands shown in above C are compared with the total intensities of both two protein bands (16 kD and 17 kD), which are determined by using the UVP Labwork software (UVP Inc.).

[Fig. 4(B), number 3], which destroyed the predicated stem-loop structure, translation of 16 kD ACAT1-NTP initiated from the second 5′-ORF-AUG$_{1415–1417}$ was significantly increased to about 20% of total ACAT1-NTPs [Fig. 4(C,D), lane 3]. These results also confirmed the existence of a stable upstream stem-loop structure in the region of nucleotides 1340 to 1396. Deletion of the predicated stem-loop structure region (Δ1355–1384) as shown in Fig. 4(B) (number 4) also evidently enhanced the translation of 16 kD ACAT1-NTP to about 15% of total ACAT1-NTPs [Fig. 4(C,D), lane 3], while the partial sequence (Δ1362–1379) deletion of the predicated stem-loop structure [Fig. 4(B), number 5] had no obvious change for the expression of both 17 and 16 kD ACAT1-NTPs [Fig. 4(C,D), lane 5].

These evidences demonstrated that a stable upstream stem-loop structure (nt 1355–1384) enhanced the selection of the first 5′-ORF-AUG$_{1397–1399}$ as a main start codon for the translation initiation of human ACAT1 mRNA, in addition to A$_{1394}$ at the –3 position, which is the same site exactly as Kozak sequence. It also implied that a smaller stem-loop structure which beared the same modulation function as the whole stem-loop structure [Fig. 4(C,D), comparing lane 5 with lane 1] could remain active despite the partial sequence deletion [Δ1362–1379, Fig. 4(B), number 5 & Fig. 4(A)].

Enzymatic activity of ACAT1 initiated respectively from the two adjacent 5′-ORF-AUGs

The above results illustrated that both of the two adjacent 5′-ORF-AUGs of human ACAT1 mRNA could be used as start codons for translation initiation of ACAT1 proteins but the first 5′-ORF-AUG$_{1397–1399}$ was selected as a main start codon for ACAT1 expression. So, the 50 kD ACAT1 protein, described previously [4–6], ought to be translated from the first 5′-ORF-AUG$_{1397–1399}$, and the ACAT1 protein initiated the translation from the second 5′-ORF-AUG$_{1415–1417}$ was predicated about 49 kD due to the difference of 6 amino acid residues (MVGEEK) encoded by the in-frame coding sequence of its upstream 18 nucleotides [Fig. 1(A)]. Thereby, it is interesting to investigate whether the enzymatic activities of 50 kD and 49 kD ACAT1s are different. Firstly, expression plasmids

containing the whole predicted cDNA K1 ORF sequence with or without relevant mutants were constructed and transfected into AC29 cells individually. The translation of relevant mRNAs [Fig. 5(A)] produced in the transfected cells could be initiated from the two 5′-ORF-AUGs [Fig. 5(A), number 1], the second 5′-ORF-$AUG_{1415–1417}$ alone [Fig. 5(A), number 2], the first 5′-ORF-$AUG_{1397–1399}$ alone [Fig. 5(A), number 3], or an inserted upstream AUG [Fig. 5(A), number 4]. The Western blot results indicated that the translation of 50 kD ACAT1 protein could be initiated from the first 5′-ORF-$AUG_{1397–1399}$ alone [Fig. 5(B), lanes 3] and 49 kD ACAT1 protein from the second 5′-ORF-$AUG_{1415–1417}$ alone [Fig. 5(B), lane 2]. The normalized ACAT1 activities indicated the similarity between 50 kD [Fig. 5(B), lane 3] and 49 kD [Fig. 5(B), lane 2] ACAT1s.

The determined enzymatic activities depicted no obvious activity difference between 50 kD and 49 kD ACAT1s, even with a difference of 6 amino acid residues (MVGEEK). This result was consistent with early results that the active site of ACAT1 was located in its C-terminal [18,19]. Therefore, it was considered that the two adjacent 5′-ORF-AUGs in human ACAT1 mRNA might have evolved at some point during evolution. The partial sequence alignment analysis of ACAT1 or ACAT1-related genes from several species [Fig. 5(D)] shows that there are two 5′-ORF-AUGs existing in the highly-evolved human and monkey genes but only one 5′-ORF-AUG in the genes of rat and yeast (relevant to the first 5′-ORF-$AUG_{1397–1399}$) as well as in mouse (relevant to the second 5′-ORF-$AUG_{1415–1417}$). This indicates that two adjacent 5′-ORF-AUGs might occur together in the ACAT1 gene of highly-evolved species.

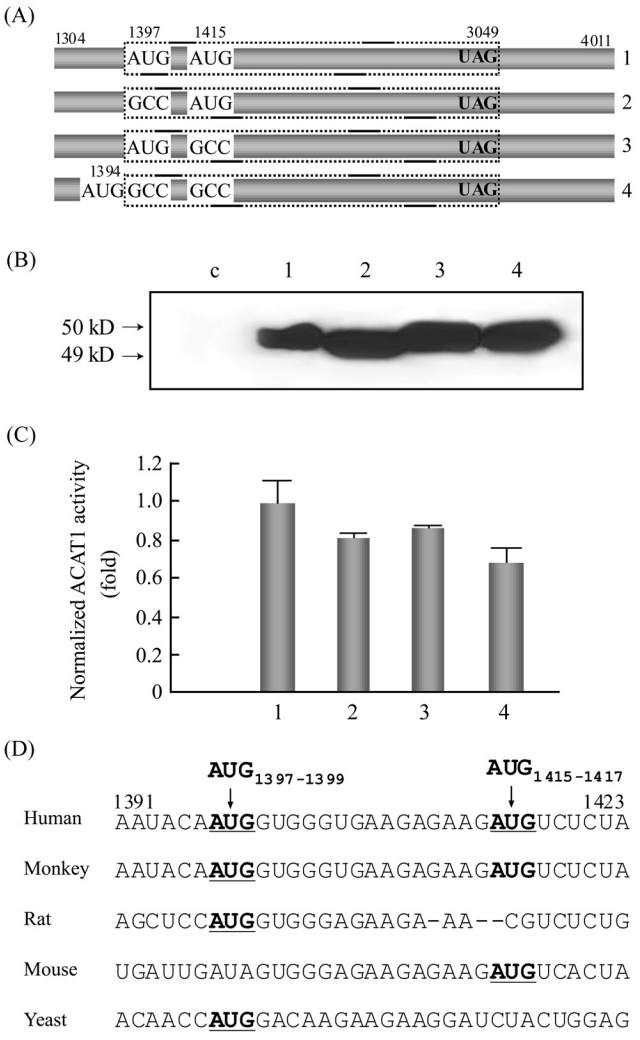

Fig. 5 Enzymatic activity assay of ACAT1 proteins initiated from two adjacent 5′-ORF-AUGs

(A) Schematic diagram of the partial ACAT1 mRNAs (nt 1304–4011, Number 1 to 4) corresponding to cDNA K1 sequences respectively inserted into expression plasmids pcDNA3A1D4, pcDNA3A1D4M60, pcDNA3A1D4M61 and pcDNA3A1D4M51. Number 1, wild type; Number 2 and 3, two AUG codons were individually replaced by mutated codon GCC (underlined); Number 4, a special control with that both of AUG codons were replaced by mutated codons GCCs (underlined) and the in-frame codon1394 replaced by a AUG codon just upstream of the mutated codon GCC1397 (underlined). (B) Western blot analysis. Samples of lane 1 to 4 were individually prepared from AC29 cells transfected with the plasmids expressing wild type and mutants relative to number 1 to 4 in (A). Sample of lane c was prepared from AC29 cells transfected with vector pcDNA3, and used as control. The detailed experimental performances are described under "Materials and Methods". (C) Normalized ACAT1 enzyme activity. The ACAT1 activities in AC29 cells transfected with the plasmids expressing wild type and mutants relative to number 1 to 4 in above A were determined and normalized by the protein amounts shown in (B). The ACAT activity determination was described in "Experimental Procedures". The data represent one of two separate experiments with the same result. (D) Partial sequence alignment analysis of ACAT1 and ACAT1-related genes from human, monkey, rat, mouse and yeast. The 5′-ORF-AUGs in different species are in bold and underlined.

Discussion

The experimental evidences presented in this work demonstrated that both of the two 5′-ORF-AUGs (the first 5′-ORF-$AUG_{1397–1399}$ and the second 5′-ORF-$AUG_{1415–1417}$) in human ACAT1 mRNA could be used as start codons for the translation initiation, and the first 5′-ORF-$AUG_{1397–1399}$ was selected as a main start codon (more than 95%) consistent with the start codon in the predicted ORF (1397–3049) of human ACAT1 mRNA corresponding to cDNA K1 [4].

For the translation initiation from the AUG site, the upstream secondary structure was usually inhibitory by interfering with ribosome binding to mRNA or by blocking ribosome scanning on mRNA [11,12]. However, in

the case of the two adjacent 5′-ORF-AUGs in human ACAT1 mRNA, a stable upstream stem-loop structure (nt 1355–1384) enhanced the selection of the first 5′-ORF-$AUG_{1397–1399}$ as a main start codon for the translation initiation of human ACAT1 mRNA, in addition to A_{1394} at the –3 position which is the same site exactly as Kozak sequence. Destruction and deletion of this upstream stem-loop structure [Fig. 4(A), numbers 3 and 4] decreased the amount of 17 kD ACAT1-NTP initiated at the first 5′-ORF-$AUG_{1397–1399}$. Meanwhile, a smaller stem-loop structure that bears the same modulation function as the whole stem-loop structure [Fig. 4(C,D), comparing lane 5 with lane 1] could remain active despite the partial sequence deletion [Δ1362–1379, Fig. 4(B), number 5 & Fig. 4(A)]. It has been described in some literatures that the stem-loop structures performed their functions by recruiting special factors [20,21], hinting that the modulation of upstream stem-loop structure on AUG selection could be fulfilled by recruiting special protein factors binding to the stem-loop structure. To our knowledge, little is known about how the upstream stem-loop structure modulates the start codon selection of adjacent multi-5′-ORF-AUGs of the mRNA. The result that a stable upstream stem-loop structure modulated the start codon selection might possess a crucial significance in the translation initiation from a adjacent multi-5′-ORF-AUGs of the mRNA. Elucidating this can also enrich the understanding of the translation initiation of eukaryotic mRNAs.

It was also clarified that the translation of the 50 kD ACAT1, reported early in human cells and transfected AC29 cells [5,6], is initiated from the first 5′-ORF-$AUG_{1397–1399}$. Translation initiated from the two adjacent 5′-ORF-AUGs deduced the 6 amino acid residues difference (MVGEEK) between the 50 and 49 kD products, which were rarely distinguished in SDS-PAGE [Fig. 5(B)], while 17 and 16 kD ACAT1-NTPs could be easily separated (Fig. 3 and 4). Notwithstanding the 6 amino acid residues difference at their N-termini, 50 and 49 kD products showed almost the same ACAT activities, consisting with the finding that the active site of ACAT1 is in its C-terminal [18,19]. So, the two adjacent 5′-ORF-AUGs might guarantee the expression of cellular ACAT1, a key and exclusive kind of enzyme catalyzing cholesteryl ester from free cholesterol and fatty acid. Yet, two adjacent 5′-ORF-AUGs of ACAT1 mRNA are found to exist in human and monkey, but only one of AUG codon relative to these two adjacent 5′-ORF-AUGs occurs in the relatively lower-evolved rat, mouse and yeast [Fig. 5(D)], demonstrating that the occurrence of two adjacent 5′-ORF-AUGs of ACAT1 mRNA in human and monkey may have happened in evolution. From another point of view, it was also indicated more importance of cellular cholesterol homeostasis and its correlative regulation in highly-evolved human and monkey, which also might be beneficial for intelligence evolution.

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