Caveolin-1 isoforms are encoded by distinct mRNAs

Identification of mouse caveolin-1 mRNA variants caused by alternative transcription initiation and splicing

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Received 28 October 1999; received in revised form 3 December 1999

Edited by Avril Somlyo

Abstract By searching the EST database with the known cDNA sequence encoding α-caveolin-1 (full-length: FL), we found a variant having a hitherto unknown sequence in place of the first exon (5′-end variant: 5′V). The expression level of 5′V mRNA was equivalent to that of FL mRNA. The entire sequences of FL and 5′V mRNA were determined by 3′- and 5′-RACE analysis; their sizes were 2484 bp and 2533 bp, respectively, and the sequences were identical except for the region of the first exon. By Northern blotting, FL and 5′V mRNAs showed the same tissue distribution, and were intensely expressed in the lung, heart, and skeletal muscle. Analyzing the protein production from these mRNAs using green fluorescent protein as a tag, we found FL mRNA to produce the α-isoform predominantly, but to form little β-isoform. The production of the β-isoform from 5′V mRNA was also demonstrated. By sequence analysis of the first intron of the caveolin-1 gene, a TATA box was found at 28 bp upstream of the transcription initiation site for 5′V mRNA. This is the first demonstration of caveolin-1 mRNA variants generated by alternative transcription initiation, and it indicates that the two isoforms of caveolin-1 are produced from two distinct mRNAs.

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Key words: Caveolin-1 isoform; mRNA variant; Alternative transcription initiation

1. Introduction

Caveolae are invaginations of the plasma membrane and are seen abundantly in adipocytes, endothelial cells, smooth muscle cells, and type I pneumocytes. These structures are believed to play a role in transcytosis of macromolecules [1], and recent studies have revealed that they are also involved in cholesterol homeostasis, intracellular signaling, and cell transformation [2–5]. Caveolin-1 was identified as a major component of caveolae [6], and was demonstrated to have many functions such as cholesterol binding and transport [7,8], inhibitory interaction with signaling molecules [9], and suppression of oncogenic transformation [10]. Moreover, de novo formation of caveolae was induced by the exogenous expression of caveolin-1 [11]. These findings indicate the important roles of caveolin-1 for caveolar function and formation.

There are two isoforms of caveolin-1, termed α and β. They are identical except for the additional 31 amino acids of the α-isoform at its N-terminus, and were shown to be produced by alternative translation initiation [12]. As to these isoforms, the differential distribution of these isoforms [12], as well as the specific phosphorylation of the α-isoform in v-Src transformed cells [13,14] and that of the β-isoform by insulin treatment of 3T3-L1 cells [15], has been reported. However, it is not clear whether the isoforms have distinct roles in cell functions, or how the expression of both isoforms is regulated.

In the present study, we identified several mRNA variants of caveolin-1 by sequence database analysis and 5′- and 3′-RACE. In particular, a variant lacking the first exon (5′-end variant: 5′V) was shown to be generated by alternative transcription initiation, and to be expressed at a level comparable to that of the mRNA having the first exon (full-length: FL). The analysis of protein production from both FL and 5′V cDNAs revealed that caveolin-1 isoforms are generated from distinct mRNAs in vivo. A possible promoter region for the 5′V mRNA transcription was also identified within the first intron of the mouse caveolin-1 gene. These data provide a molecular basis for the regulatory mechanism of the expression of caveolin-1 isoforms.

2. Materials and methods

2.1. Cell culture

Pam212 cells [16] were kindly provided by Dr. Koji Hashimoto (Ehime University, Japan). 3Y1 cells [17] were obtained from Kimura's 3Y1 library at RIKEN Cell Bank (Tsukuba, Japan). Neuro2a and COS7 cells were obtained from the Japanese Cancer Resources Research Bank (Tokyo, Japan). Cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagles minimum essential medium (Nihon-seiyaku Co.) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 0.05 mg/ml streptomycin.

2.2. RNA and DNA extraction

Total RNA and genomic DNA of culture cells and of BALB/c mouse tissues were extracted by TRIZOL reagent (Life Technologies, Inc.). The amounts of RNA and DNA were estimated by OD_{260}. The $OD_{260/280}$ ratio of the total RNA was 1.7–1.8 for all of the samples.

2.3. Primers

The sequences of primers used were as follows, with primers having the /F designation being sense primers and those with /R being antisense primers. Primers for FL caveolin-1: ex1/F 5′-AAATCACAGCCCAGGGAAACC-3′; a-start/F 5′-ACGATGTCTGGGGGCAAATAC-3′; b-start/F 5′-CAAGGCCATGGCAGACGAG-3′; ex1/R 5′-GATGTCCCTCGGAGTCCACG-3′; ex2/R 5′-CTTGACCACGTCGTCGTTGAG-3′; stop/R 5′-TCATATCTCTTTCTGCGTGCTGAT-3′. Primers for 5′V: 5′V/F1 5′-TAGCAAAAGTTGTAGCGCCAG-3′; 5′V/F2 5′-CTTTTCTTCCCACCGCTGTTGC-3′; intl/R 5′-GTAGAGATGTCCCTGTGAGG-3′. 5′V/F1 and 5′V/F2 were generated according to the expressed sequence tag (EST) sequences AA655345, AA726617 and AA726618. Primers for RACE

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PII: S0014-5793(99)01730-5

[18]: double adapter-oligo dT (DA-dT) 5′-AAGGATCCGAATCGC-TAGCTAATACGACTCACTATAGGGATTTTTTTTTTTTTTTT-TTTT-3′; outer primer (Po) 5′-AAGGATCCGAATCGC-3′; inner primer (Pi) 5′-CGCTAGCTAATACGACTCAC-3′. The locations of caveolin-1-specific primers are shown in Fig. 1A.

2.4. Polymerase chain reaction (PCR)

For RT-PCR, total RNA was reverse-transcribed by SuperScript™ II reverse transcriptase (Life Technologies, Inc.) with random hexamer primers, and amplified with the following primer sets: a-start/F and ex2/R or stop/R for FL; 5′V/F1 or 5′V/F2 and ex2/R or stop/R for 5′V. For 3′-RACE, total RNA was reverse-transcribed by AMV reverse transcriptase XL (Takara Shuzo Co., Ltd.) or SuperScript™ II reverse transcriptase with DA-dT primer and amplified by LA Taq™ DNA polymerase (Takara Shuzo Co., Ltd.) with primers Po and a-start/F for FL, or Po and 5′V/F1 for 5′V. The PCR products were further amplified with nested primers Pi and b-start/F for FL, or with Pi and 5′V/F2 for 5′V. For 5′-RACE, total RNA was reverse-transcribed with stop/R, poly(A) was added to the end of the first-strand cDNA, and PCR was performed with primers DA-dT and ex2/R as the first round of amplification. In the second-round PCR, Pi and ex1/R for FL, or Pi and int1/R for 5′V were used. To obtain the DNA fragment of the first intron of the mouse caveolin-1 gene, we amplified mouse genomic DNA with primers a-start/F and int1/R.

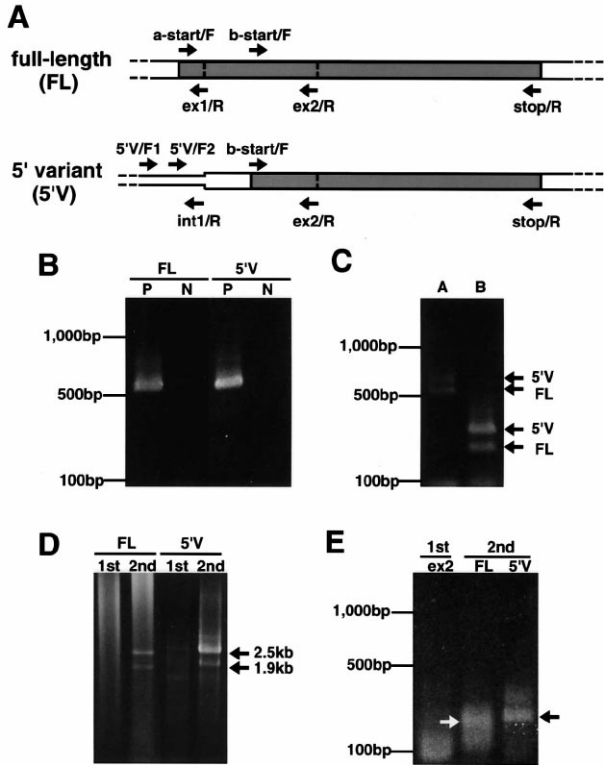

Fig. 1. RT-PCR, 3′-RACE, and 5′-RACE analyses of mouse caveolin-1 mRNA variants. A: Schematic representation of mouse caveolin-1 mRNA variants FL and 5′V, and primer sites used for the amplification. Shadowed boxes indicate the coding region for α-caveolin-1 in FL and β-caveolin-1 in 5′V, respectively. The vertical broken lines indicate the splicing sites of exons. B: RT-PCR amplification of mouse caveolin-1 mRNAs, FL (primer set: a-start/F and stop/R) and 5′V (primer set: 5′V/F2 and stop/R). The expected size of the products was 551 bp and 574 bp, respectively. Both products were detected in Pam212 cells (P) but not in Neuro2a cells (N). C: RT-PCR amplification of mouse caveolin-1 mRNAs by use of a common antisense primer stop/R (lane A) or ex2/R (lane B) and a mixture of two sense primers, a-start/F and 5′V/F1. The result shows the comparable expression of both FL and 5′V mRNA in Pam212 cells. The expected sizes of the PCR products are 646 and 551 bp in lane A, and 293 and 198 bp in lane B for FL and 5′V mRNA, respectively. D: 3′-RACE of FL and 5′V mRNA. Primer sets used for the amplification were as follows: FL 1st: a-start/F and Po; FL 2nd: b-start/F and Pi; 5′V 1st: 5′V/F1 and Po; 5′V 2nd: 5′V/F2 and Pi. PCR products of approximately 2.5 kb and 1.9 kb were obtained for both FL and 5′V. E: 5′-RACE of FL and 5′V mRNA. In the first round of PCR, DA-dT and ex2/R were used as primers. In the second round, the amplification was performed with a combination of a common primer Po and a primer specific for FL (ex1/R) or for 5′V (int1/R). The obtained DNA fragment was approximately 210 bp for FL and 240 bp for 5′V.

2.5. Sequence analysis

All PCR products were sequenced directly or after subcloning in pT7Blue-T vector (Novagen). The database sequence search was performed using BLAST 2.0 (http://www.ncbi.nlm.nih.gov/). Assembly and analysis of the sequences were achieved with a DNASTAR software package (DNASTAR Inc). The alignment of multiple sequences was performed with Clustal W [19]. The prediction of promoter sites and transcription factor binding sites was performed with the Promoter Prediction by Neural Network (NNPP) program [20] and with Transcription Element Search Software (TESS) using the TRANSFAC database [21] (http://www.hgsc.bcm.tmc.edu/).

2.6. Northern blotting

The expression of mRNA variants of caveolin-1 was examined by Northern blotting using Mouse Multiple Tissue Northern Blot (Clontech). The cDNA probes were generated by PCR using the following primers: a-start/F and stop/R for ORF (common: 537 bp); 5′V/F1 and int1/R for 5′V (140 bp); and ex1/F and ex1/R for FL (117 bp). The positions of the probes are presented in Fig. 2. The probe for β-actin supplied by Clontech was used as a control. The probes were labeled with [α-^{32}P]dCTP, and the hybridization was carried out for 3 h in ExpressHyb™ solution (Clontech) at 65°C for FL or at 68°C for other probes. Signals were visualized by exposing the blots to X-ray film for 2–6 days.

2.7. Protein expression from FL and 5′V caveolin-1 mRNA

The cDNA construct encoding the chimeric protein of α-caveolin-1 and green fluorescent protein (GFP) having no 5′ untranslated region (UTR) sequence (FL1) was produced previously [22]. The cDNAs corresponding to the bp 59–614 sequence of FL cDNA (FL2: with 22 bp of 5′UTR) and bp 31–663 of 5′V cDNA (5′V: with 191 bp of 5′UTR) were subcloned into pEGFP-N1 (Clontech) for the expression of the chimeric proteins. COS7 and 3Y1 cells were transiently transfected with the cDNA constructs with SuperFect (Qiagen). For Western blot analysis, the transfected COS7 cells were dissolved in an SDS sample buffer; and the proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The blotted membranes were probed with mouse monoclonal anti-caveolin-1 antibody (C060, Transduction Laboratories) or rabbit polyclonal anti-GFP antibody (Clontech), and the signal was detected with a SuperSignal Chemiluminescent System (Pierce). For fluorescence microscopy, the transiently transfected 3Y1 cells were fixed with 3% formaldehyde for 5 min, and the images were captured by a chilled CCD camera (Hamamatsu Photonics) with the operating program IPLab Spectrum (Signal Analytics).

3. Results and discussion

By the database sequence search, a cDNA sequence of caveolin-1 lacking the first exon (5′V) was found to be registered in mouse and human EST databases (for example, AA655345 in mouse and AA376123 in human). It contained a hitherto unknown sequence in place of the first exon of FL caveolin-1 mRNA. The expression of FL and 5′V mRNA was detected by RT-PCR in Pam212 cells, but not in Neuro2a cells (Fig. 1B). The approximate expression levels of FL and 5′V were estimated by RT-PCR using a common antisense primer (stop/R or ex2/R) and a mixture of two sense primers specific to FL (a-start/F) and 5′V (5′V/F1). As a common primer is assumed to prime to both cDNAs with the same efficiency, the amount of the products should reflect the ratio of FL and 5′V mRNAs expressed in cells. RT-PCR products from both FL

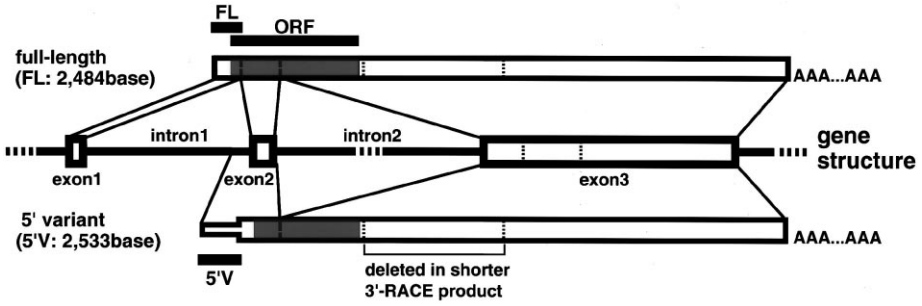

Fig. 2. Schematic representation of mouse FL and 5′V caveolin-1 mRNAs with the gene structure. The nucleotide sequence data of FL and 5′V mRNAs will appear in the nucleotide sequence database DDBJ/EMBL/GenBank with the accession numbers AB029929 and AB029930, respectively. The gene structure of mouse caveolin-1 was demonstrated by Engelman et al. [25]. Shadowed boxes indicate the coding region for α-caveolin-1 in FL and for β-caveolin-1 in 5′V. The vertical broken lines indicate the splicing sites. The vertical dotted lines within the third exon indicate the alternative splicing sites causing the 620-bp deletion. The regions of cDNA fragments used as probes for Northern blotting are also indicated with solid bars (ORF for pan caveolin-1 mRNA: FL for FL mRNA; 5′V for 5′V mRNA).

and 5′V mRNAs were detected as bands of similar intensity (Fig. 1C).

As ESTs contain only partial sequences, it was unclear whether FL and 5′V mRNA have any other difference in their overall sequences. The molecular cloning of the mammalian caveolin-1 gene has been accomplished for human (Z18951), mouse (U07645), rat (Z46614), dog (Z12161), and cattle (U86639) genes. However, the sequences are limited to those just around the coding region; and thus a large part of the mRNAs, whose size was approximately 2.5–3.0 kb by Northern blotting of mouse [15] and human [23] samples, has not been identified yet. For the identification of the entire sequences of FL and 5′V mRNA, we performed 3′- and 5′-RACE. As 3′-RACE products, when reverse-transcribed with AMV reverse transcriptase XL, major (2.5 kb) and minor (1.9 kb) products were obtained from both FL and 5′V mRNA (Fig. 1D, arrows). Sequencing revealed that the products from both FL and 5′V were identical in sequence except for the 5′-end region. The 2.5-kb product had the 1867 bp of 3′UTR sequence with a putative polyadenylation signal AATAAA at position 2465 in AB029929. The 1.9-kb product was revealed to be an alternative splicing variant of the 2.5-kb species with a deletion of 620 bp in the 3′UTR (bp 639–1258 in AB029929) having the GT-AG intron character. In the samples reverse-transcribed with SuperScript II, additional bands of 1.4 kb and 0.8 kb were detected in both mRNAs (data not shown); they were alternatively polyadenylated at 1372 and at 774, respectively. Taken together, FL and 5′V mRNA share the common sequence in the downstream region of the second exon, and show heterogeneity of their 3′UTR, which is caused by alternative splicing and polyadenylation. As to the 3′UTR of human caveolin-1 mRNAs, the corresponding splice variants are not expressed; but instead, a 0.8-kb mRNA generated by early polyadenylation was registered in the EST database and also detected by Northern blotting [23]. In recent studies, the involvement of the 3′UTR sequences in the intracellular localization, stability, and translational regulation of mRNA has been suggested [24]; however, the significance of the 3′UTR heterogeneity in caveolin-1 mRNA has not been determined yet.

As 5′-RACE products, bands with an approximate size of 210 bp for FL and 240 bp for 5′V were obtained (Fig. 1E, lanes FL and 5′V, respectively). By the sequence analysis, products containing 80, 73, 60 and 48 bp of 5′UTR sequence were obtained from FL. On the other hand, the products from 5′V contained 158 bp of the upstream sequence from the 5′-end of the second exon, and the sequence revealed that the 5′V mRNA putatively encodes the β-isoform of caveolin-1 and has 221 bp of 5′UTR sequence. The success of the primer extension to the end was confirmed by the fact that an additional G was detected at the end of all of the 5′-RACE products, indicating the reverse transcription of the capping guanine nucleotide. While our study was in progress, the sequence specific to 5′V was reported to be the intron sequence of the caveolin-1 gene [25], and we confirmed it by genomic PCR amplification (data not shown). Taken together, we identified the full sequence of FL and 5′V caveolin-1 mRNA, and the sequence was confirmed by referencing many mouse EST sequences; they were available from Genbank/EMBL/DDBJ under accession number AB029929 for FL (2484 bp) and AB029930 for 5′V (2533 bp) cDNA. The gene and cDNA structure of mouse caveolin-1 are schematically presented in Fig. 2.

The tissue distribution of both mRNAs was examined by Northern blotting (Fig. 3). Using a common probe, ORF, we detected caveolin-1 mRNAs as major (2.7 kb) and minor (2.1 kb) bands intensely expressed in the lung, skeletal muscle, and heart, and weakly in the kidney, testis, brain, and spleen (in decreasing order), but not in the liver. The sizes of the mRNAs were consistent with the sequences of FL and 5′V mRNA, and the lower minor band was considered to correspond to the alternative splicing variant having the 620-bp deletion in the 3′UTR region. Northern blotting with the specific probes for 5′V (140 bp) and FL (117 bp) was also performed. The tissue distribution as well as the apparent sizes of the two mRNAs were the same (Fig. 3, FL and 5′V), although the specific probe for FL mRNA did not generate intense signals, probably due to the shortness of the probe. Although the existence of the splicing variant was not shown for FL mRNA in Northern blotting, the 620-bp deletion in the 3′UTR was equally detected in both FL and 5′V mRNAs by RT-PCR (data not shown). Although the tissue distribution was apparently the same for the two mRNAs, there remains a possibility that the mRNAs are differentially regulated, or that their expression ratio is diverse among different cell types. Previous studies showed that caveolin-1 gene expression is modulated by cell transformation with activated mitogen-activated protein kinase signaling [26], cellular free cholesterol [27,28], and cyclic AMP signals [29]. However, unfortunately, because the probes used in the ex-

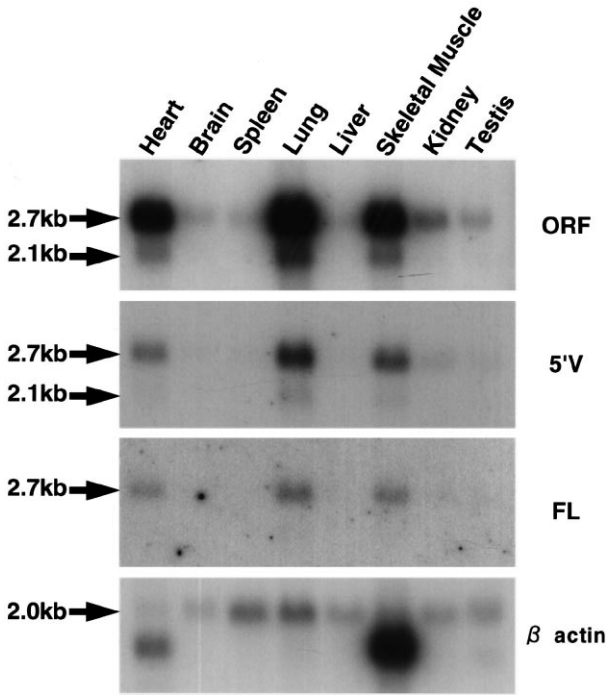

Fig. 3. Northern blot analysis of mouse caveolin-1 mRNA variants. The top panel shows the distribution of mouse caveolin-1 mRNA detected with the ORF probe, which recognizes both FL and 5′V mRNAs. Two bands, 2.7 kb and 2.1 kb, were detected in highly expressing tissues such as heart, lung and skeletal muscle. The second and third panels show the result with 5′V- and FL-specific probes, respectively. The apparent size and the tissue distribution were identical for the two mRNAs. The bottom panel is a control blot with the β-actin probe.

periments recognize both FL and 5′V mRNA, and the sizes of the two mRNAs are apparently the same, no information is available concerning the differential regulation of these two mRNAs. At the protein level, for example, regulation apparently specific to the α-isoform has been suggested in cell cycle progression [30]. Examination of whether FL and 5′V mRNAs are differentially regulated, leading to the production of the protein isoforms in different ratios, may provide a clue to elucidate the physiological roles of these isoforms.

The two isoforms of caveolin-1, α and β, have been believed to be produced by alternative translation initiation from a single mRNA [12], but the molecular mechanism that regulates the expression of the two isoforms has been unknown. The protein production from the caveolin-1 mRNA variants was examined by the transient expression of GFP chimeras in COS7 cells (Fig. 4A). As reported previously [12], both α- and β-caveolin-1/GFP were produced by transfection with the cDNA construct having no 5′UTR sequence (Fig. 4A, FL1). However, the construct having 22 bp of the 5′UTR sequence including the Kozak sequence gave rise to preferentially the α-caveolin-1/GFP with little β-isoform (Fig. 4A, FL2), suggesting that FL caveolin-1 mRNA dominantly generates the α-isoform in vivo. The efficient production of the β-isoform from 5′V mRNA was also confirmed (Fig. 4A, 5′V). The present study clearly demonstrates that the two isoforms are most likely generated by the translation of the two distinct mRNAs, FL and 5′V, rather than by alternative translation initiation of FL mRNA, indicating that the regulatory mechanism for the isoform expression may be ascribed to the transcriptional regulation of FL and 5′V mRNAs. However, this result does not exclude the possible contribution of an alternative translation initiation mechanism to isoform production in vivo. Further studies are necessary to clarify the precise mechanism regulating the relative ratio of the two isoforms. The cellular localization of the two isoforms was observed by fluorescence microscopy; both α- and β-caveolin-1/GFP were seen to exist in cell edges, and the localization was indistinguishable from each other in 3Y1 (Fig. 4B) and COS7 cells (data not shown).

To analyze the structure of the promoter for the 5′V mRNA transcription, we obtained the genomic sequence of the first intron of the mouse caveolin-1 gene by PCR and sequenced it (accession number AB029931). It contained the whole 5′-end sequence of 5′V mRNA, and the NNPP program also predicted the transcription start from this position. The partial sequence of the first intron is compared with that of the human gene [31] in Fig. 5. A putative TATA box was found at −28 from the transcription initiation site (Fig. 5, boxed). The upstream sequence from the transcription start site was highly conserved between human and mice (84% identity in −1 to −116), suggesting the critical function of this region for the transcriptional regulation. In addition, some putative transcription factor binding sites have been predicted based on a TRANSFAC database search in this intron region. For example, the putative binding site for apolipoprotein A1 regulatory protein-1 was found at −107 bp, close to the promoter region (Fig. 5, shown by a broken line); and further upstream, that for sterol regulatory element binding protein-1 was predicted at −230 bp. The possible involvement of these transcription factors is consistent with the putative functions of caveolin-1 in cholesterol homeostasis [2]. The putative sterol-responsive elements upstream of the first exon was assumed to be involved in the regulation of caveo-

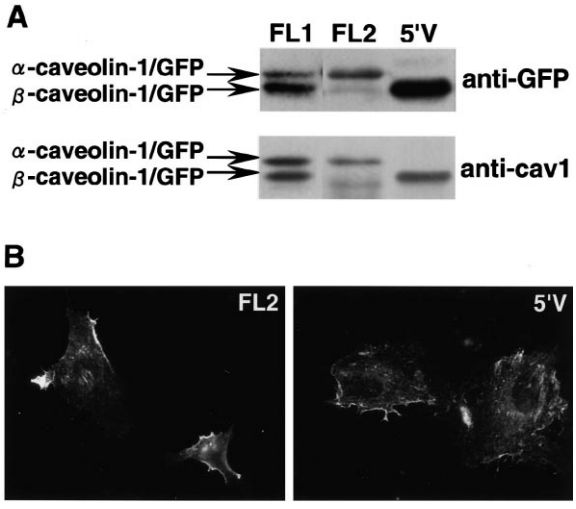

Fig. 4. Analysis of protein production from FL and 5′V mRNA tagged with GFP. A: Western blot analysis of the chimeric proteins transiently expressed in COS7 cells. Anti-GFP antibody (anti-GFP) and anti-caveolin-1 antibody (anti-cav1) were used for the detection. While both α- and β-caveolin-1/GFP were comparably produced from the construct having no 5′UTR sequences (FL1), α-caveolin-1/GFP was predominantly expressed from the construct having 22 bp of 5′UTR sequence (FL2). β-Caveolin-1/GFP was efficiently produced from the 5′V/GFP constructs (5′V). B: Fluorescence photographs of α- and β-caveolin-1/GFP (FL2 and 5′V) in 3Y1 cells. The distribution of α- and β-caveolin-1/GFP was not distinguishable.

Fig. 5. Promoter structure of an alternative transcription initiation site for mouse 5′V caveolin-1 mRNA in comparison with the human sequence. The transcription initiation site determined by 5′-RACE in mice is indicated as +1. The putative TATA box at −28 is indicated by a box. More upstream, around −90, possible GC boxes exist. A motif for the putative apolipoporotein A1 regulatory protein recognition predicted by the TRANSFAC database is underlined with a broken line. Identical nucleotides between human and mouse are indicated by asterisks. The whole sequence of the first intron of mouse caveolin-1 is available in the DDBJ/EMBL/GenBank database with the accession number AB029931.

lin-1 expression by free cholesterol [32]. Our data indicate that possible transcription regulatory elements are also present within the first intron. The analysis of the transcriptional activity of the intron region is the next object of our study.

In conclusion, we identified a novel caveolin-1 mRNA variant lacking the first exon, and demonstrated that the α and β isoforms of caveolin-1 are generated by the translation of distinct mRNAs, indicating that the expression of the caveolin-1 isoforms is regulated at the transcriptional level. Further analysis of the regulation of caveolin-1 gene expression should lead to the clarification of the biological roles of caveolin-1 isoforms in cell functions.

Acknowledgements: We would like to thank Dr. Akiko Iizuka-Kogo, National Institute for Basic Biology, for helpful discussion and technical advice; Professor Jun Takeda, Gunma University, for valuable suggestions; and Dr. Ryuji Nomura, in our laboratory, for helpful discussion.

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